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Note

In-vitro antifungal susceptibility testing of lanoconazole and luliconazole against *Aspergillus flavus* as an important agent of invasive aspergillosis

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ABSTRACT

Introduction: The incidence of *Aspergillus* infections has recently increased remarkably in certain tropical and sub-tropical countries, with *Aspergillus flavus* being identified as the leading cause of infections after *A. fumigatus*. Lanoconazole (LAN) and luliconazole (LUL) are currently approved for topical treatment of cutaneous fungal infections. We aimed the *in-vitro* antifungal susceptibility testing of two imidazole, LAN and LUL against A. *flavus*.

Methods: One hundred and eighty-seven clinical and environmental *A. flavus* were tested originating from different climate zones of Iran between 2008 and 2015. The identification of all isolates was confirmed by using PCR-sequencing of *β-tubuline* ribosomal DNA gene. *In-vitro* antifungal susceptibility test was performed using CLSI guidelines against LAN, LUL, itraconazole (ITC), voriconazole (VRC), posaconazole (POS), Isavuconazole (ISA), amphotericin B (AMB), 5-flucytosine (5FC), caspofungin (CAS) and anidulafungin (AFG). The minimum inhibitory concentration (MIC) and minimum effect concentration (MEC) values were evaluated according to CLSI M38-A2 guidelines.

Results: The geometric mean MICs for tested antifungals, in increasing order, were: 0.009 μg/mL for LUL (ranging from 0.004 to 0.062), 0.02 μg/mL for LAN (ranging from 0.004 to 0.125), POS (0.10), ISA (0.16), ITC (0.24), VRC (0.27), AMB (1.8) and 5FC (63.06) μg/mL. The mean value of MECs for AFG and CAS were 0.06 and 0.07, respectively.

Conclusion: Overall, LUL and LAN showed the lowest MIC against all isolates of *A. flavus*. Further studies are required to evaluate the *in-vivo* efficacy of these agents, and the possibility of using these agents in systemic infections.

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Table 1
In vitro antifungal susceptibility testing of *A. flavus* isolates against ten drugs as determined by CLSI M38-A2 broth microdilution method.

	Antifungal agent									
	LAN	LUL	AMB	ITC	VRC	5FC	POS	ISA	CAS	AFG
Range (μg/ml)	0.004–0.125	0.004–0.062	1–4	0.032–2	0.062–2	≥64	0.032–0.5	0.032–1.0	0.032–0.5	0.032–0.5
MIC_{50}/MEC_{50} (μg/ml)	0.016	0.008	1	0.25	0.25	≥64	0.062	0.25	0.032	0.032
MIC_{90}/MEC_{90} (μg/ml)	0.064	0.032	2	0.5	0.5	≥64	0.5	0.5	0.125	0.125
GM	0.02	0.009	1.8	0.24	0.27	63.06	0.10	0.16	0.07	0.06

LAN (lanoconazole) and LUL (luliconazole), AMB (amphotericin B), 5FC (5-flucytosine), ITC (itraconazole), VRC (voriconazole), POS (posaconazole), ISA (isavuconazole), CAS (caspofungin), AFG (anidulafungin), GM (geometric mean).

Table 2
In vitro antifungal susceptibility testing of *A. flavus* strains isolated from different sources.

Samples (No. of strains)	Antifungal drug	MIC_{50}/MEC_{50} μg/ml	MIC_{90}/MEC_{90} μg/ml	GM μg/ml
Bronchoalveolar lavage (62)	LAN	0.016	0.016	0.02
	LUL	0.008	0.016	0.01
	AMB	1	2	1.24
	ITC	0.25	0.5	0.22
	VRC	0.25	0.5	0.31
	5FC	≥64	≥64	58.4
	POS	0.062	0.25	0.09
	ISA	0.25	0.25	0.14
	CAS	0.062	0.25	0.05
	AFG	0.016	0.032	0.04
Onychomycosis (14)	LAN	0.032	0.064	0.03
	LUL	0.008	0.016	0.01
	AMB	1	2	1.1
	ITC	0.25	0.5	0.22
	VRC	0.25	0.5	0.26
	5FC	≥64	≥64	64
	POS	0.062	0.25	0.08
	ISA	0.062	0.25	0.19
	CAS	0.062	0.25	0.06
	AFG	0.016	0.032	0.05
Sinusitis (13)	LAN	0.032	0.032	0.02
	LUL	0.008	0.008	0.008
	AMB	0.5	2	0.9
	ITC	0.125	1	0.19
	VRC	0.25	1	0.20
	5FC	≥64	≥64	64
	POS	0.062	0.5	0.07
	ISA	0.25	0.5	0.14
	CAS	0.032	0.25	0.06
	AFG	0.016	0.25	0.05
Cutaneous (10)	LAN	0.032	0.032	0.02
	LUL	0.008	0.008	0.008
	AMB	1	4	1.3
	ITC	0.25	0.5	0.20
	VRC	0.25	0.5	0.17
	5FC	≥64	≥64	64
	POS	0.062	0.125	0.06
	ISA	0.25	1	0.15
	CAS	0.032	0.25	0.06
	AFG	0.016	0.032	0.07
Other clinical samples* (11)	LAN	0.032	0.032	0.02
	LUL	0.008	0.008	0.008
	AMB	2	4	1.8
	ITC	0.5	1	0.4
	VRC	0.5	1	0.5
	5FC	≥64	≥64	64
	POS	0.125	0.25	0.1
	ISA	0.5	1	0.18
	CAS	0.064	0.5	0.09
	AFG	0.064	1	0.08
Environmental (77)	LAN	0.016	0.016	0.02
	LUL	0.008	0.016	0.01
	AMB	2	4	1.9
	ITC	0.25	1	0.24
	VRC	0.25	1	0.51
	5FC	≥64	≥64	64
	POS	0.5	1	0.19
	ISA	0.5	1	0.16
	CAS	0.032	0.25	0.1
	AFG	0.064	1	0.09

LAN (lanoconazole) and LUL (luliconazole), AMB (amphotericin B), 5FC (5-flucytosine), ITC (itraconazole), VRC (voriconazole), POS (posaconazole), ISA (isavuconazole), CAS (caspofungin), AFG (anidulafungin), GM (geometric mean). *Including; otomycosis (n = 4), sputum (n = 3), nasal discharge (n = 2) and lung biopsy (n = 2).

Table 3
In vitro MICs of three isolates of *A. flavus* with high MICs against ITC, VRC and AMB.

N0	Origin	Accession number	History of antifungal use	Antifungal agent									
				LAN	LUL	AMB	ITC	VRC	5FC	POS	ISA	CAS	AFG
ABS117	**Bronchoalveolar lavage**	KY288682	50–150 mg fluconazole	0.016	0.008	16	2	2	≥64	0.25	1	0.25	0.125
ABS136	**Bronchoalveolar lavage**	KY288698	50–150 mg fluconazole	0.004	0.004	4	2	2	≥64	0.5	2	0.5	0.25
ABS23	**Hospital environment**	KY288768	-	0.032	0.016	64	2	1	≥64	0.25	2	0.25	0.5

The frequency of invasive aspergillosis (IA) has increased remarkably in immunocompromised patients [1]. After *Aspergillus fumigatus*, *A. flavus* is the second etiological agent of aspergillosis in some area with tropical climate. Iran has an essentially hot temperature that favors the growth of thermophilic filamentous fungi including *A. flavus* [2].

The treatment of IA is difficult because *Aspergillus* species show different *in-vitro* antifungal susceptibility profiles [3,4]. Azole antifungals such as voriconazole (VRC), itraconazole (ITC), posaconazole (POS), and Isavuconazole (ISA) are currently recommended for treatment of various *Aspergillus* diseases [5]. However, azole resistance is increasingly reported in environmental and clinical strains of *Aspergillus* species worldwide [6,7]. Although azole resistance has been predominantly been reported in *A. fumigatus*, resistance to voriconazole has been documented in *A. flavus*, with corresponding mutations in the *cyp51* gene [8]. Treatment of azole-resistant aspergillosis is difficult as pan-azole-resistant phenotypes predominate [9,10].

Alternative treatment options for azole-resistant infection are limited, including echinocandins or polyenes. Thus, there is a need to investigate the activity of novel antifungal agents against different species of *Aspergillus* as this may provide leads to increase treatment options in refractory cases. LUL and LAN are two new imidazole drugs, with broad-spectrum activities against a variety of opportunistic fungi including *Candida*, *A. fumigatus*, *Malassezia* and *Trichophyton* spp [11]. These drugs have been approved by the US Food and Drug Administration (FDA) for topical treatment of dermatophytosis [12].

Recently, LUL and LAN were shown a potent *in-vitro* activity against azole-resistant *A. fumigatus* in comparison with other antifungal drugs [13]. However, there is no data on the activity of LUL and LAN against *A. flavus*. Therefore, we compared the *in-vitro* activity of LAN and LUL with eight main antifungal agents including amphotericin B (AMB), flucytosine (5FC), ITC, VRC, POS, ISA, caspofungin (CAS) and anidulafungin (AFG), against 187 clinical and environmental strains of A. *flavus* isolated within Iran.

One hundred and eighty seven clinical (58.8%) and environmental (41.2%) isolates of *A. flavus* were tested. The identification of all isolates was confirmed by direct DNA sequencing of the partial beta tubulin gene.

In-vitro susceptibility testing was performed according to the Clinical & Laboratory Standards Institute (CLSI) M38-A2 guidelines [14]. Final concentrations of the following antifungal agents ranged from 0.004 to 4 μg/ml: LAN and LUL (Nihon Nohyaku Co, Osaka, Japan); 0.016–16 μg/ml: AMB (Bristol-Myers-Squib, The Netherlands), ITC, VRC (Sigma-Aldrich, USA), POS, and ISA (Schering-Plough, The Netherlands); 0.032–32 μg/ml: CAS and AFG (Pfizer, The Netherlands); 0.064–64 μg/ml: 5FC (Sigma-Aldrich, USA). Conidial suspensions were harvested after isolates were subcultured on potato dextrose agar for 5–7 days at 35 °C and were suspended in normal saline containing 0.025% Tween 20. The inocula were then prepared spectrophotometrically and further diluted in normal saline in order to obtain a final inoculum concentration of 0.4×10^4 to 5×10^4 CFU/ml. For polyenes and azoles, the Minimum inhibitory concentrations (MICs) were defined visually as the lowest drug concentration which resulted complete inhibition of growth, while, for CAS and AFG, the minimum effective concentrations (MECs) were determined microscopically, confirming to the lowest antifungal concentration at which irregular, tiny, branched hyphae were detected compared to the long, unbranched hyphae in positive controls wells. The ranges and geometric means (GMs) of the MICs and MECs were determined after 48 h of incubation at 35 °C. *Paecilomyces variotii* (ATCC 22319), *Candida krusei* (ATCC 6258) and *C. parapsilosis* (ATCC 22019) were used as quality controls.

AFST showed that both LUL and LAN exhibited the lowest MICs against *A. flavus*, in comparison with ITC, VRC, POS, ISA, CAS, AFG, AMB and 5FC (Table 1). MICs of LUL and LAN against all strains ranged from 0.004 to 0.062 and 0.004–0.125 μg/ml, respectively, compared to 1–4 μg/ml for AMB, 32–64 μg/ml for 5FC, 0.032–2 μg/ml for ITC, 0.062–2 μg/ml for VRC, 0.032–1.0 μg/ml for ISA, 0.032–0.5 μg/ml for POS. The MECs of CAS and AFG versus all isolates ranged from 0.032 to 0.5 μg/ml. The GM MIC values for LUL, LAN, ITC, VRC, POS, ISA, 5FC, AMB and MEC values for CAS and AFG across all isolates were reported in ascending order as: LUL 0.009, LAN 0.02, AFG 0.06, CAS 0.07, POS 0.10, ISA 0.16, ITC 0.24, VRC 0.27, AMB 1.8 and 5FC 63.06 μg/mL (Table 2).

Three isolates of *A. flavus* (1.5%) (two from BAL specimens and one from the hospital environment) showed MICs of ≥2 μg/ml against ITC, VRC and ISA. The MICs of these isolates against AMB were 4, 16 and 64 μg/ml, respectively (Table 3).

In the present study the three isolates of *A. flavus*, which showed high MICs (≥2 μg/ml) against ITC, VOR and AMB had the lowest MICs against LUL and LAN (Table 3). These three isolates were from patients who were receiving FLU (150 mg as a single dose or as multiple doses). Similarly, in a previous study by Abastabar et al. [13], LUL and LAN showed the lowest MICs versus sensitive and resistant *A. fumigatus* strains in comparison with those of some other antifungals. The MIC_{50} values of LUL and LAN against *A. flavus* in the current study (0.008 and 0.016 μg/ml, respectively) were higher than those of *A. fumigatus* (0.001 and 0.002 μg/ml, respectively), which was reported by Abastabar et al. [13]. In our study, the MIC GM of LUL was lower than that of LAN against all *A. flavus* strains. Although there is no a preparation for systemic use of these drugs, but *in-vivo* data in animal models have proved that these drugs are highly effective for treatment of IA in comparison with other antifungal agents [15].

The analysis of our data revealed a significant decrease within the MICs of LAN and LUL against *A. flavus* in comparison with the main antifungal drugs in treatment of aspergillosis. However, LAN and LUL are currently used for cutaneous infections caused by *Aspergillus* species but further studies are required to evaluate the *in-vivo* efficacy of these agents, and the possibility of using them in systemic fungal infections.

Conflicts of interest

P.E.V has served as consultants to and have received research grants from Astellas, Basilea, Gilead Sciences, Merck, and Pfizer. S.S has received research grant from Astellas Pharma B.V. All the others have no conflict of interests.

Ethical statement

The Ethics Committee of Mazandaran University of Medical Sciences (code: 1392/12/14)) approved this research and the written informed consent was obtained from the patient or next of kin.

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Authorship

All authors meet the ICMJE authorship criteria. S.M.O. and M. T.A. performed all tests and wrote the draft. M.T.H. designed and managed the research, and edited the final manuscript. S. S. and H.Z. referred the patients and did acquisition of data. V.M. performed data analysis. M.A. performed the molecular techniques. A.H. and A.M. obtained and cultured environmental and clinical isolates. S.A. performed antifungal susceptibility test and the collection of data. P.E.V. and S.M.S. revising the manuscript critically for important intellectual content.

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