ELSEVIER

Diagnostic Microbiology and Infectious Disease
47 (2003) 000–000

DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE

www.elsevier.com/locate/diagmicrobio

In vitro interactions of licensed and novel antifungal drugs against *Fusarium* spp

Montserrat Ortoneda[a], Javier Capilla[a], F. Javier Pastor[a], Isabel Pujol[b], Josep Guarro[a,*]

[a]*Unitat de Microbiologia, Facultat de Medicina, Hospital Universitari de Sant Joan de Reus, Universitat Rovira i Virgili, Reus, Spain*
[b]*Laboratori de Microbiologia, Hospital Universitari de Sant Joan de Reus, Universitat Rovira i Virgili, Reus, Spain*

Received 25 April 2003; received in revised form 27 August 2003

Abstract

We have studied the in vitro interactions of amphotericin B (AMB) with terbinafine (TBF), itraconazole, voriconazole (VCZ), albaconazole, and ravuconazole (RVZ), as well as TBF combined with the same azoles, against 11 isolates of *Fusarium* spp. using the fractional inhibitory concentration index. The highest percentage of synergistic interactions was observed for the combinations AMB-RVZ, TBF-VCZ, and TBF-RCZ.

Fusarium spp. are opportunistic filamentous fungi that cause severe infections in humans, particularly in patients with hematologic malignancies or hematopoietic stem cell transplant recipients (Guarro and Gené, 1995; Boutati and Anaissie, 1997; Pontón et al., 2000). *F. solani*, *F. oxysporum*, and *F. verticillioides* are the species of this genus most frequently found in disseminated infections (Guarro and Gené, 1995). These fungi show in vitro and in vivo resistance to practically all the available antifungal drugs. Despite its nephrotoxicity and its low efficacy, amphotericin B (AMB) is still the drug of choice for the clinical treatment of fusariosis (Boutati and Anaissie, 1997). Unfortunately, the novel antifungal agents tested have not proved to be more active in vitro (Capilla et al., 2001; Espinel-Ingroff, 2001; Pfaller et al., 2002), and there is little clinical experience with them (Ernst, 2001). In the last 2 years, there has been an increasing tendency to determine the in vitro activity of combinations of drugs against opportunistic fungi (Ryder and Leitner, 2001; Sugar, 2001; Perea et al., 2002; Meletiadis et al., 2003). Only the interactions AMB-caspofungin and nikkomycin-FK463 have been investigated against *Fusarium* spp. (Chiou et al., 2001; Arikan et al., 2002).

In this study, we evaluated the in vitro interaction of 6 antifungal drugs in pair combinations against 11 strains of *Fusarium* spp.

Eleven clinical isolates of *Fusarium* spp. (five isolates of *Fusarium solani*, three isolates of *F. verticillioides*, and three isolates of *F. oxysporum*) were tested. *Paecilomyces variotii* ATCC 36257 was included in each batch of tests as a reference strain to ensure quality control. The isolates were stored in potato dextrose agar (PDA) slants covered with paraffin oil, subcultured on PDA plates, and incubated at 30°C for 7 to 10 days.

Antifungal agents were obtained as pure powders. AMB (USP, Rockville, MD), itraconazole (ITZ) (Janssen Pharmaceutica, Beerse, Belgium), voriconazole (VCZ) (Pfizer Inc., Madrid, Spain), albaconazole (ABZ) (J. Uriach & Cía., Barcelona, Spain), ravuconazole (RVZ) (Bristol-Myers Squibb Company, New Brunswick, NJ), and terbinafine (TBF) (Novartis, Basel, Switzerland) were dissolved in DMSO and diluted in RPMI 1640 buffered with MOPS.

Inocula were prepared by scraping the surface of the fungal colonies from the agar plates with a loop and suspending them in sterile saline solution. The suspensions were then filtered through sterile gauze to remove hyphae. The filtrates were vortexed and adjusted with a spectrophotometer to 68–70% transmittance at 530 nm. The final inoculum sizes ranged from 0.4 to 3×10^4 conidia/ml verified by plating serial dilutions on PDA plates.

Drug interactions were assessed by a checkerboard design to provide a matrix of all possible dose combinations of the paired drugs within the required concentration range. Each microplate included single-drug minimum inhibitory concentration (MIC) determinations, performed using the

* Corresponding author. Tel.: +977-759359; fax: +977-759322.
E-mail address: umb@fmcs.urv.es (J. Guarro).

0732-8893/03/$ – see front matter
doi:10.1016/j.diagmicrobio.2003.09.003

Table 1
Distribution of the FICI values for the three species of *Fusarium* tested

	F. solani (5[a])			*F. verticillioides* (3)			*F. oxysporum* (3)			All isolates (11)		
	S[b]	A	I	S	A	I	S	A	I	S	A	I
AMB/ITZ[c]	3	2	0	0	3	0	1	2	0	4	7	0
AMB/VCZ	0	4	1	1	2	0	2	1	0	3	7	1
AMB/ABZ	0	4	1	0	3	0	0	3	0	0	10	1
AMB/RVZ	2	3	0	3	0	0	1	2	0	6	5	0
AMB/TBF	1	3	1	0	3	0	2	1	0	3	7	1
TBF/ITZ	0	0	5	2	1	0	2	1	0	4	2	5
TBF/VCZ	2	2	1	3	0	0	3	0	0	8	2	1
TBF/ABZ	0	0	5	3	0	0	2	1	0	5	1	5
TBF/RVZ	3	2	0	3	0	0	3	0	0	9	2	0

[a] number of strains tested.
[b] S, synergistic; A, additive or subadditive; I, indiferent.
[c] AMB, amphotericin B; ITZ, itraconazole; VCZ, voriconazole; ABZ, albaconazole; RVZ, ravuconazole and TBF, terbinafine.

parameters outlined in the NCCLS guidelines (National Committee for Clinical Laboratory Standards, 2002), to minimize variations. AMB and TBF were placed in the rows of the trays with final concentrations that ranged from 0.125 to 8 and 0.5 to 32 mg/L, respectively. Azoles were placed in the columns of the trays with final concentrations that ranged from 0.03 to 16 mg/L. When AMB and TBF were tested in combination, TBF was placed in the columns with a final concentration that ranged from 0.06 to 32 mg/L. The MIC of all drugs was defined as the lowest drug concentration that produced 100% inhibition of visible fungal growth after 48 h of incubation at 35°C. No trailing endpoints were observed for any of the drugs. For purposes of calculation, off-scale MIC values were converted to the next higher dilution.

The nature of the interaction between two drugs was defined by means of the fractional inhibitory concentration index (FICI), which was calculated using the following formula: $FICI = MIC_A{}^{comb}/MIC_A{}^{alone} + MIC_B{}^{comb}/MICB^{alone}$, and rounded to the nearest 0.1 unit. We defined the interaction as synergistic if FICI <1, additive if FICI $= 1$, subadditive if between 1 and 2, indifferent if FICI $= 2$, and antagonistic if FICI > 2 (Perea et al., 2002).

For all the species, AMB when tested alone showed a MIC_{90} (MIC value at which 90% of the isolates of *Fusarium* spp. were inhibited) of 4 mg/L, and MIC values ranged from 0.5 to 4 mg/L. All the azoles showed a MIC_{90} of 32 mg/L, and MIC values ranged from 16 to 32 for ITZ, VCZ and ABZ, and from 2 to 32 for RVZ. TBF showed a MIC_{90} of 64 mg/L, and MIC values ranged from 8 to 64 mg/L. These values confirmed the generalized resistance of *Fusarium* spp. (Pujol et al., 1997; Pfaller et al., 2002). Only AMB and RVZ showed relatively low MICs against one strain of *F. solani* (MIC of 0.5–2 mg/L) and one of *F. verticillioides* (MIC of 2 mg/L), respectively.

The in vitro interactions of the 6 antifungal drugs tested in this study are shown in Table 1. Twenty-nine percent of the tests using AMB combined with the other drugs were synergistic, 65.5% were additive or subadditive, and 5.5% indifferent. Synergy of AMB combined with RVZ was observed for 6 of the 11 strains tested. The most remarkable combinations were AMB-ITZ against *F. solani*, AMB-RVZ against *F. verticillioides*, and AMB-VCZ and AMB-TBF against *F. oxysporum*. In general, the MICs of these azoles in combination decreased dramatically (up to 10 2-fold dilutions, often from an off-scale endpoint), while AMB MICs decreased very slightly (one or two twofold dilutions) or remained the same.

AMB and azoles have traditionally shown an antagonistic mutual effect, especially when added simultaneously (Polak, 1999). Although no antagonistic effect was observed in our study most of the combinations with AMB were additive or subadditive, and the number of favorable interactions was low. Only the combination of AMB with RVZ rendered mainly synergistic interactions. There are no other studies in which the effect of AMB combined with RVZ was evaluated against *Fusarium* or any other filamentous fungi.

Fifty-nine percent of the tests using TBF combined with the azoles were synergistic, 16% additive, and 25% indifferent. TBF combined either with RVZ or VCZ produced more than 70% synergistic interactions against the 11 strains of *Fusarium* tested. By species, the most remarkable combinations were TBF-RVZ against *F. solani* and TBF with any of the azoles against *F. verticillioides* and *F. oxysporum*. In those cases, both TBF and azole MICs decreased to up to four 2-fold dilutions, often from off-scale values. Even though there has been no previous study on the combination of TBF and azoles against *Fusarium* spp., in vitro synergy between such drugs has been reported against other filamentous fungi, such as *Aspergillus* spp. (Ryder and Leitner, 2001) and *Scedosporium prolificans* (Meletiadis et al., 2003). This positive interaction may be due to the combined effect of TBF and the azoles on different targets in the ergosterol biosynthesis pathway.

Several authors have indicated that the concentrations at which the drugs in combination exert their effect are at least as important as the presence of synergistic interactions,

because MICs of combined drugs below their respective achievable levels in serum could indicate a greater chance of success for in vivo studies (Meletiadis et al., 2003; Ryder and Leitner, 2001). In our study, potentially achievable MIC values in serum were only observed for AMB-RVZ against two strains of *F. solani* and one strain of *F. verticillioides*, and for TBF-ABZ and TBF-RVZ against two strains of *F. verticillioides* (Ernst, 2001; Bekersky et al., 2002; Meletiadis et al., 2003). However, the clinical relevance of these findings is unknown at present.

In summary, the combinations AMB-RVZ, TBF-VCZ, and TBF-RVZ displayed mainly synergistic interactions against *Fusarium* spp. Further studies both in vitro and in vivo are warranted to further elucidate the potential use of these combination therapies.

Acknowledgments

The authors thank A. Moreno for her technical assistance. This work was supported by a grant from Fondo de Investigaciones Sanitarias from the Ministerio de Sanidad y Consumo of Spain (PI 020114).

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