

In Vitro Synergy of Caspofungin and Itraconazole against *Aspergillus* spp.: MIC versus Minimal Effective Concentration End Points

Itamar Shalit,^{1,2} Yona Shadkchan,² Zmira Samra,^{2,3} and Nir Osherov^{2*}

Schneider Children's Medical Center of Israel,¹ Sackler School of Medicine,² and Rabin Medical Center, Petach Tikvah,³ Tel Aviv University, Tel Aviv, Israel

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Caspofungin and itraconazole were studied alone and in combination against 31 clinical isolates of *Aspergillus* spp. according to NCCLS M38-P guidelines. MICs and microscopic minimal effective concentrations (MECs) were recorded, and synergy was calculated by using both end points. Synergy or synergy to additivity was found in 30 of 31 isolates by using MIC end points. With MEC end points no synergy was found and indifference was detected in 26 of 31 strains.

Caspofungin is the first member of a new class of antifungal agents, the echinocandins, licensed for clinical use. It is indicated for the treatment of invasive aspergillosis in patients who are refractory to or intolerant of other therapies (4). Aspergillosis remains a major cause of morbidity and mortality among immunocompromised hosts. Current therapeutic modalities, including the introduction of new agents, are still associated with significant mortality (9). Thus, any combination therapy that may enhance antifungal activity should be actively pursued. Caspofungin exerts its activity by inhibiting synthesis of 1,3- β -D-glucan, an essential homopolysaccharide in the cell walls of many pathogenic fungi. This mode of activity is unique, making caspofungin a potential candidate for combination therapy with other classes of antifungal agents for the treatment of aspergillosis. Preliminary studies have shown in vitro synergy or additivity between caspofungin and amphotericin B against approximately 50% of *Aspergillus* spp. (2) and between caspofungin and amphotericin B or fluconazole against *Cryptococcus neoformans* (3). In the present study we performed in vitro synergy studies with caspofungin and itraconazole against isolates of *Aspergillus* species obtained from immunocompromised patients with invasive aspergillosis. In vitro activity of caspofungin against *Aspergillus* spp., as determined by using the NCCLS M38-P microdilution methodology, is characterized by a high MIC (>16 μ g/ml) against most isolates (1, 2). In contrast, it was known that, since the introduction of the echinocandin cilofungin, much lower concentrations of members of this class of drugs lead to significant disruption of hyphae and to decreased turbidity and visual growth (5). These observations led to a new definition of minimal effective concentration (MEC) as the lowest concentration of drug causing abnormal growth characterized by short abundant branchings (2, 7). In the present study we analyzed synergy utilizing both MIC and MEC end points and compared the results obtained by both methods.

In vitro susceptibility and checkerboard assays were performed on 31 clinical isolates of *Aspergillus* (*A. fumigatus* [*n* =

13], *A. niger* [*n* = 6], *A. flavus* [*n* = 6], and *A. terreus* [*n* = 6]). Control strains (*A. fumigatus* strain AF293 and *A. niger* ATCC 16404) were tested in every experiment as internal controls. Caspofungin (Merck Research Laboratories, Rahway, N.J.) and itraconazole (Janssen Pharmaceutica, Titusville, N.J.) were used for susceptibility studies. Drug interactions were assessed by checkerboard assays using the NCCLS M38-P microdilution methodology (8) after 24 h of incubation in standard 96-well sterile flat-bottom polystyrene plates (Corning). The final concentrations of the antifungal agents ranged from 0.008 to 128 μ g/ml for caspofungin and from 0.03 to 2 μ g/ml for itraconazole. Each well received 100 μ l of the diluted drug concentrations. Dilutions were made in RPMI 1640 medium containing 0.165 M MOPS (morpholinepropanesulfonic acid) buffer at pH 7.0. Conidial inocula were counted with a hemocytometer, prepared at a concentration of 2.5×10^4 CFU/ml in RPMI 1640-0.165 M MOPS, pH 7.0, and added at 100 μ l/well (final volume of each well, conidia, and drugs, 200 μ l). The MIC was the lowest drug concentration resulting in complete inhibition of hyphal growth (2). The MEC was the lowest drug concentration resulting in aberrant hyphal growth, as previously described (2). Plates were scanned both visually and microscopically with an inverted microscope at low ($\times 40$) magnification. The results were used to determine the fractional inhibitory concentration (FIC; in micrograms per milliliter) of the combination of caspofungin and itraconazole for each clinical isolate. FICs were calculated for both MIC and MEC end point measurements taken from the microwell with the lowest concentration of the drug combination needed to achieve the respective end points. The FIC of a drug for an individual isolate was calculated as the MIC or MEC of the drug when used in combination with another drug divided by the MIC or MEC of the drug when used alone. The FIC index (FICI) value was calculated by adding the FIC of caspofungin to the FIC of itraconazole for a particular isolate. FICI values were interpreted as follows: FICI \leq 0.5, synergistic; $0.5 < \text{FICI} \leq 1$, synergistic to additive; $1 < \text{FICI} \leq 4$, indifferent; FICI > 4 , antagonistic.

The MICs, MECs, and FICI values obtained for each of the isolates at 24 h are shown in Table 1. The MICs of caspofungin for *A. fumigatus*, *A. flavus*, and *A. terreus* (MIC \geq 128 μ g/ml) were at least two- to four-fold higher than that for *A. niger* (16

* Corresponding author. Mailing address: Department of Human Microbiology, Sackler School of Medicine, Tel Aviv University, Ramat-Aviv 69978, Tel Aviv, Israel. Phone: 972-3-640-9599. Fax: 972-3-640-9160. E-mail: nosherov@post.tau.ac.il.

TABLE 1. MIC and FICI results for the *Aspergillus* spp. at 24 h

<i>Aspergillus</i> sp. (<i>n</i>) and strain	End point	Concn ^a (µg/ml) of ^d :			FICI	Result ^b	<i>Aspergillus</i> sp. (<i>n</i>) and strain	End point	Concn (µg/ml) of:			FICI	Result
		CAS (alone)	ITZ (alone)						CAS (alone)	ITZ (alone)			
<i>A. fumigatus</i> (13) AF293	MIC	128	1	0.25	S	AN2	MIC	16	1	0.75	Ad		
	MEC	0.008	0.25	2.0	I		MEC	0.015	0.25	1.12	I		
	AF1	MIC	128	0.5	0.37	S	AN3	MIC	32	1	0.31	S	
		MEC	0.015	0.25	1.12	I		MEC	0.015	0.25	1.12	I	
	AF2	MIC	128	0.5	0.25	S	AN4	MIC	32	0.5	0.75	Ad	
		MEC	0.015	0.25	1.5	I		MEC	0.03	0.125	1.25	I	
	AF3	MIC	128	0.5	0.25	S	AN5	MIC	16	1	0.75	Ad	
		MEC	0.015	0.25	2.25	I		MEC	0.03	0.5	0.56	Ad	
	AF4	MIC	128	1	0.25	S	<i>A. flavus</i> (6) Afl1	MIC	>128 ^c	0.5	0.25	S	
		MEC	0.06	0.25	3.0	I		MEC	0.015	0.25	2.12	I	
	AF5	MIC	128	0.5	0.25	S		Afl2	MIC	>128	0.5	0.25	S
		MEC	0.015	0.25	2	I			MEC	0.008	0.125	4.0	I
AF6	MIC	128	0.5	0.25	S	Afl3		MIC	>128	0.25	0.25	S	
	MEC	0.03	0.25	1.5	I			MEC	0.03	0.06	2.5	I	
AF7	MIC	128	1	0.25	S	Afl4	MIC	>128	0.25	0.25	S		
	MEC	0.015	0.25	1.12	I		MEC	0.03	0.06	3.0	I		
AF8	MIC	128	0.5	0.25	S	Afl5	MIC	>128	0.25	0.25	S		
	MEC	0.015	0.25	2.25	I		MEC	0.008	0.06	2.5	I		
AF9	MIC	128	0.5	0.25	S	Afl6	MIC	128	0.5	0.25	S		
	MEC	0.008	0.25	1.12	I		MEC	0.015	0.125	3.0	I		
AF10	MIC	128	0.5	0.37	S	<i>A. terreus</i> (6) AT1	MIC	128	0.5	0.25	S		
	MEC	0.008	0.25	1.12	I		MEC	0.015	0.03	1.5	I		
AF11	MIC	64	0.5	0.25	S		AT2	MIC	128	0.25	0.25	S	
	MEC	0.008	0.125	1.25	I			MEC	0.03	0.06	1.0	Ad	
AF12	MIC	128	0.5	0.25	S		AT3	MIC	128	0.5	0.25	S	
	MEC	0.03	0.25	2.12	I			MEC	0.125	0.5	0.5	Ad	
<i>A. niger</i> (6) AN ^e	MIC	16	1	0.75	Ad	AT4	MIC	128	0.5	0.25	S		
	MEC	0.03	0.125	1.25	I		MEC	0.06	0.06	1.0	Ad		
AN1	MIC	16	0.5	1.25	I	AT5	MIC	128	0.5	0.25	S		
	MEC	0.015	0.25	1.12	I		MEC	0.06	0.06	1.5	I		
AN2	MIC	16	1	0.75	Ad	AT6	MIC	128	0.5	0.25	S		
	MEC	0.015	0.25	1.12	I		MEC	0.03	0.125	0.75	Ad		

^a MIC at 24 h or MEC at 24 h observed by microscopy.

^b S, synergistic; Ad, additive; I, indifferent.

^c The off-scale MIC of >128 µg/ml was converted to the next highest concentration, 256 µg/ml, for FICI calculation.

^d CAS, caspofungin; ITZ, itraconazole.

^e American Type Culture Collection.

< MIC < 32). In contrast, MECs of caspofungin for all strains tested were similar (0.008 < MEC < 0.06 µg/ml), and all were far below the MICs. Both MICs and MECs of itraconazole for all 31 strains tested were similar (0.25 < MIC < 1; 0.03 < MEC < 0.5).

When FICIs were calculated by using MICs, caspofungin and itraconazole showed synergy for all *A. fumigatus*, *A. flavus*,

and *A. terreus* strains (*n* = 25). For *A. niger* synergy or synergy to additivity was found in five strains and indifference was found in one (Table 2). In contrast, when FICIs were calculated by using MECs, no synergy (FICI ≤ 0.5) was observed for any of the strains. In five strains the combination showed synergy to additivity (among them four of six strains of *A. terreus*). For the majority of strains (26 of 31) the combination

TABLE 2. Overall results obtained for *Aspergillus* spp. at 24 h by using the two end points

<i>Aspergillus</i> sp. (n)	No. of isolates showing indicated synergy result ^b at end point ^a :							
	MIC				MEC			
	S	Ad	I	An	S	Ad	I	An
<i>A. fumigatus</i> (13)	13	0	0	0	0	0	13	0
<i>A. niger</i> (6)	1	4	1	0	0	1	5	0
<i>A. flavus</i> (6)	6	0	0	0	0	0	6	0
<i>A. terreus</i> (6)	6	0	0	0	0	4	2	0

^a MIC at 24 h or MEC at 24 h observed by microscopy.

^b S, synergistic; Ad, synergistic to additive; I, indifferent; An, antagonistic.

showed indifference (Table 2). Notably, antagonism was not detected in any of the strains tested by using either MIC or MEC end points.

In vitro and in vivo studies of caspofungin activity against *Aspergillus* using standard MIC methods and animal models resulted in some unusual observations. Arikan et al. (1) have shown that measurements of caspofungin MICs by NCCLS methods lead to very high values (MICs > 16 µg/ml). However, by using a microscopic end point of aberrantly growing hyphal tips, referred to as the MEC, values that were significantly lower than the MICs were obtained. The MECs correlated with a 50% reduction in turbidity in a microdilution assay and a prominent visual decrease in growth and were considered valid end points for assessment of caspofungin activity against *Aspergillus*.

In their rabbit model of aspergillosis, Petraitiene et al. (10) found that caspofungin treatment improved animal survival and reduced organism-mediated pulmonary injury. However, a paradoxical increase in CFU of *Aspergillus* per gram and an increased galactomannan antigen index in the successfully treated animals were observed. Thus, the correlation between in vitro and in vivo activity of caspofungin and the quantification of *Aspergillus* or its modified hyphae are problematic and difficult to interpret.

In our study, caspofungin MICs and MECs for *Aspergillus* spp. were in agreement with the findings of Arikan et al. (2). In their study they found synergy or synergy to additivity between caspofungin and amphotericin B against more than 50% of *Aspergillus* spp. tested by using MIC and MEC end points for their FICI analysis. There were no major discrepancies between FICI values found when MICs were used as end points and those found when MECs were used. In contrast, in our study, there was a significant difference between the FICI values depending on the end point used. Whereas MIC end points resulted in synergy for the majority of strains, MEC end points showed mainly indifference.

It is not clear which end point better predicts in vivo drug combination outcome. Preliminary studies using caspofungin and another azole derivative, voriconazole, showed in vitro synergy for 45.8% of 48 *Aspergillus* spp. tested and additivity for 41.6% (S. Perea, G. Gonzales, A. W. Fothergill, W. R. Kirkpatrick, M. G. Rinaldi, and T. F. Patterson, Abstr. 101st

Gen. Meet. Am. Soc. Microbiol, abstr. F-87, p. 372, 2001). In another in vitro study, using a radiometric assay and a growth inhibition end point, the combination of caspofungin and voriconazole showed additivity but not synergy (E. K. Manavathu, L. T. Ganesan, J. L. Cutright, and P. H. Chandrasekar, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. 125, 2001). In the only in vivo animal study reported, voriconazole was as effective as the combination with caspofungin in terms of survival; however, synergy was found when tissue fungal burden was assessed (6).

The mechanism explaining our findings of discrepancy in interpretation using MIC versus MEC end points is not clear. It might be speculated that, while low concentrations of caspofungin affect normal growth of *Aspergillus*, higher concentrations of the drug are needed to cause sufficient damage to the cell wall, thereby enabling itraconazole to confer its activity on the cell membrane of the organism at a lower concentration.

To further analyze the potential use of the above drug combination, it is imperative to perform an in vivo animal study of invasive aspergillosis assessing carefully the potential synergy or additivity of caspofungin and itraconazole. Only such studies will enable us to assess which in vitro study is more closely related to in vivo findings and what should be the recommendation for clinicians treating immunocompromised patients with invasive aspergillosis.

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