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ORIGINAL RESEARCH

In vitro Activity of Isavuconazole and Comparators Against Clinical Isolates of Molds from a Multicenter Study in China

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Purpose: Monitoring antifungal susceptibility patterns for new or established antifungals is imperative. Antifungal resistance is frequent in molds, frequently leading to invasive mold infections (IMIs) in immunocompromised patients with high morbidity and mortality. Limited availability of effective antifungals for treatment of IMIs in China is an enormous challenge. The purpose of this study was to monitor in vitro antifungal resistance profiles of mold isolates from China, with a particular focus on evaluating in vitro isavuconazole (ISA) activity against these isolates, contributing to the treatment guidance in clinics.

Methods: We evaluated the in vitro activity of ISA and its comparators (voriconazole [VOR] and amphotericin B [AMB]) against 131 clinical isolates of *Aspergillus* spp. (n = 105) and *Mucorales* order (n = 26) collected between 2017 and 2020 from China.

Results: ISA and VOR exhibited similar in vitro activity against *Aspergillus* spp., with minimum inhibitory concentration $(MIC)_{50}$ of 1 μg/mL and MIC_{90} of 2 μg/mL, respectively. Overall, AMB was less active than azoles against *Aspergillus* spp. (MIC_{50}: 4 μg/mL, MIC_{90}: 8 μg/mL). Against the *Mucorales* order, ISA demonstrated MIC_{50} of 0.5 μg/mL and MIC_{90} of 1 μg/mL; however, one strain each of *Mucor circinelloides* and *Syncephalastrum racemosum* were resistant to ISA (MICs: >8 μg/mL). VOR exhibited little or no activity (MIC_{50}: 8 μg/mL, MIC_{90}: >8 μg/mL) against the *Mucorales* order, whereas AMB had MIC_{50} and MIC_{90} of 1 μg/mL.

Conclusion: This was the first multicenter, in vitro study conducted in China and demonstrated the excellent activities of ISA against most species of the *Mucorales* order. MIC indicated an advantage over currently available azole antifungals, positioning ISA as a potential alternative to VOR for clinical management of IMIs. As with other antimicrobials, clinicians should employ stewardship and best practices in relation to potential resistance to new azole antifungals.

Keywords: *Mucorales* order, *Aspergillus* species, antifungal susceptibility testing, isavuconazole, resistance

Introduction

Invasive mold infections (IMIs), including invasive aspergillosis (IA) and mucormycosis, cause considerable morbidity, mortality, and economic burden predominantly among immunocompromised individuals worldwide.[1–3] IMIs mainly affect immunocompromised patients such as those with hematological malignancies, organ transplants, and those prescribed long-term steroid therapy.[4–6] Besides, mucormycosis is also involved in angio-invasion with higher mortality than IA; and infection is increasingly reported in patients with uncontrolled diabetes mellitus.[3,6–8]

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Like other countries, China is also facing an increase in the incidence of IMIs due to rapid economic development and an aging population. This increases the burden on public health and socio-economic systems, and raises health-care costs.[3,8] Azoles, mainly voriconazole (VOR), are recommended as primary antifungals for IA treatment.[9] However, the emergence of azole-resistant *Aspergillus fumigatus* is gradually being reported worldwide and is associated with point mutations, particularly in the *cyp51* gene.[10,11] Amphotericin B (AMB) formulations are used as the mainstay of treatment for mucormycosis,[12] despite being associated with a high frequency of treatment-related adverse effects such as nephrotoxicity.[13] Therefore, the treatment of IMIs remains a huge challenge worldwide.

The extended-spectrum triazole antifungal, isavuconazole (ISA), was approved in 2015 by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of IA and mucormycosis.[14,15] Previous studies have reported the excellent activity of ISA against a broad range of yeasts and molds, with lower toxicity than VOR and fewer drug-drug interactions than other azoles.[16,17] Considering that ISA is currently unavailable in China, studies on in vitro antifungal activity of ISA against yeast and mold isolates from the region are lacking. We participated in the 2020 Analysis of Resistance in Antifungals (ARIA) Study conducted by IHMA, Europe Sarl. This was the first multicenter study conducted in China to evaluate the in vitro antifungal activity of ISA and its comparators (VOR and AMB) against molds including those belonging to *Aspergillus* spp. and the *Mucorales* order. The purpose of the current study was to monitor in vitro antifungal resistance profiles of mold isolates from China between 2017 and 2020, with a particular focus on evaluating in vitro ISA activity against these isolates to contribute to the treatment guidance in the clinics. In those species from which resistant isolates were identified, genetic sequencing along with an alignment for known genetic determinants of resistance (CYP 51s) with reference strains was performed to identify potential associations with ISA resistance.

Materials and Methods

Ethics Statement

This study was approved by the Human Research Ethics Committee of Peking Union Medical College Hospital (HS-2468). An informed consent waiver was granted by Peking Union Medical College Hospital prior to ethics approval, since only in-vitro susceptibility testing of strains isolated from patients was conducted, and no patient-specific information was collected. The results were only used for evaluation of antifungal agents, and no clinical diagnoses were made.

Organisms

A total of 131 clinical filamentous isolates were collected from 2017–2020 under the China Hospital Invasive Fungal Surveillance Net – North China Program (CHIF-NET). These strains were originally isolated from a total of 8 participating regions of northern China, including Beijing, Tianjin, Inner Mongolia, Hebei, Heilongjiang, Jilin, Liaoning, and Shanxi Provinces. All strains were sub-cultured on Sabouraud dextrose agar (SDA) (Thermo Fisher Scientific) and incubated at 28°C for 3–7 days.

Species Identification

All isolates were identified by combining conventional morphological methods and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), using VITEK® MS (bioMérieux, Marcy-l'Étoile, France) and M-Discover 100 MALDI-TOF MS (Zhuhai Meihua Medical Technology Co., Ltd). One unidentified isolate and two *Mucor* spp. strains were subsequently identified using molecular sequencing of the internal transcribed spacer (ITS) and/or D1/D2 regions of ribosomal DNA. Nucleotide sequences were then homologically aligned with reliable sequences in the GenBank database (https://www.blast.ncbi.nlm.nih.gov/Blast.cgi) using two reference sequences of *M. circinelloides* 1006PhL (*CYP51F1* and *CYP51F5* genes).

Antifungal Susceptibility Testing

In vitro antifungal susceptibility testing of the 131 isolates was performed by the Department of Clinical Laboratory, Peking Union Medical College Hospital (PUMCH), China using the Clinical and Laboratory Standards Institute (CLSI)

broth microdilution method (BMD) as described in the CLSI standard M38-A3.[18] Frozen 96-well panels provided by IHMA containing three antifungal agents: ISA (0.008–8 μg/mL), VOR (0.008–8 μg/mL) and AMB (0.06–8 μg/mL) were used. After these strains were cultured on SDA and incubated at 28°C for 3–7 days, an adequate amount of conidia was collected using 1 mL of sterile saline with 0.1% Tween-20 (Sigma: P1379). Subsequently, the turbidity of the stock suspension was adjusted to an A_{530} of between 0.09 and 0.13 for *Aspergillus* spp. or 0.15 and 0.17 for *Mucorales* order.[18] Thereafter, the suspension was diluted 50-fold in Roswell Park Memorial Institute (RPMI) 1640 (+MOPS) culture medium, after which 100 μL of inoculum was added to the wells of the antifungal panels to give a final inoculum between approximately 2×10^3 to 2.5×10^4 colony forming unit (CFU)/mL and the desired test concentrations of antifungal agents in a total volume of 200 μL.[18]

The minimum inhibitory concentration (MIC), 50% MIC (MIC_{50}) and 90% MIC (MIC_{90}) were determined as defined in the CLSI M38-A3 document.[18] For *Aspergillus* spp., the MICs for ISA and its comparators were observed after 46–50 h of incubation at 35°C. Due to the rapid growth of *Mucorales* isolates, the incubation time was shorter, and MICs for ISA and its comparators were recorded between 21 h and 26 h.[18] *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, *Aspergillus flavus* ATCC®204304™ and *A. fumigatus* ATCC®MYA-3627™ were used as quality control and reference strains.[19]

Analysis of *Mucor circinelloides* CYP51s (Sterol 14-Demethylase) Amino Acid Sequences

The homologous sequences of *Mucor circinelloides CYP51s* (Gene ID: HMPREF1544_03888.1 and HMPREF1544_08704.1) were obtained from FungiDB database (http://FungiDB.org). The paralogs of *CYP51s* were named *CYP51F5* or *CYP51F1* according to Nelson's database nomenclature.[20] Using the BLAST program (tblastn), *M. circinelloides* 1006PhL was identified as the reference strain from *Mucor* species (GenBank No. AOCY00000000.1); and the amino acid sequences of *A. fumigatus* CYP51A (GenBank protein ID: AAK73659.1) and CYP51B (GenBank protein ID: AAK73660.1) were used as query sequences. Species-specific primer sequences are presented in Table 1. The sequences of *CYP51F5* and *CYP51F1* were amplified by Beijing Ruibio BioTech Co., Ltd (Beijing, China). Subsequently, CYP51s amino acids sequences of *M. circinelloides* strains (M35 and M49) were translated using CLC Sequence Viewer software (version 8.0).

To find potential substitutions of CYP51s amino acids likely affecting azole resistance in *M. circinelloides*, alignment using *Saccharomyces cerevisiae* S288C Erg11 (reference strain; CYP51 sterol 14-demethylase) was compared with the CYP51s amino acids sequences of *M. circinelloides* strains (M35 and M49).

Data Analysis

Although several studies have evaluated epidemiological cut-off values (ECVs) for AMB, posaconazole, and itraconazole against a limited number of isolates of *Mucorales* order,[21] ECV values have not been formally established for ISA and VOR against isolates of *Mucorales* order or may be inapplicable to the interpretation of drug susceptibilities in China due to lack of clinical data in the population from China. Therefore, the MIC_{50} and MIC_{90} were calculated for evaluating in vitro activities of these antifungal agents against isolates of *Mucorales* order. However, ECVs are formally established for VOR, ISA, and AMB against some major *Aspergillus* spp., such as *A. fumigatus, A. flavus, A. niger*, and *A. terreus*[22] and were used for susceptibility testing of these organisms.

Results

Species Distribution

A total of 131 clinical isolates were included, comprising 4 different *Aspergillus* complexes (n = 105) and 5 different species of the *Mucorales* order (n = 26) (Table 2). The *A. fumigatus* complex consisted of 71.4% (75/105) of all *Aspergillus* strains, followed by *A. niger* complexes (10.5%, 11/105), *A. flavus* complexes (9.5%, 10/105), and *A. terreus* complexes (8.6%, 9/105). For strains from the *Mucorales* order, the predominant genus was *Rhizopus* (50%, 13/26), of which 84.6% (11/13) were *R. oryzae/R. arrhizus*, followed by *R. microspores* and *R. stolonifera* (1 isolate each). Other

Table 1 Molecular Description of *CYP51* Homologous Genes Related to Azoles-Resistance in *Mucor circinelloides*

Genes	Gene ID	Location	Total Length (nt)	CDS Length (nt)	Total Length (aa)	*A. fumigatus* Homologues Genes	*A. fumigatus* Homologues Proteins	Functions
CYP51F1	HMPREF1544_03888	KE123937 :33,460.35,232(+)	1773	1539	512	*CYP51B* (GenBank: AF338660.1)	CYP51B (Protein ID: AAK73660.1)	Lanosterol 14-alpha demethylase
CYP51F5	HMPREF1544_08704	KE124036: 54,965.56,622(+)	1658	1533	510	*CYP51A* (GenBank: AF338659.1)	CYP51A (Protein ID: AAK73659.1)	Lanosterol 14-alpha demethylase

common genera were *Rhizomucor* spp. and *Lichtheimia* spp. (15.4%, 4/26 each). For *Rhizomucor* spp., *R. pusillus* isolates were the most prevalent (n = 3), followed by *R. miehei* (n = 1), which were identified by using molecular sequencing. For *Lichtheimia* spp., all isolates were identified as *Lichtheimia ramosa* (n = 4). *Syncephalastrum* spp.

Table 2 MIC Distributions for Isavuconazole Against *Aspergillus* spp. and Species of *Mucorales* Order Using CLSI Broth Microdilution Methods

Species (No. Tested)	No. of Isolates at MIC (μg/mL)												
	≤0.008	0.016	0.03	<0.06	0.06	0.12	0.25	0.5	1	2	4	8	>8
***Aspergillus* spp. (105)**					1	5	1	26	**56**	15	1		
A. fumigatus complex (75)								24	**45**	6			
A. niger complex (11)									3	**7**	1		
A. flavus complex(10)									**8**	2			
A. terreus complex (9)					1	**5**	1	2					
***Mucorales* (26)**					2		3	**9**	8	2		1	1
***Mucor* spp. (2)**								1				1	
Mucor circinelloides (2)								1				1	
***Rhizopus* spp. (13)**					1		2	**4**	5	1			
R. oryzae/R. arrhizus (11)					1		1	3	**5**	1			
R. microsporus (1)								1					
R. stolonifer (1)							1						
***Rhizomucor* spp. (4)**							1	1	2				
Rhizomucor pusillus (3)							1		**2**				
Rhizomucor miehei (1)								1					
***Syncephalastrum* spp. (3)**								**2**					1
Syncephalastrum racemosum (3)								**2**					1
***Lichtheimia* spp. (4)**					1			1	1	1			
Lichtheimia ramosa (4)					1			1	1	1			

Note: Numbers in bold are median MIC values.
Abbreviations: CLSI, Clinical and Laboratory Standards Institute; MIC, minimum inhibitory concentration.

comprised 11.5% (3/26) of all strains from the *Mucorales* order, and all of them were identified as *S. racemosum*. *Mucor* spp. comprised only 2 isolates, which were identified as *M. circinelloides*.

Specimen Distribution

The lower respiratory tract (LRT, i.e., sputum, bronchoalveolar lavage fluid, tracheal aspirate) (75.6%, 99/131) was the primary culture source, followed by secretions (9.2%, 12/131), swabs (i.e., ear, nasopharyngeal, cornea, and skin 6.9%, 9/131), bodily fluids (i.e., blood, pleural fluid, cerebrospinal fluid, and abdominal dialysate; 3.8%, 5/131), pus (n = 3), tissue (n = 2), and stool (n = 1). Of note, more than half (61.5%, 16/26) of the isolates of *Mucorales* order originated from LRT, with sputum (n = 13) as the most frequent culture source. Of all the isolates of *R. arrhizus*, one was cultured from blood.

Antifungal Susceptibilities

Isavuconazole Activity Against *Aspergillus* spp. and *Mucorales* Order

MIC distributions for ISA against *Aspergillus* spp. and species of *Mucorales* order are shown (Table 2). Among the *Aspergillus* isolates, MICs for ISA ranged from 0.06–4 μg/mL (Table 3). The median MIC (mMIC) for ISA among all *Aspergillus* spp. was 1 μg/mL, with a low mMIC of 0.12 μg/mL for *A. terreus* complexes, a high mMIC of 2 μg/mL for *A. niger* complexes, and the same mMIC value of 1 μg/mL for *A. fumigatus* complexes (constituted the majority of the isolates identified) and *A. flavus* complexes (Table 3). Based on the ECVs available, a large proportion of *Aspergillus* strains in this study were wild-type (WT; MIC ≤ECV; range, 80.0–100.0%) (Table 3).

In this study, the activity of ISA against isolates of *Mucorales* order was slightly higher than observed for *Aspergillus* spp. (MIC_{50}: 1 μg/mL), with a lower MIC_{50} at 0.5 μg/mL (Table 3). Compared with *Aspergillus* spp., a lower mMIC value of 0.5–0.75 μg/mL was observed for *Rhizopus* spp., *Rhizomucor* spp., *Syncephalastrum* spp., and *Lichtheimia* spp. (Table 3). Higher mMIC values of 1 μg/mL were observed for *R. oryzae/R. arrhizus* and *R. pusillus* (Table 3).

Activity of Isavuconazole and Comparators (Voriconazole and Amphotericin B Against *Aspergillus* spp. and *Mucorales* Order

All MICs of quality control (QC; *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258) and reference strains (*A. flavus, A. fumigatus*) were within expected limits (Supplementary Table 1). MIC distributions for VOR and AMB against *Aspergillus* spp. and species of *Mucorales* order are shown (Tables 4 and 5). The overall MIC_{50} and MIC_{90} for both azoles against *Aspergillus* spp. (n = 105) were 1 μg/mL and 2 μg/mL, respectively (Table 3). WT susceptibility to ISA was 80% and to VOR was 100% among *A. flavus, A. niger*, and *A. terreus* complexes. MIC_{90} for both azoles was lower (1 μg/mL) against *A. fumigatus* complexes (n = 75) with a WT susceptibility of 90%. Of all *Aspergillus* spp. studied, *A. terreus* complex (n = 9) was the most susceptible to ISA and VOR with an MIC_{50} of 0.12 μg/mL and MIC_{90} of 0.5 μg/mL. AMB was less active than both azoles against *Aspergillus* spp., with an MIC_{50} of 4 μg/mL and MIC_{90} of 8 μg/mL, particularly against *A. fumigatus* complexes (89.3% of non-WT were less susceptible).

Compared with *Aspergillus* spp., ISA demonstrated better activity against the isolates of *Mucorales* order (n = 26) and *Rhizopus* spp. with an MIC_{50} of 0.5 μg/mL (Table 3). However, VOR lacked any meaningful activity against the *Mucorales* order with both MIC_{50} and MIC_{90} values of >8 μg/mL across all species (Table 3).

Overall, from in vitro data, AMB was the most active fungistatic-agent against the *Mucorales* order with an MIC range of 0.12–2 μg/mL, compared with an MIC range of 0.25–>8 μg/mL for *Aspergillus* spp. (Table 3). Additionally, MIC_{50} and MIC_{90} values for *Mucorales* order were much lower (1 μg/mL) than that of *Aspergillus* spp. (MIC_{50}: 4 μg/mL; MIC_{90}: 8 μg/mL) (Table 3). Both strains of *M. circinelloides* (M35 and M49) studied were resistant to VOR (MIC >8 μg/mL). However, ISA demonstrated strong activity against *M. circinelloides*-M49 (MIC = 0.5 μg/mL) and no activity against *M. circinelloides*-M35 (MIC = 8 μg/mL) (Table 3). Notably, among three strains of *S. racemosum* included, ISA demonstrated an MIC of 0.5 μg/mL for two strains; and no in vitro activity against one of strain with an MIC of >8 μg/mL (Table 3).

Table 3 Antifungal Activity of Isavuconazole and Comparator Antifungal Agents (Voriconazole and Amphotericin B) Against *Aspergillus* spp. and Species of *Mucorales* Order Collected in China Between 2017 and 2020

Antifungal Drugs	ISA (μg/mL)							VOR (μg/mL)							AMB (μg/mL)						
Genus (No. of Isolates Tested)	**MIC Range**	**mMIC**	**MIC**	**MIC_{50}**	**MIC_{90}**	**WT* (%)**	**Non-WT* (%)**	**MIC Range**	**mMIC**	**MIC**	**MIC_{50}**	**MIC_{90}**	**WT* (%)**	**Non-WT* (%)**	**MIC Range**	**mMIC**	**MIC**	**MIC_{50}**	**MIC_{90}**	**WT* (%)**	**Non-WT* (%)**
***Aspergillus* spp. (105)**	**0.06–4**	**1**	**–**	**1**	**2**	**–**	**–**	**0.06–2**	**1**	**–**	**1**	**2**	**–**	**–**	**0.25–>8**	**4**	**–**	**4**	**8**	**–**	**–**
A. fumigatus complex (75)	0.5–2	1	–	1	1	69 (92)	6 (8)	0.25–2	1	–	1	1	70 (93.3)	5 (6.7)	2–8	4	–	4	8	8 (10.7)	67 (89.3)
A. niger complex (11)	1–4	2	–	2	2	11 (100)	0	0.5–2	1	–	1	2	11 (100)	0	2–4	2	–	2	4	7 (63.6)	4 (36.4)
A. flavus complex (10)	1–2	1	–	1	2	8 (80)	2 (20)	1–2	1	–	1	2	10 (100)	0	4–8	4	–	4	8	6 (60)	4 (40)
A. terreus complex (9)	0.06–0.5	0.12	–	0.12	0.5	9 (100)	0	0.06–0.5	0.12	–	0.12	0.5	9 (100)	0	0.25–>8	2	–	2	>8	8 (88.9)	1 (11.1)
***Mucorales* (26)**	**0.06–>8**	**0.5**	**–**	**0.5**	**2**	**–**	**–**	**4–>8**	**>8**	**–**	**>8**	**>8**	**–**	**–**	**0.12–2**	**1**	**–**	**1**	**1**	**–**	**–**
***Rhizopus* spp. (13)**	**0.06–2**	**0.5**	**–**	**0.5**	**1**	**–**	**–**	**4–>8**	**8**	**–**	**8**	**>8**	**–**	**–**	**0.25–2**	**1**	**–**	**1**	**2**	**–**	**–**
R. oryzae/*R. arrhizus* (11)	0.06–2	1	–	1	1	**–**	**–**	4–>8	8	–	**8**	**>8**	**–**	**–**	0.25–2	1	**–**	**1**	**2**	**–**	**–**
R. microsporus (1)[#]	–	–	0.5					–	–	>8					–	–	0.5				
R. stolonifer (1) [#]	–	–	0.3					–	–	4					–	–	1				
***Rhizomucor* spp. (4)[#]**	**0.25–1**	**0.75**	**–**					**>8**	**>8**	**–**					**0.5–1**	**0.75**	**–**				
Rhizomucor pusillus (3)[#]	0.25–1	1	–					>8	>8	–					0.5–1	0.5	–				
Rhizomucor miehei (1)[#]	–	–	0.5					–	–	8					–	–	1				
***Mucor* spp. (2)[#]**	**0.5–8**	**4.25**	**–**					**>8**	**>8**	**–**					**0.5–1**	**1**	**–**				
Mucor circinelloides (2)[#]	0.5–8	4.25						>8	>8	–					0.5–1	1	–				
Mucor circinelloides-M49	–	–	0.5					–	–	>8					–	–	0.5				
Mucor circinelloides-M35	–	–	8					–	–	>8					–	–	1				
***Syncephalastrum* spp. (3)[#]**	**0.5–>8**	**0.5**	**–**					**>8**	**>8**	**–**					**0.25–1**	**0.5**	**–**				
Syncephalastrum racemosum (3)[#]	0.5–>8	0.5	–					>8	>8	–					0.25–1	0.5	–				
***Lichtheimia* spp. (4)[#]**	**0.06–2**	**0.75**	**–**					**4–>8**	**>6**	**–**					**0.12–1**	**0.75**	**–**				
Lichtheimia ramosa (4)[#]	0.06–2	0.75	–					4–>8	>6	–					0.12–1	0.75	–				

Notes: *Based on epidemiological cut-off values published by CLSI;[22] values reported using WT and non-WT for the mentioned target drug because no breakpoints available on *Aspergillus* species from CLSI.[22] [#]The number of the remaining isolates for *Mucorales* order was too low to calculate the MIC_{50} and MIC_{90}, and ECVs are also unavailable for *Mucorales* order from CLSI,[22] hence other isolates for *Mucorales* order are not presented.

Abbreviations: ISA, isavuconazole; VOR, voriconazole; AMB, amphotericin B; MIC, minimum inhibitory concentration; mMIC, median MIC; MIC_{50}, 50% minimum inhibitory concentration; MIC_{90}, 90% minimum inhibitory concentration; WT, wild-type.

Table 4 MIC Distributions for Voriconazole Against *Aspergillus* spp. and Species of *Mucorales* Order Using CLSI Broth Microdilution Methods

Species (No. Tested)	No. of Isolates at MIC (μg/mL)												
	≤0.008	0.016	0.03	<0.06	0.06	0.12	0.25	0.5	1	2	4	8	>8
***Aspergillus* spp. (105)**					2	4	4	33	**52**	10			
A. fumigatus complex (75)							2	31	**37**	5			
A. niger complex(11)								1	**7**	3			
A. flavus complex (10)									**8**	2			
A. terreus complex (9)					2	**4**	2	1					
***Mucorales* (26)**											5	5	**16**
***Mucor* spp. (2)**													**2**
Mucor circinelloides (2)													**2**
***Rhizopus* spp. (13)**											3	**4**	6
R. oryzae/R. arrhizus (11)											2	**4**	5
R. microsporus (1)													1
R. stolonifer (1)											1		
***Rhizomucor* spp. (4)**												1	**3**
Rhizomucor pusillus (3)													**3**
Rhizomucor miehei (1)												1	
***Syncephalastrum* spp. (3)**													**3**
Syncephalastrum racemosum (3)													**3**
***Lichtheimia* spp. (4)**											2		2
Lichtheimia ramosa (4)											2		2

Note: Numbers in bold are median MIC values.
Abbreviations: CLSI, Clinical and Laboratory Standards Institute; MIC, minimum inhibitory concentration.

Genetic Alignment of CYP51s (*M. circinelloides* Strains)

Molecular description of *CYP 51* homologous genes (*CYP51F5* and *CYP51F1*) in *M. circinelloides* is summarized in Table 1. *CYP51F5* and *CYP51F1* were originally acquired from *A. fumigatus CYP51A* and *CYP51B*, with more than 40% similarity in amino acid sequences. Nucleotide sequences were 1773 bp and 1658 bp and protein sequences were 512 and 510 amino acids in *CYP51F1* and *CYP51F5*, respectively.

We tested if there was a Y129F substitution or other amino acid mutations existed in *M. circinelloides* CYP51s which possibly affected their intrinsic resistance to ISA and VOR.

Consensus sequences for CYP51F1 and CYP51F5 were aligned against each other. The CYP51s of *M. circinelloides*-M35, *M. circinelloides*-M49, and Erg11 of *S. cerevisiae* S288C were screened for amino acid substitutions previously and confirmed as correlation to resistance to short-tailed azoles (e.g. VOR) in different fungal species (analogous to Y140F in *S. cerevisiae*).[23,24] As depicted in Figure 1, Erg11 of *S. cerevisiae* S288C and CYP51F1s of both strains of *M. circinelloides* retained a tyrosine residue (alignment of Y140 for *S. cerevisiae* and Y130 for *M. circinelloides*). On the contrary, alignment identified a phenylalanine 129 substitution (Y129F, Y140F *S. cerevisiae*) conserved in CYP51F5s of both *M. circinelloides*. There was another V (valine) to A (alanine)

Table 5 MIC Distributions for Amphotericin B Against *Aspergillus* spp. and Species of *Mucorales* Order Using CLSI Broth Microdilution Methods

Species (No. Tested)	No. of Isolates at MIC (μg/mL)												
	≤0.008	0.016	0.03	<0.06	0.06	0.12	0.25	0.5	1	2	4	8	>8
***Aspergillus* spp. (105)**							1		1	18	**62**	22	1
A. fumigatus complex (75)										8	**49**		18
A. niger complex (11)										**7**	4		
A. flavus complex (10)											**6**	4	
A. terreus complex (9)							1		1	**3**	3		1
***Mucorales* (26)**						1	2	7	**14**	2			
***Mucor* spp. (2)**								1	1				
Mucor circinelloides (2)								1	1				
***Rhizopus* spp. (13)**							1	2	**8**	2			
R. oryzae/R. arrhizus (11)							1	1	**7**	2			
R. microsporus (1)								1					
R. stolonifer (1)									1				
***Rhizomucor* spp. (4)**								2	2				
Rhizomucor pusillus (3)								**2**	1				
Rhizomucor miehei (1)									1				
***Syncephalastrum* spp. (3)**							1	**1**	1				
Syncephalastrum racemosum (3)							1	**1**	1				
***Lichtheimia* spp. (4)**						1		1	2				
Lichtheimia ramosa (4)						1		1	2				

Note: Numbers in bold are median MIC values.
Abbreviations: CLSI, Clinical and Laboratory Standards Institute; MIC, minimum inhibitory concentration.

substitution (V311 for *S. cerevisiae*) observed in CYP51F5s (293A for both *M. circinelloides)*. A total of four *CYP51s* sequences of the two *M. circinelloides* strains, M35-*CYP51A*, M49-*CYP51A*, M35-*CYP51B*, and M49-*CYP51B* have been deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/; accession numbers OM976985, OM976986, OM976987, and OM976988).

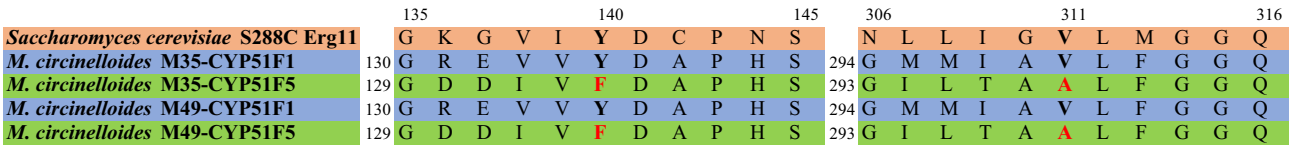

Figure 1 Amino acid sequence alignment of lanosterol 14-α demethylase between *Saccharomyces cerevisiae* S288C and tested *Mucor circinelloides* CYP51s. Alignment using *Saccharomyces cerevisiae* S288C Erg11 (CYP51) as reference, Y140 (a tyrosine residue) is marked in bold, the previously confirmed mutation as correlation to resistance to voriconazole in different fungi species (analogous to Y140F in *S. cerevisiae*), and the change to phenylalanine (129F) is seen in both *M. circinelloides* CYP51F5s, displayed in bold and red. Another amino acid mutation of V (valine) to A (alanine) (position V311 in *S. cerevisiae*, and mutated to 293A for *M. circinelloides* numbering) potentially related to resistance to voriconazole is also observed in both *M. circinelloides* CYP51F5s, displayed in bold and red.

Discussion

Isavuconazole has been indicated for the treatment of IFIs in the USA and Europe since 2015. However, due to limited data from studies in China, it is currently unavailable for the treatment of IFIs in China. This was the first multicenter study to evaluate the in vitro activity of ISA and its comparators (VOR and AMB) against clinical isolates of *Aspergillus* spp. and *Mucorales* order in China.

Both azoles exhibited a low MIC_{50} and MIC_{90} against combined *Aspergillus* spp. However, AMB was less active than the azoles against *Aspergillus* spp. with high MICs. In this study, AMB was less active against *Aspergillus* species, including *A. flavus* complexes, *A. terreus* complexes, and *A. fumigatus* complexes. It is well-documented that the majority of strains from *A. terreus* and *A. flavus* complexes are intrinsically resistant to AMB, and VOR is recommended as the first line of treatment.[25] Additionally, *A. fumigatus* isolates were less susceptible to AMB, with 89.3% of non-WT isolates susceptible to AMB. There are several possibilities for this observation. First, the CLSI range of MICs of AMB to the reference strain (*A. fumigatus* ATCC®MYA-3627™) was from 0.5–4 μg/mL,[19] thus the MIC of AMB against *A. fumigatus* in our study was in the upper limit of CLSI MIC range. Notably, a study from Canada indicated that 96.4% (188/195) of *A. fumigatus* isolates were less susceptible to AMB,[26] which was higher than our study (89.3%). This suggested that widespread AMB resistance might be gradually trending in many parts of the world including Canada and northern areas of China. Since AMB has been the recommended drug of choice for IA treatment, a large-scale and long-term use might result in an increasing trend of resistance to *A. fumigatus*. Given that the AMB susceptibility patterns could vary among geographic/ecological populations, Ashu et al. indicated that local clinicians should understand the limitations of the aforementioned recommendations,[26] and apply stewardship in the management of AMB. However, whether AMB resistance is also common in other parts of China remains to be investigated in a study with a larger sample size from various regions. Azoles demonstrated contrasting activity against isolates from the *Mucorales* order, with an MIC_{50} of 0.5 μg/mL and MIC_{90} of 1 μg/mL for ISA and an MIC_{50} of 8 μg/mL and MIC_{90} of >8 μg/mL for VOR. An MIC_{50} and MIC_{90} of 1 μg/mL was reported for AMB.

Rhizopus spp. has been reported as the most frequent cause of mucormycosis worldwide (~48–66%), followed by *Mucor* spp. (14%) and *Lichtheimia* spp. (13%)[27–29] with *R. arrhizus* being identified as the most common (33%) sub-species.[29] The present study corroborates earlier reports, in that *Rhizopus* spp. was the most predominant cause of mucormycosis, with fewer *Mucor* spp. isolated. Moreover, the sites from which isolates were cultured in the current study (i.e., LRT) were also more frequent in the prior study,[29] as also reported in an epidemiology and susceptibility study conducted in USA.[30]

In addition to an increase in the incidence of IMIs globally and in China,[1–3,8,31] a gradual increase in patients with COVID-19 co-infected with IA or mucormycosis has also been reported.[32–36] The incidence of invasive pulmonary aspergillosis in patients with COVID-19 ranged from 19.4–33.3%.[33] Considering the possibility of combined infections as well as limited availability of antifungal agents in China, development of new antifungal drugs is critical for effective treatment. Many isolates of the *Mucorales* order are resistant to the echinocandin and azole classes of antifungals.[37,38] Besides, due to the disadvantages of the currently recommended VOR and available AMB,[9,39] there is a need for drugs such as ISA which has been developed as a more effective and safer antifungal azole that may be used to treat a wide range of IMIs.[16,17]

In the current study, both ISA and AMB demonstrated considerable activity against most species of the *Mucorales* order and exhibited better activity than VOR. However, similar activities were observed between ISA and VOR against *Aspergillus* spp., with MICs ranging between 0.06–4 μg/mL. Overall, our findings in clinical isolates of molds from China were consistent with studies conducted in the Netherlands/India and Denmark (Table 6) in that ISA (MIC ranges: 0.125–≥8 μg/mL) and AMB (MIC ranges: ≤0.03–1 μg/mL) exhibited notably increased activity compared with VOR (MIC ranges: 1–≥16 μg/mL) against isolates from the *Mucorales* order. Notably, *Rhizopus* spp. strains in China were more susceptible to ISA with MICs between 0.06–2 μg/mL compared with those tested in the Netherlands/India and Denmark with MICs between 0.125–8 μg/mL (Table 6). Strains from the *Mucorales* order such as *M. circinelloides*, isolated from the US demonstrated higher MICs for ISA (2–>16 μg/mL) and AMB (≤0.03–8 μg/mL) compared with those from China (ISA: 0.5–8 μg/mL; AMB: 0.5–1 μg/mL) (Table 6).

Table 6 Review of MICs of Isavuconazole and Comparator Antifungal Agents (Voriconazole and Amphotericin B) MICs of Major *Mucorales* Order Using CLSI Broth Microdilution Methods Reported in Different Countries

Species	Country	No. of Isolates Tested	MIC Range (or MIC) μg/mL		
			ISA	VOR	AMB
Mucor circinelloides	Netherlands/India[42]	5	1–8	8–16	0.03–0.125
	USA[30]	67	2–>16	–	≤0.03–8
	Denmark[40]	13	1–8	1–>16	≤0.03–0.125
	China*	2	0.5–8	>8	0.5–1
Rhizopus arrhizus[#]	Netherlands/India[42]	25	1–8	4–16	0.03–0.5
	USA[3028]	114	0.125–4	–	≤0.03–1
	China*	11	0.06–2	4–>8	0.25–2
Rhizopus oryzae[#]	Denmark[40]	6	0.125–2	4–8	≤0.03–0.25
Rhizopus microsporus	Netherlands/India[42]	23	0.5–8	1–16	0.03–1
	USA[3028]	121	0.125–>16	–	≤0.03–1
	Denmark[40]	26	0.125–1	2–16	≤0.03–0.25
	China*	1	0.5	>8	0.5
Rhizopus stolonifer	Netherlands/India[42]	4	0.25–1	4–8	0.03–0.06
	China*	1	0.25	4	1
Rhizomucor pusillus	Netherlands/India[42]	7	1–8	8–16	0.06–1
	Denmark[40]	8	0.125–1	1–16	≤0.03–0.25
	China*	3	0.25–1	>8	0.5–1
Rhizomucor miehei	Netherlands/India[42]	1	8	16	1
	China*	1	0.5	8	1
Syncephalastrum racemosum	Netherlands/India[42]	15	0.125–8	4–16	0.03–0.5
	USA[3028]	19	0.5–>16	–	≤0.03–1
	China*	3	0.5–>8	>8	0.25–1
Lichtheimia ramosa	Netherlands/India[42]	7	0.5–8	8–16	0.06–1
	USA[30]	22	≤0.03–1	–	0.125–2
	Denmark[40]	4	0.5–2	2–>16	≤0.03
	China*	4	0.06–2	4–>8	0.12–1

Notes: For Europe also CLSI data is presented. *Data from China reported in our study. [#]*R. oryzae*/*R. arrhizus* strains were difficult to distinguish by MALDI-TOF MS, hence combined data are presented for China.

Abbreviations: ISA, isavuconazole; VOR, voriconazole; AMB, amphotericin B; MIC, minimum inhibitory concentration; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry.

Of the two *M. circinelloides* strains identified, M35 was resistant and M49 was sensitive to ISA. These results do not corroborate the results reported by Arendrup et al., in which ISA exhibited in vitro activity against isolates of *Mucorales* order except for *M. circinelloides* (13 isolates evaluated).[40] Another possible contribution to VOR resistance (short-tailed azoles) by V293A (*M. circinelloides*) substitution (V311A, *S. cerevisiae*) was reported in two different functional

analysis and structure-based studies.[41] There was a V (valine) to A (alanine) substitution (V311 for *S. cerevisiae*) observed in CYP51F5s (293A for both *M. circinelloides*). Therefore, results observed in the current study are consistent with a previous study in which the Y129F substitution in *M. circinelloides* CYP51F5 possibly affected its intrinsic resistance to VOR, with the V293A substitution also potentially playing a role. As ISA belongs to mid-tailed azoles,[41] the substitutions on Y129F and V293A in *M. circinelloides* CYP51F5 found in both M35 and M49 did not appear to be associated with ISA resistance in *M. circinelloides*-M35.

Despite *Syncephalastrum* spp. rarely causing mucormycosis and fewer cases reported,[29] ISA resistance in one *S. racemosum* strain was observed in this study. The occurrence of ISA resistance in *M. circinelloides* and *S. racemosum* suggested that there may be species-specific differences in susceptibility patterns of several species of *Mucorales* order. Therefore, rapid identification of infection causing species and improved surveillance is critical to ensure optimal treatment.

Conclusion

ISA exhibited in vitro activity against most clinical isolates from the *Mucorales* order. Among clinical isolates of *Aspergillus* spp., similar MICs were reported for ISA and other azoles (VOR). However, as with all antimicrobial agents, clinicians should employ appropriate stewardship to minimize potential resistance to antifungals, and rationally deploy new antifungals in the treatment of IA and mucormycosis.

Abbreviations

AMB, amphotericin B; BMD, broth microdilution; Clinical and Laboratory Standards Institute, CLSI; IA, invasive aspergillosis; IMI, invasive mold infection; ISA, isavuconazole; ITS, internal transcribed spacer; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; MIC, minimum inhibitory concentration; RPMI, Roswell Park Memorial Institute; SDA, Sabouraud dextrose agar; VOR, voriconazole.

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