Medical Mycology September 2008, **46**, 567–573

informa
healthcare

Comparative *in vitro* activities of posaconazole, voriconazole, itraconazole, and amphotericin B against *Aspergillus* and *Rhizopus*, and synergy testing for *Rhizopus*

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We compared the *in vitro* activities of posaconazole, voriconazole, itraconazole, and amphotericin B against clinical isolates of *Aspergillus* spp. and *Rhizopus* spp., and explored the *in vitro* interaction between posaconazole and amphotericin B against *Rhizopus* spp. Clinical strains of 82 *Aspergillus* spp. (43 *Aspergillus fumigatus*, 29 *A. flavus*, 7 *A. niger*, 2 *A. terreus*, 1 *A. nidulans*) and 11 *Rhizopus oryzae* isolates were tested in accordance with CLSI M38-A microdilution guidelines. *In vitro* activity of posaconazole against *Aspergillus* spp. was also investigated with the Etest. The combination of posaconazole and amphotericin B against *R. oryzae* isolates was investigated by the checkerboard methodology. Voriconazole was the most active drug *in vitro* against *Aspergillus* spp., followed by posaconazole, itraconazole, and amphotericin B, in order of decreasing activity. In studies with *R. oryzae* isolates, posaconazole was found to be the most potent drug followed by itraconazole and amphotericin B. Voriconazole had no meaningful activity against *Rhizopus*. Posaconazole Etest MICs (μg/ml) with *Aspergillus* spp. were found to be considerably lower than those obtained with the CLSI microdilution method (4–9 and 3–7 two-fold lower than CLSI MICs at 24 and 48 h, respectively). The interaction between posaconazole and amphotericin B was indifferent for all *R. oryzae* isolates tested; importantly no antagonism was observed.

Keywords *Aspergillus*, *Rhizopus*, Antifungal susceptibility test, checkerboard

Introduction

Aspergillus and *Rhizopus* are two genera of filamentous fungi that cause severe, frequently fatal infections in immunocompromised patients [1,2]. Treatment of invasive aspergillosis [2] and zygomycosis [3,4] is problematic and frequently associated with suboptimal therapeutic outcomes. The availability of the novel drugs, including voriconazole and posaconazole, appears to offer the potential for improved therapy for patients with these infections and necessitates the determination of comparative *in vitro* activities and clinical efficacies of the available antifungal agents in clinical use [5–10]. While voriconazole is now the drug of choice in primary treatment of invasive aspergillosis [11], it has little or no activity against *Rhizopus* and other members of the Zygomycetes [3,9,12]. Posaconazole, on the other hand, appears promising in the treatment of disseminated zygomycosis, as well as invasive aspergillosis [4,13,14]. The use of posaconazole for treatment of certain serious fungal infections (including invasive aspergillosis, fusariosis, chromoblastomycosis, mycetoma, and coccidioidomycosis) in

Received 29 March 2007; Received in final revised version 15 January 2008; Accepted 10 February 2008
This study was presented at 44th Interscience Conference on Antimicrobial Agents and Chemotherapy, 30 October to 2 November 2004, Washington, DC. Abst. no. M-1799, p. 436.
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DOI: 10.1080/13693780801975576

adult patients who remain refractory to or intolerant of commonly used antifungal drugs was approved in the European Union. Its use in treatment of oropharyngeal candidiasis and prophylaxis for *Candida* and *Aspergillus* infections in severely immunocompromised patients has been approved by FDA [15].

The development of novel antifungal drugs also raises the possibility that combinations of antifungals may provide enhanced *in vitro* and/or *in vivo* efficacy as compared to the use of single drugs against *Aspergillus* and *Rhizopus* infections [16–18]. The *in vitro* interaction of various combinations of antifungals has been explored for *Aspergillus* spp. [19–22]. *In vitro* interaction studies with R*hizopus* spp. have been carried out for the combinations of rifampin-amphotericin B, flucytosine-amphotericin B, terbinafine-amphotericin B, and terbinafine-voriconazole [23]. There are no published data on the *in vitro* combination of amphotericin B and posaconazole against *Rhizopus*.

The present study was undertaken to investigate: (i) The comparative *in vitro* activities of posaconazole, voriconazole, itraconazole, and amphotericin B against *Aspergillus* and *Rhizopus*, (ii) the agreement between Etest and microdilution assay for susceptibility testing of posaconazole against *Aspergillus*, and (iii) the *in vitro* interaction between posaconazole and amphotericin B against *Rhizopus*.

Materials and methods

Isolates

A total of 82 clinical isolates of *Aspergillus* (43 *Aspergillus fumigatus*, 29 *A. flavus*, 7 *A. niger*, 2 *A. terreus*, 1 *A. nidulans*) and 11 clinical strains of *Rhizopus oryzae* were included in the study. *Aspergillus* isolates were recovered from 50 respiratory specimens (23 sputum, 21 bronchoalveolar lavage fluid, 6 tracheal aspiration), 21 biopsy samples (9 nasal, 4 sinus, 4 skin, 2 lung, 1 lymph node, and 1 oral cavity lesion), 5 pus specimens, 4 sinonasal aspiration (2 sinus, 2 nasal), 1 blood culture and 1 bone marrow aspiration. *R.oryzae strains* were isolated from 5 biopsy samples (2 sinus, 1 mucosal, 1 skin, 1 oral cavity lesion), 4 respiratory specimens (2 bronchoalveolar lavage fluid, 2 sputum), and 2 ophthalmic specimens (1 vitreous fluid sample, 1 corneal scraping).

Conventional mycological methods were used in the identification of the species of the *Aspergillus* isolates, while the strains of *Rhizopus* were identified to the genus level [24]. The isolates of the latter were identified to species by the sequencing of the internal transcribed spacer (ITS) region of rRNA gene complex. Cycle sequencing was performed in RefGen Biotechnology Laboratory (Ankara, Turkey) using a BigDye Terminator v.3.1 cycle sequencing kit in an ABI PRISM 3100-Avant genetic analyzer (Applied Biosystems, Foster City, CA). Sequences were analyzed using Sequencing Analysis Software v.5.1 (Applied Biosystems). The obtained sequences were compared to all known sequences in the Genbank by use of BLAST (National Center for Biotechnology Information, Bethesda, MD. [http://www.ncbi.nlm.nih.gov/BLAST/]).

Candida krusei ATCC 6258 and *Candida parapsilosis* ATCC 22019 were included as quality control in susceptibility tests.

Antifungal susceptibility testing

For microdilution antifungal susceptibility testing, posaconazole (Schering-Plough Research Institute), voriconazole (Pfizer Inc.), itraconazole (Janssen Pharmaceutica), and amphotericin B (Bristol-Myers Squibb) were provided in standard powder form by the respective manufacturers. Microbroth susceptibility testing was performed as described in the CLSI document M38-A [25]. The minimum inhibitory concentration (MIC, μg/ml) values were read visually after 24 and 48 h of incubation and determined by using the endpoint of MIC-0, i.e., the complete inhibition of growth for all drugs. For the CLSI approved *Candida* QC strains, the CLSI M27-A2 guidelines were employed [26]. The MIC values were established by using the MIC-0 endpoint for amphotericin B, and MIC-2 ($\sim$50% reduction in turbidity compared to the growth control) endpoint was employed for the azoles. CLSI microdilution antifungal susceptibility testing was performed at least twice for each isolate included in the study.

Posaconazole MICs for *Aspergillus* spp. were also determined through the use of the Etest on RPMI agar plates supplemented to 2% glucose as directed by the manufacturer (AB Biodisk). The Etest MICs were read as the concentration point at which the growth on the plate intersects the Etest strip. To directly compare the Etest and CLSI microdilution values, the Etest MIC was converted to the next higher concentration which corresponds to the two-fold dilution series used in the CLSI method. Since MIC breakpoints have not been determined for the genera and the antifungal drugs tested, the microdilution and Etest results were evaluated by calculating the geometric mean (GM) MICs and MIC ranges.

In vitro *antifungal combination testing*

Checkerboard tests were done to determine the *in vitro* interaction between posaconazole and amphotericin B against *R. oryzae* isolates. Combination testing was conducted at least 2 times for each isolate included in the study. The fractional inhibitory concentration index (FICI, μg/ml) of posaconazole-amphotericin B combination for each *Rhizopus* strain was calculated as $(MIC_{drugA\ in\ combination}/MIC_{drugA\ alone})+(MIC_{drugB\ in\ combination}/MIC_{drugB\ alone})$. Checkerboard plates were read at both 24 and 48 h using the MIC-0 endpoint. FICI values were interpreted as follows: FICI ≤ 0.5 = synergistic; $0.5 <$ FICI ≤ 4 = indifferent; and FICI > 4 = antagonistic [27,28].

Results

The GM MICs and MIC ranges obtained after 24 and 48 h of incubation with *Aspergillus* and *Rhizopus* strains are shown in Table 1. The 48 h MICs were either identical to or one or two-doubling dilutions higher than the 24 h MICs for all drugs and for most of the isolates tested. In general, voriconazole was the most active drug against *Aspergillus* strains, followed by posaconazole, itraconazole, and amphotericin B in rank activity order. On the other hand, posaconazole was the most active drug against *Rhizopus* strains, followed by itraconazole, amphotericin B, and voriconazole in order of activity. The activity of posaconazole was similar against *Aspergillus* spp. and *Rhizopus*, while voriconazole had favorable activity against *Aspergillus*, but no meaningful activity against *Rhizopus* strains. Itraconazole and amphotericin B were slightly more active against *Aspergillus* spp. than *Rhizopus* isolates (Table 1).

The inter-species variability of the activity of the tested drugs against *Aspergillus* was also evaluated (Table 1). Due to the low number of the *A. nidulans* and *A. terreus* isolates, they were not included in the analysis of this inter-species variability. Posaconazole, voriconazole, and itraconazole were similarly active against *A. fumigatus*, *A. flavus*, and *A. niger*. On the other hand, amphotericin B was slightly more active against *A. niger* than *A. fumigatus* and *A. flavus*. The comparison of Etest and CLSI microdilution method for the assessment of the activity of posaconazole against *Aspergillus* showed that Etest MICs were considerably lower than those obtained with the CLSI microdilution method (4–9 and 3–7 two-fold lower at 24 and 48 h, respectively) (Table 1).

FICI values for the combination of posaconazole and amphotericin B against 11 *R. oryzae* isolates are shown in Table 2. Posaconazole and amphotericin B yielded indifferent interactions for all *R. oryzae* strains tested (FICI = 0.75–1.5 and 0.63–1.5 at 24 and 48 h, respectively). Against all strains and at both incubations times, posaconazole alone was at least as active as amphotericin B by itself. There was no evidence of antagonism between posaconazole and amphotericin B.

Discussion

The *in vitro* activities of posaconazole, voriconazole, itraconazole, and amphotericin B against clinical *Aspergillus* strains have been previously reported. Manavathu et al. [29] tested posaconazole, voriconazole, itraconazole, and amphotericin B against isolates of *A. fumigatus*, *A. flavus*, and *A. niger*. They reported that none of the drugs exhibited any significant inter-species differences in their activity. In contrast, in our hands, amphotericin B appeared to be slightly more active against *A. niger* than *A. fumigatus* and *A. flavus*. The same authors noted that posaconazole was significantly more active than voriconazole, itraconazole, and amphotericin B against both *A. fumigatus* and non-*A. fumigatus Aspergillus* spp. (including *A. flavus and A. niger*) [29]. Our results indicated that of the drugs included in the present study (posaconazole, voriconazole, itraconazole, and amphotericin B), voriconazole was the most active against *Aspergillus* spp.

In vitro activities of posaconazole, itraconazole, voriconazole, and amphotericin B against *Rhizopus* spp. have been explored by Sun et al. [6]. However, a direct comparison of their results with those of our investigation is not possible due to differences in the test parameters. Sun et al. used Antibiotic Medium 3 in studies with amphotericin B rather than RPMI 1640 as the test medium. In addition, they employed MIC-2 endpoint instead of MIC-0 for the azole antifugnals. At 48 h, the amphotericin B MICs for *R. oryzae* isolates in the present investation are considerably higher than those reported by Sun et al. (GM MIC of 3.53 vs. 0.33, respectively). There are comparative data for posaconazole, caspofungin and voriconazole tested simultaneously in Antibiotic Medium 3 and RPMI 1640 medium against *Rhizopus* [30]. The MICs obtained using Antibiotic Medium 3 tended to be lower than those obtained using RPMI-1640. There are no published reports on the influence of Antibiotic Medium 3 on amphotericin B MICs against *Rhizopus*. However, Antibiotic Medium 3 was previously shown to lower the amphotericin B MICs as compared to RPMI 1640 against *Aspergillus* spp. and *Fusarium* spp. [31]. This MIC-lowering effect of Antibiotic Medium 3 may be one of the factors that has led to the lower amphotericin B

Table 1 MICs of posaconazole, voriconazole, itraconazole, and amphotericin B against clinical *Aspergillus* and *Rhizopus* isolates.

Test isolates (number tested) Incubation time	Posaconazole CLSI microdilution		Posaconazole Etest		Voriconazole CLSI microdilution		Itraconazole CLSI microdilution		Amphotericin B CLSI microdilution	
	GM	Range	GM	Range	GM	Range	GM	Range	GM	Range
Aspergillus (Total, $n=82$)										
24 h	0.96	0.5–1	0.02	0.002–0.03	0.49	0.125–1	0.93	0.25–2	1.82	0.5–4
48h	1.01	0.5–2	0.02	0.0075–0.125	0.80	0.125–2	1.13	0.25–2	2.51	1–8
A. fumigatus (43)										
24 h	0.94	0.5–1	0.01	0.002–0.03	0.45	0.125–1	1	0.5–2	1.73	1–4
48 h	0.97	0.5–2	0.03	0.0075–0.125	0.72	0.125–2	1.21	0.5–2	2.39	2–8
A. flavus (29)										
24 h	1	1	0.02	0.0075–0.03	0.65	0.25–1	0.83	0.25–1	2.2	1–4
48 h	1.02	1–2	0.06	0.03–0.125	1	0.5–2	0.98	0.25–2	2.93	2–4
A. niger (7)										
24 h	0.91	0.5–1	0.01	0.002–0.03	0.30	0.125–0.5	0.91	0.5–2	1.21	0.5–2
48 h	1.22	1–2	0.07	0.06–0.125	0.67	0.25–1	1.35	0.5–2	1.81	1–2
A. terreus (2)										
24 h	–	1	–	0.0075–0.03	–	0.5	–	1	–	2
48 h	–	1	–	0.015–0.125	–	1	–	1	–	2–4
A. nidulans (1)										
24 h	–	1	–	0.03	–	0.125	–	1	–	2
48 h	–	1	–	0.125	–	0.25	–	1	–	2
R.oryzae ($n=11$)										
24 h	1.13	1–2	ND	ND	15.02	8 →8	2	2	2.57	2–4
48h	1.55	1–2	ND	ND	15.02	8 →8	3.75	2–8	3.53	2–8

GM, Geometric mean; ND, not determined.

MICs for *Rhizopus* reported by Sun et al. Alternatively, differences in the *Rhizopus* spp. included in the studies may also explain the differences.

The MICs found in our study and by Sun et al. are similar for posaconazole, voriconazole, and itraconazole against *Rhizopus* and posaconazole is the most active triazole, followed by itraconazole and voriconazole. The results of both of the studies confirm that voriconazole has no meaningful *in vitro* activity against *Rhizopus* [6].

Dannaoui et al. also used MIC-2 endpoint (instead of MIC-0) for susceptibility testing of the azoles [10] against *Rhizopus*. The azole MICs generated in their hands are slightly lower than our MICs (24 h, GM MIC; posaconazole: 0.27 vs. 1.13, voriconazole: 8.77 vs. 15.2; itraconazole: 0.87 vs. 2, respectively). Although the same testing conditions were used for amphotericin B susceptibility testing in the two studies, the amphotericin B MICs reported by Dannaoui et al. are also lower than those in this study (24 h, GM MIC: 0.42 vs. 2.57, respectively) [10]. These results in general suggest that amphotericin B MICs against *Rhizopus* spp. may be variable and again this may be due to the different species of *Rhizopus* tested.

In the study of Sabatelli et al. [32], *in vitro* activities of posaconazole, itraconazole, voriconazole and amphotericin B against 1,423 *Aspergillus* and 32 *Rhizopus* strains were determined by using CLSI M38-A method, with the exception of amphotericin B which was tested in Antibiotic Medium 3. Comparable to our results, posaconazole was more potent than the other triazoles against *Rhizopus* spp. Although posaconazole was found to be more active than amphotericin B in our study, amphotericin B was reported to be more active than posaconazole in their hands. For *Aspergillus*, on the other hand, voriconazole was the most active drug in our study while posaconazole was either more potent than or equivalent to itraconazole, voriconazole and amphotericin B in the Sabatelli's study.

The GM MICs reported here were higher than those reported by Cuenca-Estrella et al. [33], i.e., GM MICs for 0.80, 1.01, 1.13, 2.51 vs. 0.48, 0.10, 0.33, 0.41 for voriconazole, posaconazole, itraconazole, and amphotericin B, respectively. While voriconazole was the most active drug against *Aspergillus* spp. followed by posaconazole, itraconazole and amphotericin B in our investigation, posaconazole exhibited the most potent activity, followed by itraconazole, amphotericin

Table 2 MIC (μg/ml) and FICI results obtained for posaconazole (POS) and amphotericin B (AMB) combination against 11 *Rhizopus oryzae* isolates. All FICI results were interpreted as *indifferent* interaction.

Strain Item code	Incubation time	AMB MIC		POS MIC		FICI
		Alone	Combined	Alone	Combined	
26	24 h	2	0.5	1	0.5	0.75
	48 h	2	1	2	1	1
32	24 h	2	1	1	1	1.5
	48 h	4	1	2	1	0.75
34	24 h	4	1	1	1	1.25
	48 h	8	1	2	1	0.63
36	24 h	2	1	1	1	1.5
	48 h	4	1	1	1	1.25
48	24 h	2	1	1	1	1.5
	48 h	2	1	1	1	1.5
61	24 h	4	1	1	1	1.25
	48 h	4	1	2	1	1.25
72	24 h	2	1	1	1	1.5
	48 h	4	1	1	1	1.25
103	24 h	4	1	2	1	0.75
	48 h	4	1	2	1	0.75
107	24 h	2	1	1	1	1.5
	48 h	2	1	1	1	1.5
111	24 h	4	1	1	1	1.25
	48 h	4	1	2	1	0.75
130	24 h	2	1	2	1	1
	48 h	4	1	2	1	0.75

FICI, Fractional inhibitory concentration index; AMB, Amphotericin B; POS, Posaconazole.

B and voriconazole against 697 *Aspergillus* strains in the study of Cuenca-Estrella et al.

There have been previous comparative studies of the Etest and CLSI reference microdilution broth method with posaconazole against *Candida* spp. [34,35] and filamentous fungi [36,37]. For *Candida* species, Diekema et al. observed that Etest results correlated well with microdilution MICs except for *C. glabrata* [35]. Overall agreement between Etest and reference microdilution MICs with molds was found to be 84% [36] and 99.3% [37] for *Aspergillus* spp. and 100% for the less common opportunistic mold, except for *Penicillium* spp [36]. When a discrepancy was observed between the reference method and Etest, the Etest tended to give lower MIC values. We also reported lower MIC values with the Etest as compared to CLSI microdilution method relative to posaconazole activity against *Aspergillus* spp. In the study of Pfaller et al., the MIC results obtained by M38-A microdilution method and Etest showed that all isolates of *Aspergillus* spp. were inhibited by ≤ 1 μg/ml posaconazole at 48h. Although our posaconazole MICs obtained by CLSI M38-A microdilution method were similar to those reported by Pfaller et al., the MICs obtained by Etest method were significantly lower than those described by these authors [36].

Due primarily to their multidrug resistant nature, treatment of infections caused by zygomycetes has proven to be increasingly problematic. In recent years, the activity of antifungal combinations against these fungi has been under investigation. To our knowledge, the combination of posaconazole and other drugs against zygomycetes has not been studied. Therefore, this investigation is the first to evaluate the *in vitro* efficacy of a combination of posaconazole and amphotericin B against *Rhizopus*. Although we did not detect any synergistic interaction, it is important to note that neither was antagonism observed with the combination of these two drugs. This *in vitro* result is in accordance with the *in vivo* data reported for *A. flavus* by Najvar et al. [38]. These authors investigated the *in vivo* interaction of posaconazole and amphotericin B against *A. flavus* infection in mice and found no antagonistic interaction of these drugs in combination.

In conclusion, our results suggest that voriconazole is the most active drug *in vitro* against *Aspergillus* spp., followed by posaconazole, itraconazole, and amphotericin B. Against *Rhizopus* spp., on the other hand, posaconazole is the most active drug *in vitro* followed by itraconazole and amphotericin B. Voriconazole has no meaningful activity against *Rhizopus* spp. *In vitro* combination of posaconazole and amphotericin B yields indifferent interaction against *Rhizopus* spp but

the drugs are not antagonistic. Further *in vitro* and *in vivo* studies are required to clarify the clinical significance of these findings. The poor correlation between CLSI microdilution and Etest for posaconazole also demands further investigation and wider data set, including other medically important filamentous fungi.

Conflict of interest

Paul McNicholas is an employee of Schering Plough Research Institude. No conflict of interest for the other authors.

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This paper was first published online on iFirst on 10 March 2008.