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Screening the *in vitro* susceptibility of posaconazole in clinical isolates of *Candida* spp. and *Aspergillus* spp. and analyzing the sequence of *ERG11* or *CYP51A* in non-wild-type isolates from China

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ABSTRACT

The present study was to determine the *in vitro* activity of posaconazole (POS) against 385 *Candida* and 268 *Aspergillus* clinical isolates from China. We found that POS was active against 85.5% *Candida* and 94.4% *Aspergillus* isolates. Non–wild-type (non-WT) phenotype was found in a subset of *Candida albicans* (15.4%), *Candida tropicalis* (11.9%), *Aspergillus fumigatus* (4.1%), and *Aspergillus flavus* (17.4%) isolates. Cross-resistance to POS and other triazoles was seen. Gene sequencing showed that 4 *C. albicans*, 1 *C. tropicalis*, and 9 *A. fumigatus* isolates with cross-resistance to POS and other triazoles had mutations in *ERG11* or *CYP51A*. In conclusion, POS has potent *in vitro* activity against most of *Candida* and *Aspergillus* isolates from China. Non-WT phenotype and those with cross-resistance to POS and other triazoles exist, frequently driven by mutations of *ERG11* in *Candida* spp. and *CYP51A* in *Aspergillus* spp.

1. Introduction

Invasive candidiasis (IC) and invasive aspergillosis (IA) are major threats to patients with immunodeficiency or severe underlying disease (Kullberg and Arendrup, 2015; Segal, 2009). *Candida albicans* remains the most common cause of IC, yet non-*albicans Candida* infections have been increasing in the last 3 decades (Kullberg and Arendrup, 2015). In the same time, *Aspergillus fumigatus* is the main cause for IA, followed by *Aspergillus flavus* (Segal, 2009). Currently, fluconazole (FLC), voriconazole (VRC), itraconazole (ITA), echinocandins, and amphotericin B are the commonly used antifungal drugs in treatment of fungal infections (Bassetti et al., 2015; Kullberg and Arendrup, 2015). Posaconazole (POS) has been demonstrated to have therapeutic activity against *Candida* spp. (Torres et al., 2005). And it has been used globally for more than 10 years.

POS is a broad-spectrum triazole with antifungal activity (Moore et al., 2015). It has been shown to be effective in clinical practice for treating severely immunocompromised patients with disseminated candidiasis and aspergillosis (Dekkers et al., 2016; McKeage, 2015). Currently, susceptibilities of *Candida* spp. and *Aspergillus* spp. to POS have been determined in China in several studies (Deng et al., 2017; Fan et al., 2017;Hou et al., 2017; Pfaller et al., 2015; Xiao et al., 2015). Non–wild-type (non-WT) phenotype to POS mainly existed in non-*albicans Candida* spp., such as *Candida tropicalis*, *Candida glabrata*, and *Candida parapsilosis* (Hou et al., 2017; Pfaller et al., 2015; Xiao et al., 2015). In *A. fumigatus*, non-WT phenotype was observed recently (Deng et al., 2017), although non-WT phenotype was not determined or found in the earlier researches (Pfaller et al., 2015; Shi et al., 2010). Hence, susceptibilities of *Candida* spp. and *Aspergillus* spp. to POS need to be evaluated again in China. And susceptibility to POS, cross-resistance to POS and other triazoles, and possible mechanism of POS resistance or cross-resistance would be useful.

In this study, we evaluated the *in vitro* susceptibilities of *Candida* spp. and *Aspergillus* spp., isolated from different parts of China, to POS. We further analyzed the mutations of *ERG11* (in *Candida* spp.) and *CYP51A* genes (in *Aspergillus* spp.), encoding for the target enzyme of triazoles, of the POS non-WT isolates. These results will provide the helpful information for clinical treatment with POS in China.

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2. Materials and methods

2.1. Fungal strains and species identification

All of 385 *Candida* strains were isolated from blood samples taken from patients with IC in the China-SCAN study (Guo et al., 2013; Liu et al., 2014) and were identified using chromogenic culture media (CHROMagar, Paris, France), the API 20C AUX yeast identification kit (bioMe´rieux SA, Marcy l'E´toile, France), or ITS region and D1/D2 domain sequencing when necessary (Kurtzman and Robnett, 1997; Liu et al., 2014), including *C. albicans* (156), *C. parapsilosis* (83), *C. tropicalis* (67), *C. glabrata* (50), *Candida haemulonii* (15), *Candida guilliermondii* (6), *Candida pelliculosa* (5), *Candida inconspicua* (1), *Candida metapsilosis* (1), and *Candida norvegensis* (1). All of 268 *Aspergillus* strains were isolated mainly from sputum and bronchoalveolar lavage fluid samples of patients with aspergillosis and were identified using a combination of morphological analysis and ITS, β-tublin, and actin sequencing in Peking University First Hospital (Schoch et al., 2012), including *A. fumigatus* (222), *A. flavus* (23), *Aspergillus terreus* (11), *Aspergillus niger* (8), *Aspergillus nidulans* (2), *Aspergillus sydowii* (1), and *Aspergillus penicilloides* (1).

2.2. Susceptibility testing

Antifungal susceptibility testing was performed using broth microdilution method according to CLSI M27-A4 for *Candida* spp. and M38-A2 for *Aspergillus* spp. POS powder (Merck) was prepared in DMSO for *in vitro* testing. Minimal inhibitory concentrations (MICs) of POS for *Candida* spp. were determined after growth for 24 h at 35 °C and read as the lowest drug concentration producing a strong decrease in turbidity translating to 50% growth reduction compared with the drug-free control. MICs of triazoles for *Aspergillus* spp. were determined after 48 h incubation at 35 °C and read as the lowest drug concentrations that produced complete growth inhibition (100%). The recently revised epidemiological cutoff values (ECVs) for POS were used for *Candida* spp. and *Aspergillus* spp. to determine WT or non-WT susceptibilities (Espinel-Ingroff et al., 2010; Pfaller and Diekema, 2012). MICs of FLC, VRC, and ITA against *Candida* spp. were obtained from the China-Scan study (Liu et al., 2014). Susceptibilities of *Aspergillus* spp. to POS, VRC, and ITA were evaluated and MICs were determined in this study.

2.3. ERG11 or CYP51A sequencing

The complete sequence of coding region in *ERG11* or *CYP51A* with the promoter sequence in POS non-WT isolates was amplified by PCR and was sequenced as described previously (Feng et al., 2010; Liu et al., 2015a; Snelders et al., 2010). Sequences were analyzed through alignment with those in WT isolates to detect mutations in *ERG11* or *CYP51A*.

3. Results

3.1. In vitro susceptibilities to POS

The MICs ranges (mg/L) of common *Candida* isolates were as follows: *C. albicans* (0.007–4); *C. parapsilosis* (0.01–0.5); *C. tropicalis* (0.007–0.5), and *C. glabrata* (0.007–0.5) (Table 1). Based on the ECVs for POS, 132/156 *C. albicans*, 82/83 *C. parapsilosis*, 59/67 *C. tropicalis*, 50/50 *C. glabrata*, and 6/6 *C. guilliermondii* isolates were designated WT to POS (Table 1). However, 24 *C. albicans,* 1 *C. parapsilosis,* 8 *C. tropicalis*, and 5 *C. pelliculosa* isolates were classified non-WT to POS (Table 1).

MICs ranges (mg/L) of POS against *Aspergillus* isolates were as follows: *A. fumigatus* (0.03–16), *A. flavus* (0.125–0.5), *A. terreus* (0.06–0.125), *A. niger* (0.03–0.5), and *A. nidulans* (0.06–0.125) (Table 2). In terms of the ECVs for POS, 213/222 *A. fumigatus*, 19/23 *A. flavus*, 11/11 *A. terreus*, 8/8 *A. niger*, and 2/2 *A. nidulans* isolates were designated WT to POS (Table 2). However, 9 *A. fumigatus* and 4 *A. flavus* isolates were classified non-WT to POS (Table 2).

3.2. Cross-resistance to POS and other triazoles

MIC values of FLC, VRC, and ITA against all *Candida* isolates in this study had been determined previously in the China-Scan study (Liu et al., 2014). In terms of MICs, among 24 POS non-WT *C. albicans* isolates, 2 were cross-resistant to FLC, 3 were cross-resistant to ITA, and 6 were cross-resistant to both FLC and ITA (Fig. 1A). Among 8 POS non-WT *C. tropicalis* isolates, 1 was cross-resistant to FLC, 2 were cross-resistant to ITA, and 1 was cross-resistant to FLC and ITA (Fig. 1A). And only 1 *C. parapsilosis* isolate was non-WT to POS; at the same time, it was cross-resistant to both FLC and ITA (Fig. 1A). In order to investigate the cross-resistance among *Aspergillus* isolates, MICs of VRC and ITA were also tested in this study, and 15 *A. fumigatus* isolates were non-WT to triazoles. Among these 15 isolates, 9 were non-WT to POS. Moreover, 5 of 9 POS non-WT isolates were cross-resistant to ITA, and 4 of 9 were cross-resistant to both VRC and ITA (Fig. 1B). For *A. flavus*, just 1 isolate showed cross-resistance to both VRC and POS (Fig. 1B).

Table 1
The *in vitro* susceptibilities to posaconazole of *Candida* isolates.

Species	Isolates, *n* (%)	[a]MIC (mg/L)				[d]ECV (mg/L)	
		Range	50	90	GM	WT (%)	Non-WT (%)
Candida albicans	156 (23.9)	0.007–4	0.015	0.125	0.15	132 (84.6)	24 (15.4)
Candida parapsilosis	83 (12.7)	0.01–0.5	0.03	0.12	0.086	82 (98.8)	1 (1.2)
Candida tropicalis	67 (10.3)	0.007–0.5	0.03	0.25	0.053	59 (88.1)	8 (11.9)
Candida glabrata	50 (7.7)	0.007–0.5	0.12	0.25	0.048	50 (100)	0 (0.00)
Candida haemulonii	15 (2.3)	0.01–0.125	0.01	0.125	0.05	[c]-	-
Candida guilliermondii	6 (0.91)	0.03–0.25	[b]Δ	Δ	0.097	6 (100)	0 (0.00)
Candida pelliculosa	5 (0.77)	0.5	Δ	Δ	0.5	0 (0.00)	5 (100)
Candida inconspicua	1 (0.15)	0.01	Δ	Δ	0.01	-	-
Candida metapsilosis	1 (0.15)	0.01	Δ	Δ	0.01	-	-
Candida norvegensis	1 (0.15)	0.01	Δ	Δ	0.01	-	-

Data are presented as frequency with percentage (%).
GM = geometric means; ECVs = epidemiological cutoff values; $MIC_{50/90}$ = the minimal inhibition concentration that inhibits the growth of 50% and 90% of isolates, respectively.
[a] $MIC_{50/90}$ values are calculated for species with≥10 isolates from the same geographical region.
[b] Δ, MIC_{50} or MIC_{90} unavailable.
[c] -, ECVs unavailable.
[d] ECVs for posaconazole were used to identify non-WT isolates of *C. albicans* (ECV > 0.06 mg/L), *C. parapsilosis* (ECV > 0.25 mg/L), *C. tropicalis* (ECV > 0.12 mg/L), *C. glabrata* (ECV > 2 mg/L), *C. guilliermondii* (ECV > 0.5 mg/L), and *C. pelliculosa* (ECV > 2 mg/L) (Pfaller and Diekema, 2012).

Table 2
The *in vitro* susceptibilities to posaconazole of *Aspergillus* isolates.

Species	Isolates, *n* (%)	MIC (mg/L)				[a]ECV (mg/L)	
		Range	50	90	GM	WT (%)	Non-WT (%)
Aspergillus fumigatus	222 (34.0)	0.03–16	0.125	0.25	0.26	213(95.9)	9(4.1)
Aspergillus flavus	23 (3.5)	0.125–0.5	0.25	0.5	0.25	19(82.6)	4(17.4)
Aspergillus terreus	11 (1.7)	0.06–0.125	0.06	0.125	0.06	11(100)	0(0.00)
Aspergillus niger	8 (1.2)	0.03–0.5	Δ	Δ	0.15	8(100)	0(0.00)
Aspergillus nidulans	2 (0.30)	0.06–0.125	Δ	Δ	0.087	2(100)	0(0.00)
Aspergillus sydowii	1 (0.15)	0.125	Δ	Δ	0.125	-	-
Aspergillus penicilloides	1 (0.15)	0.125	Δ	Δ	0.125	-	-

[a] ECVs for posaconazole were used to identify non-WT isolates of *A. fumigatus* (ECV > 0.5 mg/L), *A. flavus* (ECV > 0.25 mg/L), *A. terreus* (ECV > 0.5 mg/L), *A. niger* (ECV > 0.5 mg/L), and *A. nidulans* (ECV > 1 mg/L) (Espinel-Ingroff et al., 2010).

3.3. Mutations of ERG11 or CYP51A in POS non-WT isolates

As shown in Table 3, among 4 POS non-WT *C. albicans* isolates which were cross-resistant to FLC and ITA, 1 had P56S, D116E, and V488I substitutions, while 3 had A114S and Y257H substitutions in Erg11. However, mutations in Erg11 of the other 20 POS non-WT *C. albicans* isolates were not discovered. For 8 POS non-WT *C. tropicalis*, only 1 isolate with cross-resistance to FLC had Y132F substitution in Erg11, and mutations of *ERG11* in the other 7 isolates were not found (Table 3). Among 9 POS non-WT *A. fumigatus* isolates, 5 isolates with cross-resistance to ITA had G54W substitutions in Cyp51A, 1 isolate with cross-resistance to both ITA and VRC had TR34/L98H, and the remaining 3 isolates with cross-resistance to both ITA and VRC had TR34/L98H/S297T/F495I mutations in *CYP51A* (Table 3). No mutation of *CYP51A* was detected among the 4 *C. flavus* isolates non-WT to POS.

4. Discussion

Although there were data about POS susceptibility of *Candida* spp. in some studies recently (Fan et al., 2017; Hou et al., 2017; Pfaller et al., 2015; Xiao et al., 2015), there were no data about POS susceptibility in the China-Scan study because POS was not used in China during that time (Liu et al., 2014). Hence, we tested the *in vitro* susceptibility to POS against these strains in this study. The results showed that most of clinical *Candida* isolates were WT to POS in terms of *in vitro* susceptibility. Moreover, among 15 FLC- and 25 ITA-resistant *C. albicans* isolates from the China-Scan study, 7 and 16 were still WT to POS, respectively (data not shown). Similar observation occurred in *C. tropicalis* and *C. parapsilosis* isolates. It was noteworthy that all 50 *C. glabrata* isolates in the China-Scan study were nonsusceptible to FLC, including 48 dose-dependent and 2 resistant isolates (Liu et al., 2014). And among these 50 *C. glabrata* isolates, 7 (14%) were resistant to caspofungin, and 3 were non-WT to VRC and 2 to ITA (Liu et al., 2014). Since caspofungin is the first choice for treatment of IC (Pappas et al., 2016), when the patients are infected with these *C. glabrata* isolates resistant to triazoles and caspofungin, other drugs need to be used as an alternative choice for therapy. Reassuringly, our study showed that all the *C. glabrata* isolates were WT to POS. However, non-WT phenotypes for POS were still existing, mainly among *C. albicans* (15.4%) and *C. tropicalis* (11.9%) in this study, similarly to what has been previously described (Castanheira et al., 2014). We further observed that cross-resistance to POS and other tirazoles appeared mainly among *C. albicans* (11/24 POS non-WT isolates) and *C. tropicalis* (4/8 POS non-WT isolates), consistent with the previous report (Pfaller et al., 2008b). Additionally, no cross-resistance of *C. glabrata* to POS and FLC or other triazoles was detected in this study despite it being reported elsewhere (Pfaller et al., 2008b; Xiao et al., 2015).

Triazole resistance in *Candida* spp. is usually due to mutations in *ERG11* (Feng et al., 2010; Liu et al., 2015a). It has been reported that D116E coexisting with V488I in Erg11 could contribute to FLC or/and ITA resistance (Cernicka and Subik, 2006; Rosana et al., 2015), and A114S coexisting with Y257H could confer FLC resistance in both *C. albicans* and *Saccharomyces cerevisiae* (Xiang et al., 2013; Xu et al., 2008; Ying et al., 2013). In addition, the similar structures between these 2 triazoles may contribute to cross-resistance to POS and ITA (Torres et al., 2005). Our results showed that 4 POS non-WT *C. albicans* isolates, which were cross-resistant to FLC and ITA, had mutations of Erg11 including P56S, D116E, and V488I substitutions in 1 isolate and A114S with Y257H substitutions in 3 isolates. It has been verified that P56S substitution in Erg11 could not cause triazole resistance in *C. albicans*. Hence, we speculate that D116E with V488I and A114S with Y257H might result in cross-resistance to POS and other

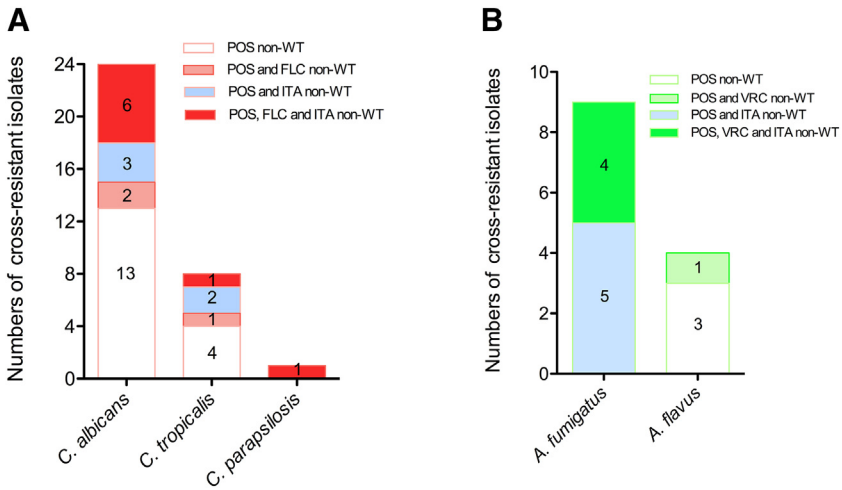

Fig. 1. Cross-resistance to posaconazole and other triazoles. POS = posaconazole; FLC = fluconazole; VRC = voriconazole; ITA =itraconazole. (A) Numbers of cross-resistance to POS and other triazoles in *Candida* spp. (B) Numbers of cross-resistance to POS and other triazoles in *Aspergillus* spp.

Table 3
Mutations of *ERG11* or *CYP51A* in posaconazole non-WT isolates.

Isolates[a]	*n*	Triazole drugs[b]	Mutation in *ERG11* or *CYP51A*
C. albicans	1	POS, FLC, ITA	P56S, D116E, V88I
	3	POS, FLC, ITA	A114S, Y257H
	2	POS, FLC	Intact[c]
	3	POS, ITA	Intact
	2	POS, FLC, ITA	Intact
	13	POS	Intact
C. tropicalis	1	POS, FLC	Y132F
	2	POS, ITA	Intact
	1	POS, FLC, ITA	Intact
	4	POS	Intact
A. fumigatus	5	POS, ITA	G54W
	1	POS, VRC, ITA	TR34/L98H
	3	POS, VRC, ITA	TR34/L98H/S297T/F495I

POS = posaconazole; FLC = fluconazole; VRC = voriconazole; ITA = itraconazole.
[a] The isolates non-WT to posaconazole.
[b] Triazole drugs to which the isolates were resistant/non-WT.
[c] Mutation in *ERG11* or *CYP51A* was not found.

triazoles in these 4 *C. albicans* isolates. However, *ERG11* in the other 20 POS non-WT isolates was intact.

Since it has been demonstrated that triazole resistance in *Candida* spp. could result from overexpression of target enzyme, upregulation of multidrug transporters, or cellular stress responses beside mutations of target enzyme (Xie et al., 2014), we speculate here that non-WT phenotype for POS and cross-resistance between POS and other triazoles in these 20 isolates are Erg11 mutation-independent alterations. Further investigation in these isolates is needed. For *C. tropicalis*, previous studies showed that FLC resistance in *C. tropicalis* resulted from Y132F substitution of Erg11 which could cause cross-resistance to FLC and ITA in *S. cerevisiae* (Jiang et al., 2013; Tan et al., 2015), and we found Y132F substitution of Erg11 in 1 isolate with cross-resistance to POS and FLC among 8 POS non-WT *C. tropicalis* isolates in this study. Hence, we speculate that Y132F substitution of Erg11 in *C. tropicalis* isolate might cause cross-resistance to POS and FLC. However, alterations of Erg11 were not found in the other 7 *C. tropicalis* isolates with non-WT MICs to POS. Non-WT phenotype for POS and cross-resistance to POS and other triazoles in the other 7 isolates might be due to the overexpression of *ERG11* or *MDR* which has been reported to confer triazole resistance in *C. tropicalis* (Jiang et al., 2013; You et al., 2017).

The results of susceptibility to POS in *Aspergillus* spp. showed that POS has activity against most of *Aspergillus* spp., even for *A. fumigatus* isolates resistant to other triazoles (6/15 triazole resistant isolates). However, non-WT phenotypes for POS were detected, mainly among *A. fumigatus* (4.1%) and *A. flavus* (17.4%), in agreement with other studies (Deng et al., 2017; Espinel-Ingroff et al., 2010; Pfaller et al., 2008a). In addition, we found that cross-resistance to POS and other triazoles appeared mainly among *A. fumigatus* (9/9 POS non-WT isolates). This result was also consistent with the previous report (Pfaller et al., 2008a).

For 9 POS non-WT isolates among 15 triazole-resistant *A. fumigatus* isolates, further sequencing showed that G54W substitution in Cyp51A existed in the 5 isolates with non-WT MICs to POS and ITA; TR34/L98H/S297T/F495I in the 3 isolates with non-WT MICs to POS (MIC, 1 mg/L), VRC (MIC, 2 mg/L), and ITA (MIC>16 mg/L); and TR34/L98H in the last one with non-WT MICs to ITA (MIC>16 mg/L), VRC (MIC, 8 mg/L), and POS (MIC, 1 mg/L). Previous studies have verified that alteration of a single amino acid, G54W, in Cyp51A could result in cross-resistance to ITA and POS in *A. fumigatus* (Mann et al., 2003; Snelders et al., 2010). And TR34/L98H combined with S297T/F495I could cause cross-resistance to VRC, ITA, and POS in *A. fumigatus* (Hagiwara et al., 2016; Liu et al., 2015b; Meis et al., 2016). Hence, mutations including G54W and TR34/L98H/S297T/F495I in *CYP51A* may result in cross-resistance to POS and other triazoles in *A. fumigatus* isolates. Snelders et al. reported that TR34/L98H alone in *A. fumigatus* could only cause resistance to ITA and elevation of VRC MIC but less than 2 mg/L (Snelders et al., 2011). In our study, 1 isolate with TR34/L98H exhibited VRC MIC 8 mg/L and POS MIC 1 mg/L. The cause of VRC MIC 8 mg/L and non-WT MIC to POS in this isolate is not obviously due to TR34/L98H mutation in *CYP51A*. And the exact mechanism is being investigated. For 6 POS WT isolates among 15 triazole-resistant *A. fumigatus* isolates, 2 isolates, which were only non-WT to VRC, had TR46/Y121F/T289A in *CYP51A*; 1 of the other 4 isolates, which were only non-WT to ITA, had M220I substitution; and the remaining 3 isolates had G54R substitution. We speculate that the mutations including TR46/Y121F/T289A, M220I, and G54R alone in *CYP51A* may not cause non-WT phenotype for POS in *A. fumigatus*.

In conclusion, POS has potent *in vitro* activity against most of *Candida* isolates from the China-Scan study and *Aspergillus* isolates in this study. Cross-resistance to POS and other triazoles, although uncommon, exists. Mutations of *ERG11* in *Candida* spp. and *CYP51A* in *Aspergillus* spp. play an important role in the cross-resistance to POS and other triazoles, but other non–*ERG11*-dependent mechanisms exist. This study provides the susceptibility profile of *Candida* spp. and *Aspergillus* spp. to POS in China and would be helpful for the treatment of both IC and IA.

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