In vitro activities of voriconazole, fluconazole, itraconazole and amphotericin B against non *Candida albicans* yeast isolates

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Summary

Antifungal susceptibility testing was performed on 197 yeast isolates from the BCCM/IHEM biomedical fungi and yeasts collection (Belgian Co-ordinated Collections of Micro-organisms / IPH-Mycology) to study the in vitro activity of voriconazole against fluconazole, itraconazole and amphotericin B. MICs of the four antifungal agents were determined by an adapted NCCLS M27-A microdilution reference method. MIC readings were visually and spectrophotometrically determined. Optical density data were used for calculation of the MIC endpoints. For amphotericin B, the MIC endpoint was defined as the minimal antifungal concentration that exerts 90% inhibition, compared to the control growth. The azoles endpoints were determined at 50% inhibition of growth. The MIC distribution of voriconazole susceptibilities showed that 193 isolates had a MIC ≤ 2 µg/ml and 185 a MIC ≤ 1 µg/ml. Cross-tabulation of voriconazole, fluconazole, and itraconazole MICs indicated that voriconazole MICs raised with fluconazole and itraconazole MICs. The in vitro data obtained in this study suggest that voriconazole may also be effective treating yeast infection in patients infected with fluconazole or itraconazole resistant isolates.

Key words Antifungal, Voriconazole, Fluconazole, Itraconazole, Amphotericin B, Yeasts

Actividad in vitro del voriconazol, fluconazol, itraconazol y anfotericina B contra levaduras no *Candida albicans*

Resumen

Se ha estudiado la sensibilidad in vitro de 197 levaduras pertenecientes a la colección “BCCM/IHEM biomedical fungi and yeasts collection” (Colección Coordinada de Micro-organismos/ ISP-Micologia) a voriconazol y se ha comparado con su sensibilidad a fluconazol, itraconazol y anfotericina B. Las CMI se determinaron visual y espectrofotométricamente según una modificación del método M27-A del NCCLS. Para la anfotericina B, el punto final fue definido como la mínima concentración que inhibe el 90% del crecimiento respecto al control. Para los azoles la CMI fue determinada considerando el 50% de inhibición del crecimiento. Ciento noventa y tres cepas presentaron una CMI de voriconazol ≤ 2µg/ml y 185 una CMI ≤ 1 µg/ml. Se observó una correspondencia entre las CMIs de voriconazol y las del resto de los azoles. Nuestro estudio sugiere que voriconazol puede ser efectivo para el tratamiento de infecciones por levaduras resistentes al fluconazol e itraconazol.

Palabras clave Antifúngicos, Voriconazol, Fluconazol, Itraconazol, Anfotericina B, Levaduras

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Voriconazole belongs to the triazole class of drugs and exhibits broad-spectrum activity at concentrations of ≤ 1 mg/l against most common fungal pathogens, such as *Candida* spp. including isolates of fluconazole-resistant *Candida krusei* [3]. It has been found to be as effective as fluconazole in esophageal candidiasis [1]. Moreover, voriconazole can be considered as salvage treatment for patients with refractory candidiasis with an overall treatment success rate of 55%. Depending on the *Candida* species, satisfactory global response rates for patients with nonesophageal candidiasis ranged from 100% for cases involving *Candida parapsilosis* isolates to 25% for cases involving *Candida glabrata* isolates [7].

The aim of this study was to compare the *in vitro* activity of voriconazole, to fluconazole, itraconazole and amphotericin B on non *Candida albicans* yeast isolates from the BCCM/IHEM biomedical fungi and yeasts collection (Belgian Co-ordinated Collections of Micro-organisms/IPH-Mycology).

Materials and methods

Yeast isolates. 84 *Candida glabrata*, 45 *Candida parapsilosis*, 19 *Candida tropicalis*, 17 *Candida krusei*, 11 *Candida lusitaniae*, three *Candida dubliniensis*, one *Pichia anomala* and 17 *Saccharomyces cerevisiae* i.e. 197 yeast isolates, were selected.

The majority of these isolates were of clinical origin, mainly from blood samples (67%). Eight percent were recovered from the environment.

The strains were maintained on Sabouraud glucose agar slants, stored at 4 °C, until used. Prior to testing, each strain was subcultured on Sabouraud agar for 24h at 35 °C to ensure high viability. Two NCCLS (National Committee for Clinical Laboratory Standards) quality control (QC) strains, *C. krusei* (ATCC 6258) and *C. parapsilosis* (ATCC 22019), were tested each time a set of clinical isolates was evaluated [4]. The minimal inhibitory concentrations (MICs) of the QC strains (not shown) were within the control limits described by Barry et al. [2].

Antifungal drugs. Fluconazole and voriconazole (Pfizer Pharmaceuticals, Belgium), itraconazole (Janssen Pharmaceutical Beerse, Belgium) were obtained from the manufacturers as reagent grade powders. Amphotericin B was purchased as the Fungizone^{R} intravenous preparation in sodium deoxycholate (Bristol-Myers Squibb Belgium S.A., Belgium). Stock suspensions were prepared as follows: amphotericin B by resuspension of a Fungizone^{R} vial in 10 ml of water, fluconazole, itraconazole and voriconazole by dissolution in dimethylsulfoxide (DMSO) at 6.5 mg/ml for fluconazole and 1.6 mg/ml for itraconazole and voriconazole. The stock solutions were frozen at –80 °C for a maximum of two months. To prepare the test microdilution plates, the stock solution of amphotericin B was first diluted to a concentration of 1.6 mg/ml. The azole stock solutions were serially diluted in DMSO in accordance with the NCCLS M27-A guidelines [4]. In a laminar flow cabinet and with a semi-automatic dispenser (Multidrop 384, Thermolabsystems Helsinki, Finland), volumes of 100 µl of sterile distilled water were first dispensed into the wells of sterile flat bottomed 96-well microdilution plates (Nunc, Rosgilde, Denmark). To ensure reproducible microdilution plates, 2 µl of individual antifungal drug dilutions were automatically dispensed in each well with a 96-well Precision Pipetting Robot (model 250, Quadra 96 CV, Tomtec, Hamden, USA), except in six growth positive control wells (drug-free). The in-house prepared microplates were stored at –80 °C in plastic bags until needed.

Table 1. Antifungal susceptibilities as determined by the NCCLS microdilution reference broth method M27A.

Species (No. of isolates)	Antifungal agent	MIC ranges (µg/ml)	MIC for 50%	MIC for 90%
Candida glabrata (84)	Amphotericin B	0.5 – 2	1	2
	Fluconazole	4 – >64	16	64
	Itraconazole	0.063 – 2	0.25	1
	Voriconazole	0.13 – 4	0.25	2
Candida parapsilosis (45)	Amphotericin B	0.5 – 2	1	2
	Fluconazole	0.25 – 64	1	2
	Itraconazole	<0.008 – 0.25	0.032	0.13
	Voriconazole	<0.008 – 0.25	0.016	0.063
Candida tropicalis (19)	Amphotericin B	1 – 2	1	2
	Fluconazole	0.5 – >64	1	4
	Itraconazole	<0.008 – 8	0.063	0.5
	Voriconazole	0.032 – 16	0.063	0.5
Candida krusei (17)	Amphotericin B	0.25 – 2	1	1
	Fluconazole	32 – >64	64	>64
	Itraconazole	0.016 – 4	0.063	0.25
	Voriconazole	0.25 – 2	0.5	1
Candida lusitaniae (11)	Amphotericin B	0.5 – 1	0.5	1
	Fluconazole	0.13 – 1	0.25	1
	Itraconazole	<0.008 – 0.25	0.032	0.063
	Voriconazole	<0.008 – 0.016	<0.008	0.016
Saccharomyces cerevisiae (17)	Amphotericin B	0.25 – 0.5	0.25	0.5
	Fluconazole	0.13 – 32	2	8
	Itraconazole	<0.008 – 0.5	0.063	0.25
	Voriconazole	<0.008 – 0.5	0.063	0.13
All organisms (193)	Amphotericin B	0.25 – 2	1	2
	Fluconazole	0.13 – >64	8	64
	Itraconazole	<0.008 – 8	0.13	0.5
	Voriconazole	<0.008 – 16	0.25	1

N.B. results for *Candida dubliniensis* (three isolates) and *Pichia anomala* (one isolate) are not included.

Antifungal susceptibility testing. The yeast suspensions were prepared according to NCCLS M27-A guidelines [4]. The optical densities of the working suspensions in sterile saline were adjusted with a densitometer to produce a 0,5 McFarland standard at a wavelength of 530 nm. 20 µl of the inoculum stock-suspension was added in 10 ml RPMI 1640 (Angus Chemical Company) (RPMI media with L-glutamine, with 0,165 M morpholinepropanesulfhonic acid but without sodium bicarbonate, buffered to pH 7,0 and sterilised by filter 0,22 µm). The final inoculum concentration in each well ranged from $5x10^2$ to $2,5x10^3$ cells/ml. The amount of glucose in the RPMI medium was doubled to 2% to support optimal growth into the medium. This glucose concentration gave more reproducible optical density (OD) readings [5]. The final DMSO-concentration in each well was 1%, which has no significant influence on yeast growth.

A negative sterility control plate was included in each run. The final concentrations of amphotericin B ranged from 8 to 0.032 µg/ml, from 64 to 0.032 µg/ml for fluconazole and from 16 to 0.008 µg/ml for itraconazole and voriconazole.

Determination of the minimal inhibitory concentrations (MICs). The plates were incubated at 35 °C. After 24h incubation, visual MIC endpoints were determined with a reading mirror. Visual endpoints were determined as described in the M27-A method. The NCCLS-recommended endpoint for azoles is the lowest drug concentration with a prominent decrease in turbidity, while for amphotericin B, the MIC is the drug concentration showing a complete inhibition of growth. Spectrophotometric readings were performed after 48h by measuring the OD at 540 nm, after agitation of the plates, with a plate reader (model 312e, Bio-Tek Instruments, Vermont, USA). The raw OD readings were converted into measurements of growth as percentages of control readings, corrected for the background OD. The azoles'inhibitory concentration that gave 50% growth reduction (IC50) was taken as the MIC endpoint. The amphotericin B's inhibitory concentration that gave 90% growth reduction (IC90) was used as the MIC endpoint.

Interpretation. NCCLS breakpoints were used for itraconazole and fluconazole. Establishing a clear correlation between amphotericin B MIC and outcome is difficult, and official NCCLS interpretative breakpoints are not available. Yet, isolates inhibited by ≤1 µg /ml of amphotericin B are considered as susceptible [5]. Official NCCLS interpretative breakpoints are unavailable for voriconazole. In accordance with other authors and Pfizer recommendations, we used a susceptible breakpoint of ≤ 1 µg/ml [3,8].

MIC50% (the MIC at which 50% of the isolates are inhibited) and MIC90% (the MIC at which 90% of the isolates are inhibited) were also calculated.

Results

Table 1 summarizes the *in vitro* susceptibilities (MIC range, MIC50%, MIC90%) to the four antifungal agents for the most representative species. Resistances to amphotericin B (MIC >1 µg/ml) were seen in some *C. glabrata, C. parapsilosis. C. tropicalis* and *C. krusei* isolates. In contrast, MICs >1 µg/ml were not observed in *C. lusitaniae*, a species that is often found resistant to this drug [10].

Resistance to fluconazole and itraconazole were mostly noted in *C. glabrata* isolates (MIC90%/fluconazole: 64 µg/ml and MIC90%/itraconazole: 1 µg/ml) and in *C. krusei* to fluconazole (MIC90%: >64 µg/ml).

C. parapsilosis (MIC90%: 0.063 µg/ml) and *C. lusitaniae* (MIC90%: 0.016 µg/ml) were the two most susceptible species to voriconazole. *C. glabrata* was the less susceptible species, with a MIC90% = 2 µg /ml.

Table 2 shows the MICs distribution of voriconazole: 185/197 isolates had a MIC ≤ 1 µg/ml and 193/197 a MIC ≤ 2 µg/ml, the-resistant isolates (MIC > 2 µg /ml) were two *C. glabrata* and two *C. tropicalis* isolates.

The results obtained in three *C. dubliniensis* and one *P. anomala* isolates were not included in table 1.

The results given in table 2 showed that *C. dublinien-sis* and *P. anomala* isolates were susceptible to voriconazole with a MIC of ≤ 0.008 µg /ml and MIC = 0.25 µg/ml respectively.

Table 3 shows the cross-tabulations of voriconazole and fluconazole or itraconazole. The voriconazole's MICs rose alone with the MIC values of fluconazole and itraconazole. In 40 strains, with fluconazole MICs of 16 and 32 µg/ml i.e.susceptible-dose-dependent isolates, the voriconazole MICs ranged between 0.25 and 1 µg/ml (i.e. 97.5% of susceptible isolates) (except for one *C. tropicalis* strain). In 26 strains highly resistant to fluconazole (MIC ≥ 64 µg/ml), the voriconazole MICs were 0.25 to 4 µg/ml. Sixteen of these isolates (61%) were completely susceptible to this antifungal with a MIC of ≤ 1 µg/ml. In 66 strains, with itraconazole MICs of 0.25 and 0.5 µg/ml i.e. susceptible-dose–dependent isolates, the voriconazole MICs ranged between 0.016 and 1 µg /ml (i.e. 100% of susceptible isolates). In 15 highly resistant strains to itraconazole (MIC ≥ 1 µg/ml), voriconazole MICs were also consistently higher, ranging from 1 µg/ml in three still susceptible isolates (20%) to 16 µg/ml.

Table 2. In vitro activity of voriconazole.

	No. of times the following inhibitory concentrations that give 50% growth reduction (IC50s) (µg/ml) were reported											
Species (No. of isolates)	≤0.008	0.016	0.032	0.063	0.13	0.25	0.5	1	2	4	8	16
Candida glabrata (84)					6	37	24	8	7	2		
Candida parapsilosis (45)	1	27	11	4		2						
Candida tropicalis (19)			6	8	1	1	1				1	1
Candida krusei (17)						3	11	2	1			
Candida lusitaniae (11)	9	2										
Candida dubliniensis (3)	3											
Pichia anomala (1)						1						
Saccharomyces cerevisiae (17)	1	1	4	6	3		2					
Total (197)	14	30	21	18	10	44	38	10	8	2	1	1

Table 3. Cross-tabulation of voriconazole vs fluconazole (a) and of voriconazole vs itraconazole (b).

		Voriconazole IC50* (µg/ml)												Total
		<0.008	0.016	0.032	0.063	0.13	0.25	0.5	1	2	4	8	16	
(a) Fluconazole IC50* (µg/ml)	<0.125	7												7
	0.25	3												3
	0.5	2	11	5	1									19
	1	2	18	13	5									38
	2		1	3	8	2	1					1		16
	4				3	3	1	1						8
	8				1	5	29	5						40
	16						7	21	3					31
	32						4	1	3				1	9
	64						1	9	3	7				20
	Off-scale							2	1	1	2			6
	Total	14	30	21	18	10	43	39	10	8	2	1	1	197
(b) Itraconazole IC50* (µg/ml)	<0.008	7	1	2	1									11
	0.016	2	10	1	5	1	2							21
	0.032	3	9	8	3		2	3						28
	0.064	2	6	3	2	2	2	4						21
	0.13		2	7	5	4	13	3	1					35
	0.25		2		2	3	18	9						34
	0.5						6	20	6					32
	1								3	6		1		10
	2									1	2			3
	4									1				1
	8												1	1
	Total	14	30	21	18	10	43	39	10	8	2	1	1	197

* IC50: inhibitory concentrations that give 50% growth reduction

Discussion

Voriconazole showed an excellent *in vitro* potency and broad-spectrum activity against all tested species: 94% of non *C. albicans* isolates were inhibited at ≤ 1 µg/ml.

These results suggest that voriconazole can be effective to treat the majority of yeast infections resistant to amphotericin B. This finding not only supports an early study suggesting that voriconazole represents a clear advance in the treatment of invasive aspergillosis with proven superiority over amphotericin B in terms of both efficacy and patient survival [3], but also suggests that this observation can be extrapolated to pathogenic yeast as well.

Susceptibilities to fluconazole and itraconazole were similar to those reported in other major surveillance systems [6,8,9]. *C. glabrata* remains the least susceptible species and *C. krusei* was once more demonstrated to be intrinsically resistant to fluconazole. Fluconazole and itraconazole resistances were observed to some *C. tropicalis* isolates, as well as fluconazole resistances to some *C. parapsilosis* isolates. No serious problem was encountered with *C. lusitaniae, C. dubliniensis, S. cerevisiae* and *P. anomala* isolates.

With the exception of *C. glabrata,* the MICs 90% for voriconazole were always ≤ 1 µg/ml, suggesting that in most cases, this drug is effective to treat yeast infections among fluconazole or itraconazole resistant isolates.

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