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CLINICAL THERAPEUTICS

Preexposure to Isavuconazole Increases the Virulence of *Mucorales* but Not *Aspergillus fumigatus* in a *Drosophila melanogaster* Infection Model

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ABSTRACT Breakthrough mucormycosis in patients receiving isavuconazole prophylaxis or therapy has been reported. We compared the impact of isavuconazole and voriconazole exposure on the virulence of clinical isolates of *Aspergillus fumigatus* and different *Mucorales* species in a *Drosophila melanogaster* infection model. In contrast to *A. fumigatus*, a hypervirulent phenotype was found in all tested *Mucorales* upon preexposure to either voriconazole or isavuconazole. These findings may contribute to the explanation of breakthrough mucormycosis in isavuconazole-treated patients.

KEYWORDS *Aspergillus fumigatus*, *Drosophila melanogaster*, *Rhizopus arrhizus*, isavuconazole

Invasive mold infections (IMI) remain a major cause of morbidity and mortality in immunocompromised patients. Whereas invasive aspergillosis, mostly caused by *Aspergillus fumigatus*, accounts for the majority of these infections, an epidemiological shift toward an increased incidence of mucormycosis has been observed, with *Rhizopus arrhizus* (*Rhizopus oryzae*) being the most frequent causative agent (1, 2). Several studies have linked this epidemiological shift to the widespread use of voriconazole (VRC), an azole with good *Aspergillus* but no *Mucorales* activity (1, 3). Isavuconazonium sulfate (ISAV) is the most recently introduced triazole exhibiting activity against a wide range of medically important fungi and a favorable toxicity profile compared to other triazoles (4). A recent study examining breakthrough mold infections in leukemia patients who were receiving oral or intravenous ISAV for prophylaxis or therapy reported that most microbiologically documented breakthrough mold infections were caused by *Mucorales* (5).

Although antifungal therapy may select for breakthrough infections caused by more resistant pathogens through direct selection pressure, drug-induced changes in pathogen virulence may be encountered (6, 7). We previously found evidence of increased virulence, angioinvasion, and inflammatory host response in invertebrate and mammalian models of mucormycosis when animals were infected with a strain of *R. arrhizus* that was passaged on VRC-containing agar but not posaconazole (7). The impact of ISAV on the virulence of *A. fumigatus* and *Mucorales* has not yet been characterized. Using a well-validated *Drosophila melanogaster* infection model (8), we sought to evaluate virulence changes of *A. fumigatus* and *Mucorales* serially passaged on agar supplemented with ISAV or VRC.

Two *A. fumigatus* isolates (internal numbers 6058 and 8171) and four *Mucorales* isolates (*Rhizopus arrhizus* 749 and 969, *Rhizomucor pusillus* 449, and *Mucor circinelloides* 518) were obtained from patients at the University of Texas M.D. Anderson Cancer Center. Preexposure of these pathogens to VRC and ISAV by serial passage on

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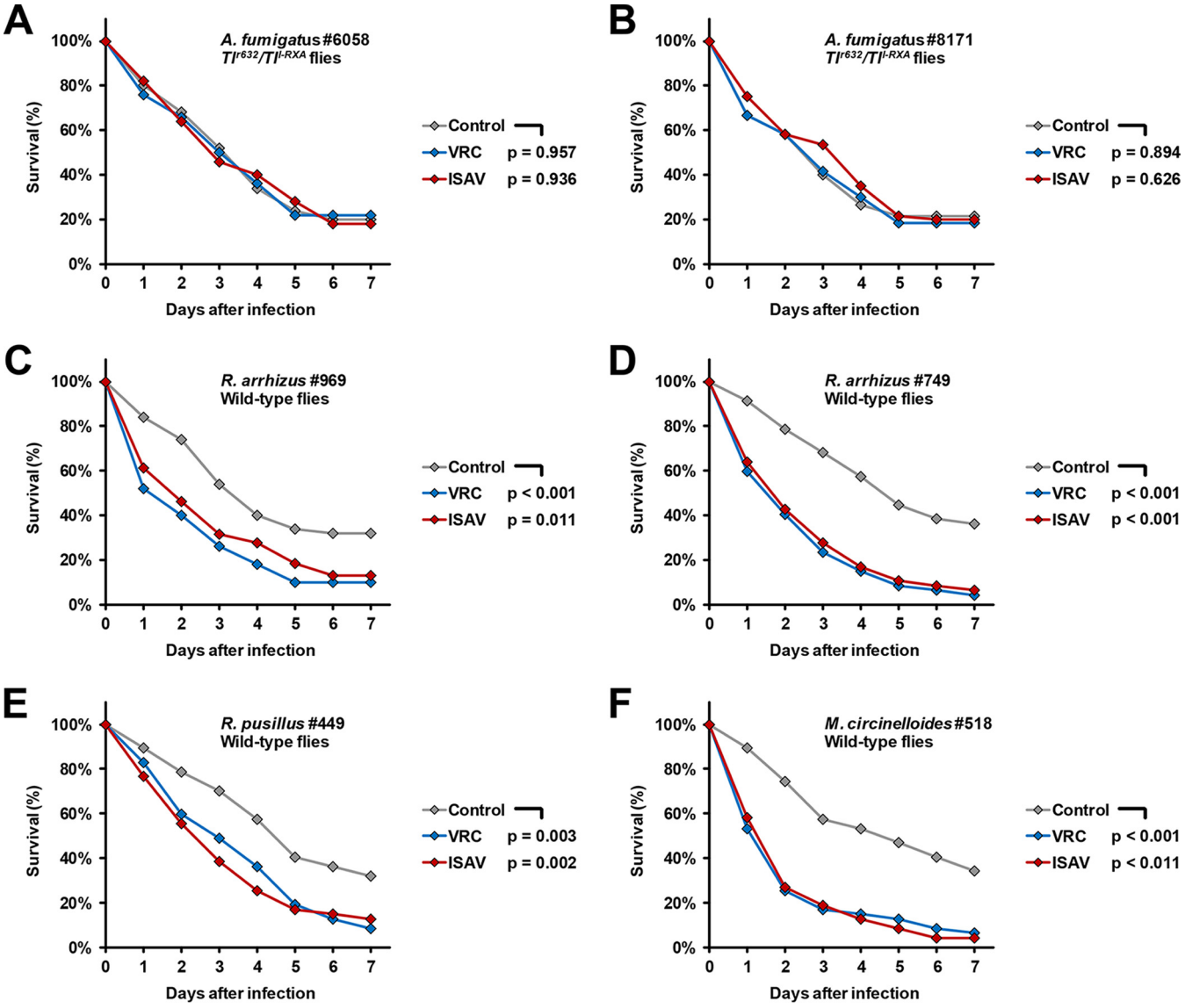

FIG 1 Survival rates of *A. fumigatus*- and *Mucorales*-infected *D. melanogaster* flies depending on fungal preexposure to azoles. Clinical isolates of *A. fumigatus* and four different *Mucorales* isolates underwent four serial passages on ISAV-supplemented, VRC-supplemented, or nonsupplemented (Control) yeast extract-agar-glucose plates. $Tl^{r632}/Tl^{l\text{-}RXA}$ *Drosophila* mutant flies and Oregon^R WT flies were infected with *A. fumigatus* and *Mucorales*, respectively. The survival of the flies was monitored for 7 days postinfection. Two independent experiments were performed, and survival rates were calculated based on cumulative data.

antifungal-supplemented yeast extract-agar-glucose plates was performed as previously described (7). Four different concentrations of each antifungal were tested (0.0625, 0.125, 0.25, and 0.5× MIC as determined according to the CLSI M38-A2 reference method) in order to define the highest fraction of MIC still yielding confluent growth after 48 h at 37°C. This concentration (usually 0.25× MIC) was used for subsequent passages (*Mucorales*: 4 μg/ml VRC, 0.5 μg/ml ISAV; *A. fumigatus*: 0.5 μg/ml VRC, 0.125 μg/ml ISAV). Controls were prepared by serial passaging of each fungus on yeast extract-agar-glucose plates not supplemented with antifungals.

For *Mucorales* infections, the dorsal side of the thorax of female Oregon^R wild-type (WT) flies was pricked with a needle dipped in a 1×10^7/ml spore solution as previously described (8). *A. fumigatus* infections (inoculum concentration, 5×10^7/ml) were performed in $Tl^{r632}/Tl^{l\text{-}RXA}$ *Drosophila* mutant flies generated by crossing thermosensitive allele of *Toll* (Tl^{r632}) flies with a null allele of *Toll* ($Tl^{l\text{-}RXA}$) flies. Survival was monitored daily for 7 days postinfection. For each isolate, two independent experiments were

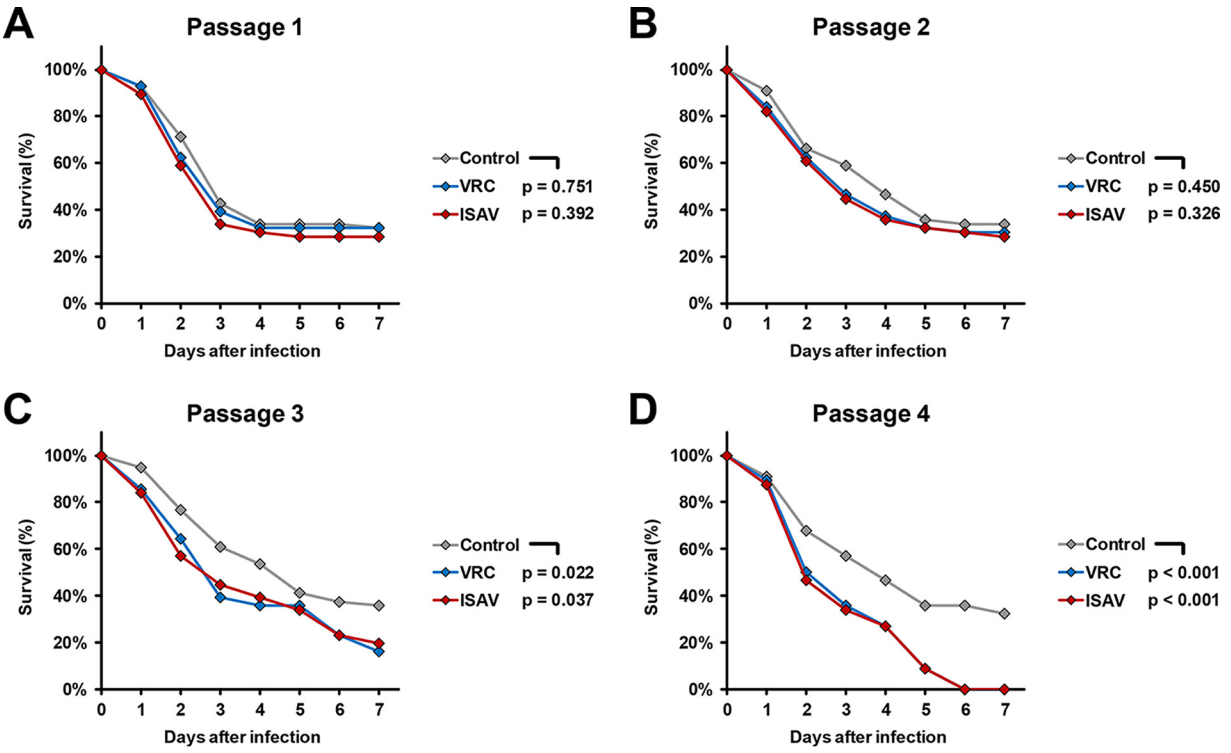

FIG 2 Survival rates of *R. arrhizus* 749-infected *D. melanogaster* flies depending on the number of passages of the fungus on azole-supplemented plates. *R. arrhizus* 749 was passaged four times on ISAV-supplemented, VRC-supplemented, or nonsupplemented (Control) yeast extract-agar-glucose plates. Oregon^R WT flies were infected after each passage. The survival of flies was monitored for 7 days postinfection. Two independent experiments were performed, and survival rates were calculated based on cumulative data.

performed ($n = 47$ to 60 per cohort). Statistical comparison of survival rates was performed using a log rank test (GraphPad Prism 7.03). A P value of <0.05 was considered significant.

For both *A. fumigatus* isolates tested, preexposure to VRC or ISAV for four passages did not result in significant differences in survival curves, with 7-day survival rates of 20 to 22% (control), 18 to 22% (preexposed to VRC), and 18 to 20% (preexposed to ISAV), respectively. The median survival times (MSTs) of *Toll*-deficient flies infected with *A. fumigatus* strain 6058 were 4 days (control), 3.5 days (preexposure to VRC), and 3 days (preexposure to ISAV), respectively (Fig. 1A). Upon infection with strain 8171, 3-day (no preexposure or preexposure to VRC) and 4-day (preexposure to ISAV) MSTs were observed (Fig. 1B).

In line with earlier findings (7, 9, 10), four passages of *R. arrhizus* 969 on VRC-supplemented plates led to reduced MST (2 days versus 4 days) and 7-day survival (10% versus 32%, $P < 0.001$) of infected WT flies compared to infection with the nonexposed control (Fig. 1C). Similarly, preexposure of *R. arrhizus* 969 to ISAV resulted in a 2-day MST and 87% mortality of infected flies during the 7-day observation period ($P = 0.011$). To preclude a strain-specific phenotype, these findings were corroborated using a second isolate (isolate 749), revealing an even more prominent decline in MST (2 versus 5 days) and 7-day survival rates (4 and 6% versus 36%) of infected flies upon fungal preexposure to VRC or ISAV (Fig. 1D). In addition, two *Mucorales* isolates from other genera were used to evaluate intergenus differences in VRC- and ISAV-induced hypervirulence. In *R. pusillus* 449, preexposure to either drug reduced the MST from 5 to 3 days, and the 7-day survival rates dropped from 32 to 9% (VRC) and 12% (ISAV), respectively (Fig. 1E). Similarly, VRC- and ISAV-exposed *M. circinelloides* caused significantly declined MST (2 versus 5 days) and a 7-day survival compared to the nonexposed serially passaged control (Fig. 1F).

To assess whether ISAV and VRC differ in their potential to cause hypervirulence

depending on the duration of exposure, we infected WT flies with serially passaged *R. arrhizus* 749 after each of four subsequent passages. Exposure to both drugs gradually increased the gap in 3- and 7-day survival rates compared to nonexposed *R. arrhizus* 749, reaching statistical significance after three passages and 100% 7-day mortality after four passages (Fig. 2).

In summary, these results indicate that, similar to VRC, ISAV induces a hypervirulent phenotype of Mucorales, whereas the virulence of *A. fumigatus* remains unaltered by ISAV. This observation, based on experiments in four *Mucorales* strains from three different species, may contribute to the explanation of breakthrough mucormycosis in patients receiving ISAV prophylaxis or therapy (5). Importantly, sufficient concordance of comparative virulence studies in the *Drosophila* model and mammalian hosts has been previously documented in experimental mucormycosis (7). The mechanism of VRC- and ISAV-associated hypervirulence of *Mucorales* remains to be determined. Based on the observation of attenuating hypervirulence upon termination of VRC exposure (7), complex epigenetic alterations, possibly associated with the plasticity of the ergosterol biosynthetic pathway, may be operative, resulting in accumulation of alternative sterols in the fungal cell membrane and leading to increased adherence to the extracellular matrix (7). In addition to the finding of enhanced virulence, further *in vivo* studies are warranted to explore whether exposure of *Mucorales* to ISAV results in a higher risk of diminished efficacy of subsequent therapy with other triazoles and other classes of antifungal drugs.

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