In vitro antifungal activity of voriconazole against dermatophytes and superficial isolates of *Scopulariopsis brevicaulis*

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Summary

We have studied the *in vitro* antifungal activity of voriconazole, fluconazole and itraconazole against 252 clinical isolates of dermatophytes and *Scopulariopsis brevicaulis* by a standardized agar diffusion method (NeoSensitabs). Several important factors such as temperature (28 ºC *vs.* 35 ºC) and incubation time (2-10 days *vs.* 18-74 h) were adapted to dermatophytes and *Scopulariopsis* requirements. Voriconazole showed an excellent activity against most species of dermatophytes, higher than itraconazole and fluconazole. However, *S. brevicaulis* isolates were highly resistant to all azoles used in this study. Voriconazole might be an interesting antifungal alternative of to refractory superficial mycoses.

Key words

Voriconazole, Itraconazole, Fluconazole, Dermatophytes, *Scopulariopsis brevicaulis,*NeoSensitabs

Actividad antifúngica in vitro de voriconazol contra dermatofitos y aislamientos superficiales de *Scopulariopsis brevicaulis*

Resumen

Hemos estudiado la sensibilidad in vitro a voriconazol, itraconazol y fluconazol de 252 aislamientos de dermatofitos y *Scopulariopsis brevicaulis*, mediante una técnica de difusión en agar (NeoSensitabs). Algunas variables experimentales, como la temperatura (28 ºC *vs* 35 ºC) y el tiempo de incubación (2-10 días *vs* 18-74 h), fueron adaptadas a los requerimientos de este tipo de hongos. Voriconazol mostró una actividad antifúngica in vitro excelente frente a la mayoría de las especies de dermatofitos estudiadas, superior a la mostrada por itraconazol y fluconazol. Sin embargo, los aislamientos de *S. brevicaulis* mostraron una elevada resistencia a todos los azoles. Voriconazol podría ser una herramienta alternativa interesante para el tratamiento de micosis superficiales recalcitrantes.

Palabras clave

Voriconazol, Itraconazol, Fluconazol, Dermatofitos, *Scopulariopis brevicaulis,* NeoSensitabs

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The treatment of the most severe and chronic superficial fungal infections, which includes tinea capitis and tinea unguium, often requires the administration of systemic antifungal treatments. Terbinafine and the orally active triazoles, fluconazole and itraconazole, have substantial activity against the etiologic agents of these diseases and are currently used in the treatment of superficial mycoses [14]. Since the management of both, dermatophytes (onychomycosis) and *Scopulariopsis brevicaulis* are of difficult management, a broad-spectrum antifungal could be useful as alternative drug treatment [22].

Voriconazole is a novel broad-spectrum triazole antifungal agent similar in structure to fluconazole and in spectrum of action to itraconazole [2]. This agent has demonstrated substantial preclinical activity, in both *in vitro* and *in vivo* models against a variety of fungi, such as dimorphic fungi, yeasts, and opportunistic filamentous fungi (including dermatophytes) [15,16,27].

The aim of the present study has been to compare the *in vitro* activities of voriconazole and two other established agents used for the treatment of dermatophytosis, fluconazole and itraconazole, against isolates of 19 different species of dermatophytes and *Scopulariopsis brevicaulis* using a standardized agar diffusion method.

A total of 252 clinical isolates of dermatophytes and *S. brevicaulis* were evaluated: 86 *Trichophyton rubrum*, 41 *Trichophyton mentagrophytes*, 34 *Microsporum canis*, 20 *Epidermophyton floccosum*, 20 *S. brevicaulis*, 13 *Trichophyton interdigitale*, 9 *Trichophyton violaceum*, 6 *Microsporum gypseum*, 6 *Trichophyton schoenleinii*, 5 *Trichophyton soudanense*, 5 *Trichophyton tonsurans*, 4 *Microsporum audouinii*, 2 *Trichophyton equinuum*, 1 *Microsporum racemosum* and 1 *Trichophyton terrestre*.

All isolates were obtained during 2003 from human specimens collected at different Spanish hospitals. Identification was based on the macroscopic and microscopic characteristics of the isolates in culture and on additional biochemical and physiological tests, including the production of red pigment on potato glucose agar, urease activity, growth in different vitamin and amino acid agars (*Trichophyton* agars), and the hair perforation test [25,31]. To ensure the purity and viability of the inoculum, all iso-lates were subcultured on antimicrobial agent-free potato dextrose agar (Biolife Italiana, Italy) at 28 ºC for 7-15 days. The strain *Paecilomyces variotti* (ATCC 36257) was included as quality control.

Antifungal susceptibility tests were performed in modified Shadomy medium (dextrose –Merck, Germany- 10 g/l, Bacto-asparagin –Difco, USA- 1.5 g/l, yeast extract –Difco- 6.7 g/l and agar 15 g/l). The medium was sterilized, and 16 ml were poured and allowed to solidify onto 10 cm diameter Petri dishes (Greiner, Spain). Sterility control of medium batches was ensured.

Voriconazole, itraconazole and fluconazole were obtained from the manufacturers as standardized tablets of 9 mm diameter (NeoSensitabs, Rosco, Denmark). Diffusible antifungal charge of tablets was 10 µg for itraconazole, 25 µg for fluconazole and 1 µg for voriconazole.

Inoculum fungal suspensions were prepared from 7 to 15 days cultures grown on potato dextrose agar at 28 ºC. Mature colonies were covered using 10 ml of 0.85% sterile saline and Tween 20 (Difco), scraping the surface with the tip of a Pasteur pipette. The resulting mixture of conidia and hyphal fragments was transferred to sterile tubes. Heavy particles were allowed to sediment for 15 to 20 min at room temperature and supernatants were mixed with a vortex for 15 s. Supernatants turbidity was spectrophotometrically measured at a wavelength of 530 nm and transmission was adjusted to 65 to 70%. Inocula were quantified by plating 10 µl of a 1:100 dilution of the adjusted inoculum (1.8×10^4 to 6×10^6 UFC/ml) on potato dextrose agar.

A 2 ml inoculum was spread over the surface of agar and plates were dried 30 min prior to placement of the antifungal tablets on the surface of the plates. After 2 to 7 days of incubation in reverse position at 28 ºC, the inhibition diameter areas around the tablets were measured. Strains were classified according with the manufacturer's criteria, as susceptible (S), susceptible-dose dependent (SDD) and resistant (R) as follows: voriconazole: $R < 14$-, $S \geq 14$ mm; itraconazole: $R =$ no inhibition area, SDD 10-15 mm and $S \geq 16$ mm; and fluconazole: $R < 14$ mm, SDD 15-21 mm, $S \geq 22$ mm. Semi-inhibited colonies inside the inhibition area were not considered.

Table. In vitro susceptibility of 252 isolates of dermatophytes and *Scopulariopsis brevicaulis* to voriconazole, itraconazole and fluconazole.

Species (No. of isolates)	Voriconazole				Itraconazole							Fluconazole					
	S		R		S		S-DD		R			S		S-DD		R	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)		No.	(%)	No.	(%)	No.	(%)
Epidermophyton floccosum (20)	20	(100)			20	(100)						20	(100)				
Microsporum auodouinii (4)	4	(100)			3	(75)			1	(25)		1	(25)			3	(75)
Microsporum canis (34)	34	(100)			29	(85)	1	(3)	4	(11)		20	(59)	3	(9)	11	(32)
Microsporum gypseum (6)	5	(83)	1	(17)	6	(100)										6	(100)
Microsporum racemosum (1)	1	(100)							1	(100)						1	(100)
Scopulariopsis brevicaulis (20)	2	(10)	18	(90)	2	(10)	2	(10)	16	(80)				1	(5)	19	(95)
Trichophyton equinum (2)	2	(100)			2	(100)						2	(100)				
Trichophyton interdigitale (13)	12	(92)	1	(8)	9	(69)			4	(31)		2	(15)	5	(39)	6	(46)
Trichophyton mentagrophytes (41)	34	(83)	7	(17)	29	(71)	1	(2)	11	(27)		8	(20)	5	(12)	28	(68)
Trichophyton rubrum (84)	80	(95)	4	(5)	74	(88)			10	(12)		42	(50)	6	(7)	36	(43)
Trichophyton schoenleinii (6)	6	(100)			6	(100)						6	(100)				
Trichophyton soudanense (6)	6	(100)			6	(100)						5	(83)			1	(17)
Trichophyton terrestre (1)	1	(100)			1	(100)								1	(100)		
Trichophyton tonsurans (5)	5	(100)			4	(80)			1	(20)		1	(20)	1	(20)	3	(60)
Trichophyton violaceum (9)	9	(100)			8	(89)			1	(11)		5	(55)			4	(45)
Total	221	(87.6)	31	(12.4)	198	(78.5)	4	(1.6)	50	(19,8)		112	(44.5)	21	(8.3)	119	(47.2)

S: Susceptible; S-DD: Susceptible-dose dependent; R: Resistant.

Overall, nearly 90% fungal isolates tested were susceptible to voriconazole (Table 1). Lower numbers of isolates were susceptible to itraconazole (~ 80%) and fluconazole (~45%). The genus *Epidermophyton* was the most susceptible to all of them, with *T. interdigitale*, *M. gypseum*, and *T. mentagrophytes* being the less susceptible species to voriconazole (7.7%, 16.7%, and 17.1% of resistant isolates, respectively). Differences in the susceptibilities of the various species of *Microsporum* and *Trichophyton* are depicted in the table. According to their higher or lesser susceptibility to voriconazole, the different species of *Microsporum* could be classified as follow: *M. audouinii* ≈ *M. canis* ≈ *M. racemosum* > *M. gypseum*. In the case of *Trichophyton* spp., the order of susceptibility was *T. rubrum* ≈ *T. violaceum* ≈ *T. schoenleinii* ≈ *T. soudanense* ≈ *T. tonsurans* ≈ *T. equinuum* ≈ *T. terrestre* > *T. interdigitale* > *T. mentagrophytes*.

The excellent anti-dermatophyte activity of voriconazole encounter in this study coincides with that of previous reports [3,15,16,18,21,24,27,32]. Voriconazole and itraconazole were more effective than *in vitro* griseofulvin against most dermatophytes tested. Voriconazole was also more potent than fluconazole against the dermatophytes isolates [32]. Voriconazole possesses strong fungicidal activity against most filamentous fungi, likely due to the high affinity of voriconazole for fungal 14-α-demethylase, an activity supported by ultrastructural and biochemical analysis. The pharmacokinetics of voriconazole in man produced sustained high blood and tissue levels following oral and intravenous applications of 50 to 200 mg/day [24].

There is only a proposed reference methodology for determining broth dilution antifungal susceptibility in filamentous fungi [26], but a reference method for the antifungal susceptibility testing of dermatophytes is not yet available, although some progress has been made [10-12,19,20]. Variations in critical technical factors, such as inoculum size (variability in the proportion of different fungal structures, such as hyphae, macroconidia, and microconidia), type of medium, incubation temperature, and time of reading, are potential factors that may explain the different results in antifungal susceptibility testing so far obtained [21,28]. Previously, Cabañes et al. [7] adapted the agar diffusion method (NeoSensitabs) for the study of antifungal susceptibility of *E. floccosum* to several antifungal drugs. In the current study, some changes affecting the incubation temperature (28 °C instead of 35 °C), and incubation time (4 to 10 days instead of 21 to 74 h), has been introduced with good results.

In the present study, most *S. brevicaulis* isolates were very resistant to the three azole antifungal agents tested. Only two isolates were susceptible to voriconazole and itraconazole. The information regarding the susceptibility of this species to antifungal agents is sparse and somewhat contradictory. Most antifungals have limited *in vitro* activity against *Scopulariopsis* species, including amphotericin B and itraconazole [1,13]. A large *in vitro* study with voriconazole showed superior activity over amphotericin B and itraconazole [16]. However, other *in vitro* studies have shown contradictory results. Cuenca-Estrella et al. [13] reported general inactivity of six different antifungals, including voriconazole, against 32 clinical isolates of *S. brevicaulis* isolated from skin and nails. Johnson et al. [23] reported the resistance in vitro to amphotericin B and itraconazole of five strains of *S. brevicaulis*. Wildfeuer et al. [32], using a different susceptibility testing procedure, reported lower average MICs of amphotericin B, itraconazole, and voriconazole for 22 isolates. Promising results include a report of terbinafine showing in vitro synergy with fluconazole, itraconazole, and voriconazole against isolates of *S. brevicaulis* [30]. It should be emphasized that interpretative breakpoints for susceptibility testing of filamentous fungi are not available and clinical studies with this organism have yet to be reported.

The present study supports and expands previous findings on the excellent activity of voriconazole against dermatophytes, using various *in vitro* susceptibility test methods. Moreover, we found that the *in vitro* activity of voriconazole against dermatophytes was superior to those activities of fluconazole and itraconazole. Voriconazole did not show a similar activity against *S. brevicaulis* and probably a combination therapy of two or more antifungal agents might be needed for proper management of the infections caused by this fungus. These data support the concept that a clinical evaluation of voriconazole as potential antifungal drug in the treatment of recalcitrant superficial mycoses due to dermatophytes, is granted.

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