In vitro synergy of caspofungin with licensed and novel antifungal drugs against clinical isolates of Fusarium spp.

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Combinations of caspofungin (CAS) with amphotericin-B (AMB), voriconazole (VRC), terbinafine (TRB) and tacrolimus (FK-506) were tested in vitro with 10 Fusarium isolates. MIC and minimal effective concentrations (MEC) were investigated in accord with the CLSI methodology. Drug interactions were assessed by the fractional inhibitory concentration index (FICI). Synergy occurred in 10/10, 9/10, 7/10 and 4/10 isolates with CAS/FK-506, CAS/TRB, CAS/AMB and CAS/VRC, respectively. Caspofungin MECs reached clinically attainable concentrations with FK-506 and TRB. Hyphal length and DiBAC staining demonstrated enhanced inhibition and killing with CAS/FK-506 and CAS/TRB. The combination of CAS/TRB and CAS/FK-506 is strongly synergistic in vitro against Fusarium spp. Our finding should be further studied in animal models of invasive infections caused by this fungus.

Keywords  Caspofungin, Fusarium, synergy, tacrolimus, terbinafine

Introduction

Fusarium species cause a broad spectrum of infections in humans, the most severe form, invasive disseminated disease, has been increasingly reported among immunosuppressed hosts [1]. Therapeutic alternatives for disseminated Fusarium infections include amphotericin B (AMB) and its lipid formulations, as well as the newer azoles, voriconazole (VRC) and posaconazole. However, therapeutic failures and mortality are still unacceptably high reaching ~ 40–70% in different series [1,2]. Most Fusarium species are resistant in vitro to many antifungal classes, but there seems to be a lack of correlation between in vitro results and in vivo outcomes [3–5]. Fusarium spp. are resistant in vitro to caspofungin (CAS), nevertheless, there are several case reports of successful clinical outcomes in patients with severe, life-threatening infections treated with this antifungal as salvage therapy following lack of response to AMB [3,4]. In addition, CAS showed improved activity compared to various AMB preparations in an animal model of invasive infection [5]. In one in vitro study, drug combinations with CAS showed synergistic activity when combined with AMB against several Fusarium solani and Fusarium oxysporum isolates [6]. In the present study we analyzed, for the first time, the in vitro activity of CAS in combination with VRC, terbinafine (TRB) and the anti-calcineurin drug tacrolimus (FK-506) against clinical isolates of Fusarium species. TRB was previously demonstrated to act alone and showed synergistic activity in combination with VRC against Fusarium [7], while FK-506 was previously found to act synergistically with CAS against other fungal pathogens [8].

In this report we demonstrate strong synergistic interactions with pathogenic isolates of Fusarium spp. between CAS and TRB and between CAS and FK-506...
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using checkerboard, hyphal length measurements and DiBAC vital staining.

Material and methods

Checkerboard synergy assay

\textit{In vitro} susceptibility testing using the checkerboard assay was performed with the following ten clinical isolates of \textit{Fusarium} collected from Israeli hospitals, \textit{F. solani} (\(n=6\)), \textit{F. oxysporum} (\(n=3\)), and \textit{F. dimerum} (\(n=1\)). CAS (Merck Research Laboratories, Rahway, NJ), AMB (Sigma Chemical Co.), VRC (Pfizer Inc. Sandwich, England), TRB (Novartis, Basel, Switzerland) and FK-506 (Sigma Chemical Co.) were tested. CAS interactions were assessed by checkerboard assays based on the CLSI (Clinical and Laboratory Standards Institute, formerly NCCLS) M38-A microdilution methodology [9]. The final concentrations of the antifungal agents ranged from 4–256 mg/l for CAS, 0.08–8 mg/l for AMB, 0.03–32 mg/l for VRC, 0.06–64 mg/l for TRB, and 0.02–20 mg/l for FK-506. The checkerboard tests were performed as described by Shalit \textit{et al.} [10]. Freshly harvested conidia were counted with a hemocytometer and diluted to a final concentration of 2.5 \(\times\) 10^7 conidia/ml in RPMI 1640 medium (Biological Industries, Beit Haemek, Israel) containing 0.165M MOPS (Sigma) buffer at pH 7.0 (RPMI-MOPS). The plates were scanned with an inverted microscope (\(\times 40\) magnification) after 48 h of incubation at 28°C in standard 96-well plates (Costar; Corning, Corning, NY). The minimal inhibitory concentration (MIC) was the lowest drug concentration resulting in complete inhibition of hyphal growth. The minimal effective concentration (MEC) was the lowest drug concentration resulting in aberrant hyphal growth (for CAS) or a prominent reduction of growth (for the other compounds) [6]. The results were used to determine the fractional inhibitory concentration index (FICI) of the combination of CAS and AMB, VRC, TRB or FK-506 for each clinical isolate. FICI values were interpreted as follows: FICI \(\leq 0.5 = \)synergy; FICI >0.5–4 = no interaction and FICI >4 = antagonism. To test reproducibility, the combinations of CAS/FK-506 and CAS/TRB were tested in five independent experiments with \textit{F. solani} 2 strain. MIC and MEC values did not diverge more than 2-fold between each of these experiments, and FICI values remained constantly synergistic.

Hyphal length measurement

Hyphal length measurements were performed by microscopic observation using a micrometer after 24 h of growth at 28°C in the presence of CAS alone or in combination with FK-506 or TRB. Conidia from \textit{F. solani} 2 were incubated in the presence of MEC or 2
\[2\] MEC concentrations of each drug alone or in combination using the checkerboard assay, as described above. Each datum point of hyphal length represents the average measurements of 50 randomly selected germlings. Error bars denote standard deviations. \(P\) values were calculated by the Student unpaired \(t\) test. These experiments were repeated three times.

DiBAC staining

DiBAC staining was used for microscopic analysis of conidia and hyphal cell death. Isolate \textit{F. solani} 2 was incubated in the presence of CAS and FK-506 or TRB at MEC and 2 \(\times\) MEC concentrations of each drug alone or in combination using the checkerboard assay, as described above. Each datum point of hyphal length represents the average measurements of 50 randomly selected germlings. Error bars denote standard deviations. \(P\) values were calculated by the Student unpaired \(t\) test. These experiments were repeated three times.

\begin{table}[h]
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\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
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Isolate* & \multicolumn{2}{c|}{CAS} & \multicolumn{2}{c|}{FK506} & \multicolumn{2}{c|}{FICI*} \\
 & MIC & MEC & MIC & MEC & MIC & MEC & MIC & MEC \\
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& Alone & Comb. & Alone & Comb. & Alone & Comb. & Alone & Comb. \\
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\textit{F. dimerum} & 9 & 256 & 64 & 64 & 8 & 40 & 0.02 & 2.5 & 0.02 & 0.26 & 0.13 & 0.26 & 0.31 \\
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\caption{Table 1 (Continued)}
\end{table}

FICI = The FICI is defined as the sum of the MIC of each drug when used in combination divided by the drug alone. FICI values were interpreted as follows: FICI \(\leq 0.5 = \)synergy; FICI >0.5–4 = no interaction and FICI >4 = antagonism.

*The clinical origin of the 10 clinical isolates was as follows: \textit{F. solani} 1 (disseminated); 2 (eyes); 3 (lungs); 4 (skin lesion); 5 (skin lesion); 6 (lung). \textit{F. oxysporum} 7 (burn wound); 8 (lungs); 9 (sinus). \textit{F. dimerum} 10 (disseminated).
**Results**

**Checkerboard synergy assay**

The MICs, MECs, and FICIs obtained with the ten isolates employed in these studies at 48 h are shown in Table 1. The MICs and MECs were between 128 and 256 mg/l and 32 and 125 mg/l, respectively (Table 1). Combination of CAS and AMB and CAS with TRB appeared synergistic with respect to the MIC (7/10 isolates and 9/10 isolates, respectively) and MEC endpoints (8/10 isolates and 6/10 isolates, respectively), whereas the combination of CAS and VRC appeared
synergistic for 4/10 (MIC) and 2/10 (MEC) of the isolates. Most strikingly, the combination of CAS and FK-506 appeared synergistic against all the isolates tested with regards to the MIC and MEC endpoints. In general, CAS MICs and MECs in combination with AMB, TRB and FK-506 decreased dramatically (four to sixteen-fold) with all isolates. Importantly no antagonism was observed for any of the isolates tested using MIC or MEC endpoints.

Hyphal length measurement

As shown in Fig. 1A, the combination of CAS and TRB and CAS with FK-506 significantly reduced hyphal length ($P < 0.001$) compared to each drug administered alone or at double the MEC concentration.

DiBAC staining

DiBAC staining showed that at MEC levels, CAS alone was a very weak antifungal agent, whereas FK-506 and TRB by themselves were intermediate in their ability to inhibit the test isolates. However, the combination of CAS and FK-506 or with TRB demonstrated 100% killing of Fusarium conidia (Fig. 1B).

Discussion

In this report we describe the combination of caspofungin (CAS) with AMB, VRC, TRB and FK-506 against 10 Fusarium isolates. Drug interactions were assessed by the checkerboard assay using both MIC and minimal effective concentrations (MEC) endpoints. Although the MEC endpoint is primarily employed to measure the sub-inhibitory efficacy of CAS, this definition has been extended to include the endpoints of other drugs used in combination with it [6,10].

Our results indicate that caspofungin shows strong in vitro synergy in combination with AMB, TRB and FK-506 and only moderate synergy with VRC (4/10 strains). For all 10 strains and all combinations, using MIC or MEC as endpoints, no antagonism was found. It is important to note that the activity of CAS in the various combinations was achieved at clinically attainable concentrations (MEC = 2–8mg/L) for the majority of strains with CAS/AMB and CAS/FK-506 combinations and in 30–40% of the isolates in combination with VRC and TRB. Moreover, the MEC concentrations of AMB, TRB and FK-506 in combination with CAS were extremely low for the majority of strains tested. These data were further supported by selected studies of hyphal length measurement and DiBAC staining, visually demonstrating the effect of CAS in combination with TRB and FK-506. Previous studies have shown synergy between TRB and azoles against Fusarium and in a single case report TRB and AMB were used successfully to treat a patient with invasive fusariosis [11]. We did not find previous studies on the combination of CAS and TRB. However their different mode of antifungal activity may indicate the potential for such synergy.

FK-506 exerts antifungal effects by inhibiting calcineurin, a conserved Ca$^{2+}$-calmodulin activated protein phosphatase involved in fungal stress responses, virulence and antifungal resistance [12]. FK-506 was found to act synergistically with CAS against Cryptococcus neoformans and various species of Aspergillus [8]. Similar synergy was found between CAS and other anti-calcineurin drugs [12] and our current finding with Fusarium are in line with these observations.

Several case reports have indicated the clinical utility of CAS in invasive fusariosis [3,4]. Although such studies provided limited scientific evidence, they showed clear clinical and microbiological improvement when CAS was added to AMB as a salvage therapy in such circumstances when AMB alone failed. Our finding of in vitro synergy between AMB and CAS are supportive of these observations. Taken together our findings imply a potential role for CAS in combination with certain antifungal agents and anti-calcineurin drugs as a novel therapeutic modality for Fusarium infections. These observations should be further studied in animal models of invasive fusariosis.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References


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