Title:

Isavuconazole activity against emerging fungal pathogens with reduced azole-susceptibility: *Aspergillus lentulus*, *Neosartorya udagawae* and *Cryptococcus gattii*

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Running title:

Isavuconazole susceptibility of *Aspergillus* & *C. gattii*

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Abstract (Words: 48)

Isavuconazole is a second-generation extended-spectrum triazole with *in vitro* activity against a wide variety of fungal pathogens. Clinical isolates of molds *Aspergillus lentulus*, *Neosartorya udagawae* and yeast *Cryptococcus gattii* VGII (implicated in the US Pacific Northwest outbreak) exhibit reduced susceptibilities to several azoles, but higher susceptibility to isavuconazole.

Introduction:

As more triazole antifungals have become available for clinical use, we have appreciated increased variability in *in vitro* susceptibilities. This has been particularly notable for specific members of the *Aspergillus fumigatus* Section *Fumigati*, such as *Aspergillus lentulus* (1), *Neosartorya udagawae* (2), and *N. pseudofischeri* (3). These genetically distinct molds are often misidentified as *A. fumigatus* (2, 4-6). Previously unrecognized as pathogenic, these species are now implicated in invasive mycoses globally. Studies have identified *A. lentulus* among culture collections in the Netherlands (7), Australia (8), Japan (4), and Spain (5, 9). In Spain, *A. lentulus* was a cause of invasive infection in 14 of 28 samples previously identified as *A. fumigatus* (5). *Neosartorya udagawae* has been implicated in invasive aspergillosis, with a peculiar predilection for disease in patients with chronic granulomatous disease (10).

Cryptococcus gattii (*Cg*) has recently emerged as a significant mammalian pathogen in the Pacific Northwest (PNW) of the United States and Canada (11). *Cg* is further distinguished into four molecular types designated VGI, VGII, VGIII, and VGIV (12-14). The molecular types are of epidemiological significance; VGII (a.k.a. AFLP6) has been implicated in the PNW outbreak, and VGIIa and VGIIb are considered, respectively, the major and minor outbreak types, along with the emergence of a novel genotype, VGIIc (14). *Cg* clinical and environmental isolates demonstrate variable susceptibilities to several classes of antifungals (15, 16), with a high proportion of isolates from the PNW showing lower susceptibilities to fluconazole (17).

Isavuconazole, a second-generation broad-spectrum triazole antifungal, is active *in vitro* against a large number of clinically important fungal pathogens (18). It is currently in phase III clinical development for treatment of both aspergillosis and candidiasis, as well as other rare fungi. There is a relative paucity of information available regarding isavuconazole activities against these less common, but emerging mold and yeast pathogens that exhibit variable sensitivity to clinically available antifungals.

Methods, Results and Discussion:

Antifungal susceptibilities were determined by the broth microdilution methods outlined in documents M38-A2 (molds) (19) and M27-A3 (yeasts) (20). For Aspergilli, minimum inhibitory concentration (MIC) endpoints of 100% inhibition (no discernible growth) were determined at 48h (19). For *Cg* isolates, fluconazole, voriconazole, itraconazole (20), and isavuconazole MICs (20, 21) were determined at 72h, using an endpoint of ≥50% reduction in growth relative to drug-free growth controls (20). The MICs were derived from two independent assays; the replicate values were identical or within one dilution. Using analysis functions of Microsoft Excel, Geometric Mean MICs, Mode MIC [the most

frequent MIC value for isolates tested; (22)], MIC_{50} (median MIC, or MIC value at which 50% of isolates tested are inhibited) and MIC_{90} (90^{th} percentile, or MIC value at which 90% of isolates tested are inhibited) were computed for each antifungal; MIC_{50}/MIC_{90} values, if intermediate, were presented as the next tested concentration on the antifungal dilution-series (23). The antifungals were obtained from manufacturers as follows: isavuconazole (Basilea Pharmaceutica International, Basel, Switzerland), voriconazole (Pfizer Inc., New York, NY), itraconazole, fluconazole, and Amphotericin B (all from Sigma-Aldrich, St. Louis, MO).

We tested 15 clinical isolates of *A. lentulus* (24), 9 clinical and 1 environmental isolates of *N. udagawae* (2, 24), obtained from various US centers, and one standard strain of *A. fumigatus* (A*f*293) (3) as a comparator. Both *A. lentulus* and *N. udagawae* exhibited relatively decreased sensitivity to itraconazole (Mode MICs 2 and 1μg/ml, respectively) and voriconazole (Mode MIC 1μg/ml, for both) compared to *A. fumigatus* [**Table 1**; see also (25)]. In contrast to these azoles, isavuconazole MICs were lower for *A. lentulus* and *N. udagawae* isolates (Mode MIC 0.25 and 0.125μg/ml, respectively), as well as *A. fumigatus* tested here (**Table 1**) and by others (26). *A. lentulus* also demonstrated decreased susceptibility to amphotericin B [range 0.5-4μg/ml; Mode MIC 2μg/ml; 93.3% of isolates showed MIC of ≥2μg/ml, data not separately shown], corroborating observations by other investigators (2, 27).

We also tested 90 *Cg* VGII isolates, comprised of 58 clinical (human and veterinary) and 14 environmental isolates of *Cg* VGIIa (28), 7 clinical and 1 environmental isolates of VGIIb (28), and 10 clinical isolates of VGIIc (29), from the PNW (Vancouver Island, BC; Washington and Oregon, USA) and California (14, 28, 30, 31). The VGIIa group included type-strains NIH444 (clinical) (30) and CBS7750 (environmental) (32); VGIIa isolates CBS10485 (33), CBS10866 (34) and RKI06/496 (35, 36) were recovered from European patients who traveled to Vancouver Island. *Cg* VGII clinical isolates exhibited a broad range (2-64μg/ml) of MICs for fluconazole, as observed by others (22). Susceptibility of these isolates was relatively higher to voriconazole and itraconazole; isavuconazole MICs were similarly low. *Cg* subtypes VGIIb and VGIIc appeared less susceptible to fluconazole than VGIIa (**Table 1**). These results corroborate well with an earlier study of 169 *C. gattii* isolates collected globally (37). There was no difference in MIC per isolate source (clinical/environmental). MICs of previously reported isolates (31) were within one dilution, except for one (B7394; data not shown).

Like other triazoles, isavuconazole inhibits fungal cytochrome-P450-lanosterol-14α-demethylase (Cyp51) associated with ergosterol biosynthesis (38), thereby destabilizing membrane integrity. However, isavuconazole uniquely possesses a side-arm which presumably offers a better orientation for the triazole ring to interact with fungal Cyp51 heme-

moiety inside its binding pocket. The resultant tight-binding likely provides isavuconazole's enhanced antifungal spectrum, including activity against fungi less sensitive to other azoles (39).

A tandem repeat (TR)/L98H point mutation in the *cyp51a* promoter of *A. fumigatus* is known to reduce its susceptibility to voriconazole (40) and isavuconazole (41). In addition, the role of the Cdr1Bp efflux transporter in non-Cyp51Ap-mediated itraconazole resistance in *A. fumigatus* was recently discovered (42). Efflux mechanisms in *A. lentulus* are yet unknown; however, its Cyp51Ap homolog appears responsible for the decreased sensitivity to azoles (43). Prior studies evaluating *A. lentulus* echinocandin susceptibilities also suggest that non-target characteristics, such as fungal cell-wall composition, architecture, and/or hydrophobicity, may impact antifungal susceptibilities (44). Isavuconazole MICs of *A. lentulus* and *N. udagawae* isolates in this study, *albeit* low, were variable (**Table 1**), and comparable to modal *A. fumigatus* MICs recently reported (26). Future studies are necessary to define the clinical significance of these findings.

Non-azole-target mechanisms may also influence *Cg* susceptibilities. *Cg* homologs of fungal efflux transporters (*Cryptococcus neoformans MDR1* and *AFR1*; *Candida albicans CDR1/CDR2*), expressed in *Saccharomyces cerevisiae*, confer higher fluconazole MICs and lower intracellular accumulation of ^{3}H-fluconazole, independent of *Cg ERG11* (45). High *in vitro* MIC values have been associated with clinical failure (46, 47). These findings have potential clinical implications, since fluconazole remains a recommended standard for treatment of cryptococcosis (48); these guidelines likely need revision based on emerging evidence that VGII *Cg* isolates may exhibit high fluconazole MICs *in vitro*. In our study, isavuconazole, as well as voriconazole and itraconazole, demonstrated *in vitro* antifungal activity that was superior to that of fluconazole (**Table 1**).

A unifying theme among these diverse fungi is the occurrence of closely related species displaying differential antifungal susceptibilities with no corresponding mechanistic explanation. Microbiology laboratories typically do not distinguish between *A. fumigatus* and *A. lentulus*, or identify species of *Cryptococcus* isolates. These findings supplement an increasing literature suggesting that more detailed species identification (including *Cg* genotyping) may better guide therapeutic decisions.

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Table 1: Summary of drug sensitivity of all Section *Fumigati and Cg VGII isolates**

		Mode MIC (µg/ml)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	MIC Range (µg/ml)	Geometric Mean MIC (µg/ml)
A. lentulus						
(n=15)	Itraconazole	1	1	2	(0.5-2)	1.097
	Voriconazole	2	2	2	(0.5-2)	1.516
	Isavuconazole	0.25	0.25	0.25	(0.063-0.5)	0.188
N. udagawae						
(n=10)	Itraconazole	1	1	1	(0.25-1)	0.660
	Voriconazole	1	1	1	(0.25-1)	0.812
	Isavuconazole	0.125	0.125	0.25	(0.031-0.25)	0.100
***Cg* all**						
(n=90)	Fluconazole	4	4	8	(2-64)	4.560
	Itraconazole	0.125	0.125	0.5	(0.031-0.5)	0.187
	Voriconazole	0.125	0.125	0.125	(0.031-0.5)	0.093
	Isavuconazole	0.031	0.063	0.125	(0.031-0.5)	0.057
VG IIa						
(n=72)	Fluconazole	4	4	8	(2-16)	3.849
	Itraconazole	0.125	0.125	0.5	(0.031-0.5)	0.177
	Voriconazole	0.125	0.125	0.125	(0.031-0.25)	0.088
	Isavuconazole	0.031	0.031	0.063	(0.031-0.125)	0.046
VG IIb						
(n=8)	Fluconazole	8	8	64	(4-64)	8.724
	Itraconazole	0.125	0.25	0.5	(0.125-0.5)	0.210
	Voriconazole	0.031	0.063	0.125	(0.031-0.125)	0.063
	Isavuconazole	0.063	0.125	0.25	(0.031-0.25)	0.088
VG IIc						
(n=10)	Fluconazole	8	8	32	(4-32)	9.190
	Itraconazole	0.25	0.25	0.5	(0.125-0.5)	0.250
	Voriconazole	0.25	0.25	0.5	(0.063-0.5)	0.189
	Isavuconazole	0.25	0.25	0.5	(0.125-0.5)	0.203

Mode MIC: Most frequently occurring MIC value in whole population tested

MIC_{50}: MIC value at which $\geq$50% of all isolates are inhibited; identical to median MIC

MIC_{90}: MIC value at which $\geq$90% of all isolates are inhibited

* MICs of single *A. fumigatus* isolate: 0.25µg/ml (voriconazole and itraconazole), 0.063µg/ml (isavuconazole)