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Sertaconazole Antifungal Profile Determined by a Microdilution Method versus Nine Topical Substances against Dermatophyte Fungi

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Key Words
Topical antifungals · Dermatophytes · Dermatophytosis

Abstract
Antifungal activity and in vitro inhibition time for sertaconazole (STZ) and 9 other topical drugs, namely amorolfine, bifonazole, clotrimazole, econazole, ketoconazole, miconazole, oxiconazole, terbinafine, and tioconazole were determined against 124 clinical isolates of dermatophyte (12 species) fungi by the microdilution method in a liquid medium and the measurement of optical density. STZ's antifungal activity was not always affected by the tested dermatophyte genus, as was the case with the remaining antifungals. In vitro antifungal activity was at the same level for all the studied azole derivatives, but, in terms of partial inhibitory concentrations, STZ starts its in vitro inhibitory activity in a shorter time than the other tested substances, particularly in those incubation periods when the growth of the dermatophyte fungi was more developed.

Introduction

Dermatophyte fungi belong to a group of microorganisms that affects keratinized tissues in humans and other groups of vertebrate animals, causing various types of superficial infections [1–3]. In the case of immunocompromised patients, these infections cause atypical manifestations and frequently severe lesions [4]. In general, dermatophytosis responds well to antifungal therapy and several topical antifungals are available for clinical practice, even though the use of systemic treatments is occasionally required [5]. The purpose of this paper has been to comparatively assess the in vitro antifungal activity and the inhibition time developed by STZ and another 9 antifungal drugs for topical use against clinical isolates of dermatophyte fungi using a standardized micromethod in a liquid medium.

Materials and Methods

In vitro antifungal activity was determined for sertaconazole (STZ) compared to amorolfine (AMR), bifonazole (BFZ), clotrimazole (CLZ), econazole (ECZ), ketoconazole (KTZ), miconazole (MNZ), oxiconazole (OXZ), terbinafine (TRB) and tio-

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conazole (TCZ) against 124 clinical isolates of dermatophyte fungi (12 species) using the microdilution method in a liquid medium standardized by the Clinical Laboratory Standard Institute (CLSI) documents M27-A2, M38-A and M38-A2 [6–9]. Drugs were used as pure substances from Sigma-Aldrich and, in the case of STZ, the substance provided by the manufacturer was used (Grupo Ferrer, Barcelona, Spain). The standardized methods include the special requirements of dermatophyte fungi and were studied by several authors, enabling the assessment of antifungal activity based on the determination of minimum inhibitory concentrations (MIC) [5–13]. These requirements were related to incubation temperature (28°C) and prolonged incubation times from 2–3 days to 4–10 days or, alternatively, until some kind of fungal development was detected. The inoculum size is considered as one of the most important experimental factors within in vitro antifungal susceptibility studies [14]. A range of final inoculum sizes between 7×10^3 and 1.5×10^4 CFU/ml was used in this study, obtained by following standardized recommendations [8, 9]. Parent solutions were prepared in 100% DMSO at a concentration of 1,600 mg/ml and were frozen at –20°C until they were used. Serial double dilutions were made following the procedure described by the CLSI reference document [6–9].

The 124 clinical isolates included the dermatophyte fungi of different genera and species: *Epidermophyton flocccosum* (n = 10), *Microsporum audouinii* (n = 2), *M. canis* (n = 26), *M. gypseum* (n = 6), *M. racemosum* (n = 1), *Trichophyton equinum* (n = 1), *T. interdigitale* (n = 19), *T. mentagrophytes* (n = 26), *T. rubrum* (n = 25), *T. terrestre* (n = 1), *T. tonsurans* (n = 2) and *T. violaceum* (n = 5). Before the trial, the isolates were reidentified by biochemical and morphological tests in culture media and also via macroscopical microscopical observations [15]. The quality control strains *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 and the reference strains *Aspergillus fumigatus* NCPF 7100 and *A. fumigatus* NCPF 7099 were included as in previous research studies on standardization and adaptation to the requirements of dermatophyte fungi [6–13].

For the reading and determination of antifungal activity, the optical density variation was obtained in each case by the spectrophotometric measurement of microplate wells (460 nm wavelength) (Multiskan FC, Thermo Fisher Scientific™ Vantan, Finland) in different incubation periods as a modification of the CLSI method. A growth index (K) was calculated based on the equation $N_1 = N_0 e^{-(kt)}$, where N corresponded to the development of the inoculum obtained by the spectrophotometric measurement of the microplate wells [16, 17]. The measurement of this optical density in the control antifungal-free wells enabled the calculation of the inhibition index of the various antifungal concentrations. The calculations of the MIC geometric means and of 50 and 90% partial inhibitory concentrations (MIC_{50} and MIC_{90}, respectively, as MIC inhibiting the 50 and 90% of isolates) of the isolates were also obtained. MICs in the case of azole antifungal drugs corresponded to the wells that showed an 80% reduction in inocula growth with respect to the control wells. In the case of the nonazole antifungals, it was a 100% inhibition based on the criteria used in prior in vitro activity studies [12, 13]. Statistical analysis of data was performed by using the Student t and one-way ANOVA tests ($p < 0.05$).

Table 1. In vitro antifungal activity of 10 substances for topical use (μg/ml) against 124 clinical dermatophyte fungi isolates (geometric mean)

	2 days			3 days		
	MIC	MIC_{50}	MIC_{90}	MIC	MIC_{50}	MIC_{90}
AMR	0.017	0.015	0.015	0.021	0.015	0.062
BFZ	0.27	0.015	0.062	0.9	0.062	≥16
CLZ	0.023	0.015	0.062	0.023	0.015	0.25
ECZ	0.022	0.015	0.031	0.035	0.015	0.5
KTZ	0.023	0.015	0.031	0.045	0.015	2
MNZ	0.026	0.015	0.125	0.053	0.015	4
OXZ	0.022	0.015	0.062	0.048	0.015	1
STZ	0.024	0.015	0.062	0.062	0.015	1
TCZ	0.024	0.015	0.062	0.04	0.015	2
TRB	0.019	0.015	0.015	0.022	0.015	0.015

Results

Highest mean K indexes for the total of the dermatophyte fungi isolates studied (n = 124) in control wells was obtained in incubation periods of 3–4 days and 4–5 days (0.0111 and 0.0131, respectively). These values went down from 5 days of incubation and even below values detected between 2–3 days (0.0091). Within 3–5 days of incubation, the best antifungal activity rate corresponded to the 3-day period rather than to the 5-day period. Increased inhibition percentages caused by different antifungal concentrations were detected when the growth indexes were obtained. However, even though inhibition percentages were higher in incubation periods longer than 5 days, the growth indexes decreased among the antifungal-free inocula. This observed effect depended both on the substance and the genus and species of the tested dermatophyte fungus.

Partial inhibitory concentrations (MIC_{50}) showed that the activity of the substances used in this study was similar at 2–3 days of incubation among azole antifungals, but differences were obtained when comparing MIC_{90} among CLZ, ECZ, OXZ and STZ, since they were the antifungals with the greatest activity among the azole derivates when considering this value (table 1). Considering these substances as equal with respect to the partial MICs, lower STZ concentrations proved to be capable of inhibiting inocula in a shorter incubation period when the inocula had a higher growth index; this could be related to the fact that STZ inhibitis the ergosterol biosynthesis and, as a second added effect, binds to nonsterol lipids in the cell membrane as other azoles do, which al-

Table 2. Mean inhibition index values in different incubation times of 10 topical antifungals against dermatophyte fungi (n = 124)

Incubation days	AMR	BFZ	CLZ	ECZ	KTZ	MNZ	OXZ	STZ	TCZ	TRB
2–3	0.01871	0.01156	–0.0076	0.0241	0.01691	0.00921	0.04376	0.07385	0.032	0.01111
3–4	0.006	0.01469	0.02503	0.01572	0.01098	0.0204	–0.0091	–0.00174	0.01181	0.00592
4–5	0.0192	0.01950	0.01996	0.00797	0.02016	0.01041	0.02004	0.02047	0.0185	0.0111
5–6	–0.0185	–0.01247	0.00266	–0.01781	0.00275	–0.02485	–0.19665	–0.02253	0.00262	–0.01753
6–7	0.00024	0.01799	–0.01224	0.01815	–0.00986	0.0204	0.18032	0.00931	–0.00443	0.00014
7–8	0.00675	0.00428	0.00823	0.00937	–0.00124	0.00662	–0.03773	0.01227	0.00737	0.00651
8–9	–0.00789	–0.00015	0.00857	0.00136	0.00646	0.0021	0.0088	0.00193	0.00422	–0.00659
9–10	–0.0243	–0.01327	–0.00455	–0.00368	–0.00368	–0.0116	–0.12139	–0.00162	–0.00349	–0.02342

ters the cell viability [18]. Thus, the highest mean inhibition rate of inocula (between 2–3 and 4–5 days of incubation) was that produced by STZ, which also occurred at 7–8 and 9–10 days (table 2). In addition, overall for STZ, the greatest inhibition index occurred between 2–3 days of incubation (fig. 1), as also happened with ECZ, TCZ and TRB. However, TRB did not show the same high inhibition index during that time when compared to STZ (table 2).

A statistically significant difference was observed in favor of inhibition percentages and rates produced by STZ over the incubation period. When studying the rates for each test concentration, the maximal rate that was obtained at 2–3 days of incubation was also achieved for all the test concentrations, except for 0.031 μg/ml, which corresponded to the period of 4–5 days. During that incubation time, inhibition indexes were highest with 0.5 μg/ml STZ (0.0329). A paradoxical effect occurred when obtaining low inhibition rates at higher concentrations compared to lower ones as in the case of OXZ (0.02879–0.07168 at ≥16 and ≤0.015 mg/l, respectively) and TCZ (0.01986–0.05559 at ≤0.015 and 0.5 mg/l, respectively). However, OXZ concentrations of 0.062 and 0.031 mg/l caused maximum inhibition over very long incubation periods of 6 and 9 days, respectively. On the other hand, none of the CLZ concentrations caused any of the best inhibition indexes between 2 and 3 days of incubation as when delayed until 9–10 days. Concentrations of 4 mg/l MNZ and 0.25 mg/l ECZ were necessary to get the maximum effect at 3 days. In the case of ECZ, concentrations <0.25 mg/l produced the greatest inhibition indexes at 8–9 days. The comparison of the inhibition levels for BFZ and KTZ revealed STZ to have a better antifungal profile because in order to obtain the same effect achieved by STZ at 2–3 days, higher concentrations of BFZ and KTZ were necessary. This was also the case with >0.25 mg/l TRB. In the case of TRB, concentrations of 0.5 and 8 mg/l produced a maximum inhibition at 6–7 and 4–5 days, respectively.

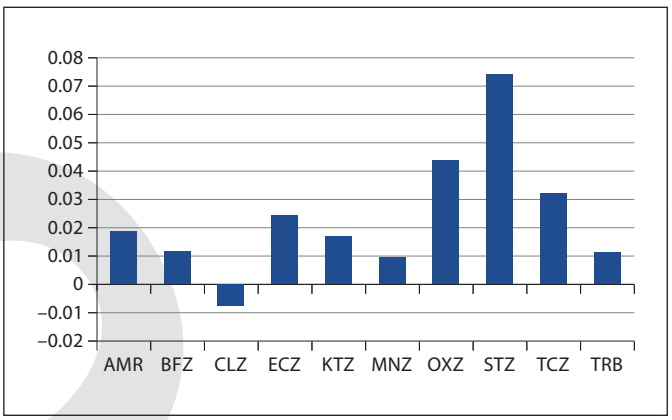

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Fig. 1. Comparison of mean inhibition index values at 2–3 days of 10 topical antifungals against dermatophytes.

The comparison of mean inhibition percentages and their variations over time was made for the 10 substances (table 3). At 3 days of incubation, the best mean inhibition percentage was produced by OXZ and STZ at the lowest trial concentration (0.015 mg/l), and was also achieved with concentrations of between 0.062 and 1 mg/l of OXZ, STZ and TRB (table 3). These same OXZ and STZ concentrations showed the highest inhibition indexes.

When comparing in vitro antifungal activity based on MIC_{90} and MIC_{50}, this was influenced by the incubation period, since statistically significant differences could be observed between substances at certain incubation periods (2, 3, 4 and 5 days in the case of BFZ, CLZ, ECZ, KTZ, MNZ, STZ and TRB, 3 and 4 days for OXZ and AMR and

Table 3. Inoculum development inhibition percentages for the 10 antifungals tested at different test concentrations (n = 124)

Concentration μg/ml	AMR	BFZ	CLZ	ECZ	KTZ	MNZ	OXZ	STZ	TCZ	TRB
≥16	25.84	25.64	24.22	35.53	34.05	34.57	40.19	32.86	40.85	34.32
8	13.58	27.46	23.54	35.2	33.04	37.18	41.35	26.57	38.44	28.32
4	13.4	28.97	24.8	34.58	32.33	32.21	41.16	31.25	32.01	33.36
2	11.5	22.7	20.73	30.38	20.91	26.44	35.67	30.89	30.51	31.54
1	3.67	20.04	22.03	25.48	19.62	25.29	35.58	27.07	22.91	24.84
0.5	0	19.12	18.99	23.26	17.62	18.23	32.77	27.71	17.54	27.62
0.25	4.31	16.27	16.43	20.26	16.65	18.02	32.56	25.41	23.22	26.28
0.125	0.53	10.84	13.34	18.79	15.08	19.06	28.98	23.66	14.07	22.47
0.062	5.55	14.36	14.02	1.02	8.26	13.57	27.32	18.93	17.16	20.17
0.031	0	11.98	13.34	20.5	9.15	13.38	28.1	13.94	14.71	16.81
≤0.015	5.03	4.16	0.9	11.68	8.88	7.41	19.72	15.75	11.42	9.01

These percentages are expressed as arithmetic mean of all isolates at 3 days of incubation.

2–3 days for TCZ). The differences between STZ and MNZ were also statistically significant at 5 days of incubation, but not at 3 days. Nevertheless, antifungal activity by STZ and MNZ remained in the same range at 2 and at 3 days (table 1).

The partial inhibitory concentrations of the antifungals tested (MIC_{50} 0.015 mg/l) showed some differences: while the MIC_{90} of STZ was 1 mg/l, the MIC_{90} of other substances was higher (TCZ and KTZ 2 mg/l, MNZ 4 mg/l and BFZ ≥16 mg/l). STZ and OXZ showed the same MIC_{90} at 3 days. When inhibition percentages were compared at 5 days, the concentration of 0.015 mg/l of STZ inhibited 29.8% of isolates above what was obtained by MNZ (25.8%) and BFZ (26%).

The genus and species of the dermatophyte fungus were seen to affect the in vitro activity of antifungals at 3 and at 8 days of incubation, since a statistically significant difference was obtained between the groups studied. Furthermore, it was possible to establish a species classification based on susceptibility to STZ, *M. audouinii* < *T. rubrum* < *M. canis* < *T. interdigitale* < *E. floccosum* < *M. gypseum* < *T. violaceum* with a statistically significant difference with respect to *T. mentagrophytes* < *T. tonsurans*. These interspecies differences were more widespread over the various incubation periods for the rest of the antifungals, as in the case of OXZ after 5 days of incubation, all incubation periods for TCZ (although with no statistical difference), after 4 days for CLZ and TRB and after 3 days for MNZ, BFZ and KTZ. Susceptibility to STZ was also homogeneous between species, and the indicated profile was maintained after 5 or 7 days of incubation.

The most active antifungals against *T. rubrum* and *T. interdigitale* in vitro were AMR, CLZ, ECZ, OXZ, STZ and TRB (table 4). There were no statistically significant differences among the antifungals tested and their in vitro activity against *E. floccosum*, even though different MICs and partial inhibitory concentrations characteristic for each substance were observed (table 4). Against *M. canis*, in vitro activity by ECZ, KTZ and STZ was in the same range, although it was below that of CLZ, MNZ, OXZ and TCZ. *T. terrestre* isolates showed reduced susceptibility to all the substances, but the reduced number of isolates prevented the calculation of partial inhibitory concentration values, as was also the case with *M. aoudouinii*, *M. racemosum*, *M. gypseum*, *T. equinum*, *T. tonsurans* and *T. violaceum* (table 4).

Considering the dermatophyte genus, the in vitro antifungal activity of STZ and OXZ was different from the other azole derivatives studied, regardless of the incubation time, the in vitro activity being *Microsporum* spp. > *Epidermophyton* sp. > *Trichophyton* spp. for both and *Microsporum* spp. > *Trichophyton* spp. > *Epidermophyton* sp. for the remaining azoles. AMR was active in the next sequence *Epidermophyton* sp > *Microsporum* spp. > *Trichophyton* spp. However, no statistically significant differences were evident between the activity that developed against *Epidermophyton* at 3 or 5 days, but they were displayed against *Microsporum* spp., as was the case between BFZ and the remaining substances. Against *Microsporum* spp., STZ was the only antifungal more or equally active than MNZ and KTZ (at 3 days) (table 4).

Table 4. Partial inhibitory concentrations of 10 antifungals against clinical dermatophyte fungi isolates

	AMR		BFZ		CLZ		ECZ		KTZ		MNZ		OXZ		STZ		TCZ		TRB	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
E.F. (n = 10)	0.015	0.015	0.015	1	0.015	1	0.015	4	0.015	2	0.015	8	0.015	0.125	0.015	2	0.015	4	0.015	2
M.A. (n = 2)	0.015		0.015		0.015		0.015		0.015		0.015		0.015		0.015		0.015		0.015	
M.C. (n = 26)	0.015	0.015	0.015	4	0.015	0.015	0.015	0.25	0.015	0.5	0.015	0.125	0.015	0.125	0.031	0.5	0.015	0.125	0.015	0.015
M.C. (n = 6)	0.018		0.39		0.024		0.054		0.08		0.03		0.098		0.07		0.06		0.06	
M.R. (n = 1)	0.015		0.015		0.015		0.015		0.015		0.015		0.015		0.015		0.015		0.015	
M.R. (n = 35)	0.015	0.015	0.015	4	0.015	0.031	0.015	0.25	0.015	0.5	0.015	0.125	0.015	0.25	0.031	0.5	0.015	0.125	0.015	0.015
T.E. (n = 1)	0.015		2		0.015		0.5		2		0.015		1		1		0.5		0.015	
T.I. (n = 19)	0.015	0.062	0.015	16	0.015	1	0.015	1	0.015	4	0.015	4	0.015	4	0.015	1	0.015	8	0.015	0.5
T.M. (n = 26)	0.015	0.031	0.061	16	0.015	1	0.015	2	0.015	0.5	0.015	8	0.031	2	0.031	16	0.015	0.5	0.015	0.015
T.R. (n = 25)	0.015	0.25	0.015	8	0.015	0.25	0.015	0.015	0.015	0.5	0.015	2	0.015	0.25	0.015	0.25	0.015	0.5	0.015	0.015
T.T.T. (n = 1)	8		16		16		2		4		16		8		16		16		4	
T.T. (n = 2)	0.125		4		0.021		0.061		0.5		0.04		1.41		2.82		0.7		0.015	
T.V. (n = 5)	0.015		0.039		0.026		0.015		0.015		0.015		0.015		0.18		0.015		0.015	
T.V. (n = 79)	0.015	0.06	0.015	16	0.015	0.5	0.015	0.5	0.015	2	0.015	4	0.015	1	0.015	4	0.015	2	0.015	0.015
Mean (n = 124)	0.015	0.062	0.015	16	0.015	0.25	0.015	0.5	0.015	2	0.015	4	0.015	1	0.015	2	0.015	2	0.015	0.015

The concentrations (in µg/ml) are expressed at 3 days of incubation. Values of species with more than 10 isolates correspond to the geometric means MIC (in µg/ml.)
E.F. = *E. floccosum*; M.A. = *M. audouinii*; M.C. = *M. canis*; M.G. = *M. gypseum*; M.R. = *M. racemosum*; T.E. = *T. equinum*; T.I. = *T. interdigitale*; T.M. = *T. mentagrophytes*; T.R. = *T. rubrum*; T.T. = *T. tonsurans*; T.T.T. = *T. terrestre*; T.V. = *T. violaceum*.

Discussion

The CLSI M38-A2 document determined the standardization of experimental conditions for the antifungal susceptibility study of dermatophyte fungus to antifungals. A consensus was reached on some experimental factors, such as inoculum size, temperature and incubation time [12, 13]. Previously published data show the validity of MIC obtained at 3–5 days of incubation [12, 13]. The data that we obtained with the microdilution method with a spectrophotometric measurement to determine the MICs made it possible to comparatively measure the activity of the antifungals at the time of the highest inoculum growth index, which was completely different from what was proposed by Alió et al. [19]. Using the macromethod, these authors obtained growth indexes with inoculum sizes greater than for 8 days of incubation and using an incubation temperature suboptimal for dermatophyte development. For a 3-day incubation time, some studies describe the best antifungal activity of substances as the most demonstrational, whereas in other cases this is assigned to 8 days [14, 19]. The differences may be attributed to the use of macromethods for which the best growth rates are obtained with longer incubation times, and also to the optical density wavelength, which in our case was 460 nm instead of 570 nm [17]. Due to the characteristic mechanism of each substance, in vitro antifungal action may be determined in the incubation period when the greatest cell growth index takes place [4, 20].

In our study, this phase was between 2 and 5 days of incubation. Longer periods generated a lower growth index, which may be associated with the loss of the culture medium's nutritional properties and/or the appearance of toxic metabolites, which in some papers is related to the appearance of fungal resistance elements [21, 22].

The susceptibility profile of dermatophyte fungi seems to be genus/species-dependent as previously described for BFZ, STZ and TRB, but with the use of nonstandardized culture media [10, 11, 21–23]. Our data suggest that the antifungal concentration is important because different inhibition percentages are obtained for inocula and isolates, which in some cases are not related to an increase in the concentration. In addition, in some cases, a variation in those values occurs at each concentration over the incubation periods, thereby reducing the inhibition effect and with no statistically significant differences on the effect of different concentrations or of different antifungals in the same incubation period.

In vitro antifungal activity for STZ was at the same level as that for TCZ, MNZ, ECZ and KTZ and above BFZ in terms of partial inhibitory concentrations, as in some previous studies [11]. STZ in vitro activity was especially important in this incubation period when the maximum K occurred, and STZ was the antifungal that first initiated the in vitro antifungal activity. STZ was particularly active in vitro against *T. rubrum* isolates, in comparison to BFZ and KTZ. The same occurred against *E. floccosum* isolates, but in this case STZ was more active in vitro than CLZ, TCZ and BFZ. A similar STZ profile was already established in previous studies that did not compare such a broad spectrum of topical substances. There are differences among studies; in our study, the incubation periods were shorter [11, 14, 16]. In the previous studies, STZ was already described as active against *E. floccosum*, *M. canis*, *T. rubrum* and *T. tonsurans* [11, 14, 16].

Due to the differences found in the antifungal susceptibility profiles and in vitro activity times of dermatophyte genera and species, the accurate identification of the etiological agent in the laboratory is of vital importance. STZ antifungal activity does not seem to be genus-dependent and, even though its activity levels are not different from the levels of other azole drugs, it is possible to find differences similar to the ones that were exposed, i.e. constituting differential characteristics among them. In this sense, STZ is capable of initiating in vitro antifungal action before other antifungals for topical use.

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