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Fluconazole, caspofungin, voriconazole in combination with amphotericin B

Research Article

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Abstract: Combined antifungal therapy has been suggested to enhance the efficacy and reduce the toxicity of antifungal agents. The aim of the study was to investigate the *in vitro* synergistic activity of caspofungin, voriconazole, and fluconazole with amphotericin B against ten isolates of *Candida parapsilosis* and *Candida albicans* strains which were resistant to azoles or amphotericin B. Three different antifungal combinations (amphotericin B [AP] – caspofungin [CS], amphotericin B – fluconazole [FL], and AP – voriconazole [VO]) were evaluated for *in vitro* synergistic effect by the microdilution checkerboard and E-test methods. For the majority of strains, the combination test showed indifferent activity. *Via* the E-test method, synergistic activity was seen in 3 strains in response to AP-CS combination treatment and in one strain after administration of AP-FL; however, no synergy was observed in response to combination treatment with P-VO. Antagonistic activity was the result in 1 strain treated with AP-CS as well as in 6 strains treated with AP-FL and AP-VO combinations. *Via* the microdilution test, no synergistic activity was seen after treatment with all 3 combinations. Antagonistic activity was the result in 2 strains with AP-CS, in 6 strains with AP-VO and in 5 strains with AP-FL combinations. Agreement between the checkerboard and E-test methods was observed to be approximately 72%. These combinations may be used in the case of antifungal resistance.

Keywords: *Antifungals • chequerboard • E-test*

1. Introduction

The use of combined antifungal therapy has been suggested to promote the efficacy and lower the toxicity of antifungal agents by the administration of lower doses of toxic agents. Due to an effort by ergosterol in the mechanisms of action of amphotericin B (AP) and the azoles, use of this combination has been encouraged [1]. Drug interaction models were used to analyze the *in vitro* interaction of different antifungal drugs against various fungal species using the microdilution checkerboard and E-test methods. Drug interaction was classified as synergistic, additive, indifferent or antagonistic on the basis of the fractional inhibitory concentration (FIC) index. The FIC index is the sum of the FICs for each drug; the FIC is defined as the MIC of each drug when used in combination divided by the MIC of the drug when used alone [2].

The aim of this study was to investigate the *in vitro* interaction of caspofungin (CS), voriconazole (VO) and fluconazole (FL) with AP against eleven isolates of *Candida parapsilosis* and *Candida albicans* strains resistant to azoles or AP.

2. Material and Methods

2.1. Antifungal susceptibilities

Broth microdilution and E-test methods were performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) and E-test manufacturer's recommendations (AB Biodisk, Sweden). The final

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concentrations of the antifungal agents ranged from 0.25 to 128 μg/ml for FL and VO; for AP and CS, they ranged from 0.03 to 16 μg/ml.

2.2. Antifungal combinations

AP – CS, AP – FL and AP – VO combinations were compared.

2.3. Checkerboard microdilution

The final concentrations of the antifungal agents ranged from 0.25 to 128 μg/ml for FL and VO, 0.03 to 16 μg/ml for AP and CS. Inocula were prepared spectrophotometrically and further diluted in order to obtain final concentrations ranging from $0.4x10^4$ to $5x10^4$ CFU/ml. Each microdilution well containing 100 μl of the diluted drug concentrations of both antifungals was inoculated with 100 μl of the diluted inoculum suspension (final volume of each well, 200 μl). The trays were incubated at 35°C, and the results were read after 24 hours visually and spectrophotometrically with a spectrophotometric microtiter plate reader (ELx800, BioTek, USA). MIC endpoints were determined as the first concentration of the antifungal agent, either alone or in combination, at which the turbidity in the well was less than 80% of that in the control well. Drug-free and fungus-free controls were included; quality control was ensured by testing *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 [3].

2.4. E-test studies

An inoculum equal to a 0.5 McFarland turbidity standard was prepared from each *Candida* isolate, and 10 μl of the suspension was inoculated onto RPMI agar plates. E-test strips of FL, VO, AP and CS were stored at −20°C until use. The E-test strips were applied to the inoculated culture plates separately by using a template, as recommended by the manufacturer, and the plates were incubated at 35°C for 48 hours under aerobic conditions. For testing, an E-test strip of an antifungal (drug A) was applied to the surface of RPMI agar plates and left for 1 hour at room temperature. Afterwards, the strip was removed and another strip (drug B) was applied onto the imprint of strip A. The plates were incubated at 35°C for 48 hours under aerobic conditions; subsequently, MIC levels of each drug and combination were interpreted [4].

2.5. FIC index model

The non-parametric approach is based on the fractional inhibitory concentration index model expressed with the following equation,

$$\varepsilon FIC = FIC_A + FIC_B = Comb_A / MIC_A + Comb_B / MIC_B$$

where MIC_A and MIC_B are the concentrations of the drugs A and B when acting alone and $Comb_A$ and $Comb_B$ are the concentrations of the drugs A and B at the iso-effective combinations [5].

The results of combination tests according to FIC index were interpreted as follows: synergistic ($\varepsilon FIC \leq 0.50$), additive ($\varepsilon FIC > 0.50$ and ≤ 1.0), indifferent ($\varepsilon FIC > 1.0$ and ≤ 4.0), and antagonistic ($\varepsilon FIC > 4.0$).

All susceptibility tests were performed in duplicate; results were accepted only when there was not more than a one-step difference in values. If this was the case, the higher value was reported.

2.6. Agreement between the FIC index in the checkerborad microdilution and the E-test

The interactions found by the FIC index in the checkerboard microdilution were compared with those found by the E-test approach for the AP-CS, AP-FL and AP-VO combinations. The percentage agreement between both methods for both combinations was determined [6].

3. Results

Two different approaches and 3 distinct drug combinations were used in this study to investigate the interactions between AP and each of three other antifungal agents against clinical isolates of *C. albicans and C. parapsilosis*. For the majority of strains, the combination tests showed indifferent activity.

Via the E-test method, synergistic activity was seen in three strains in AP-CS combination and in one strain subjected to AP-FL combination treatment; however, no synergy was observed in response to treatment with the AP-VO combination. Antagonistic activity was the result in one strain treated with AP-CS, in 6 strains treated with AP-FL and in 5 strains treated with AP-VO. Additive activity was seen in 1 strain after AP-VO combination treatment.

Via the microdilution test method, no synergistic activity was seen in the 3 combinations. Antagonistic activity was the result in 2 AP-CS strains with, in 6 AP-VO strains and in 5 AP-FL strains. Additive activity was seen in 3 strains treated with AP-CS (Table 1).

In our study, the rate of agreement between the checkerboard and E-test methods was approximately 72%. The rates of agreement obtained with the combinations AP-CS, AP-FL and AP-VO were approximately 33%, 66%, and 75%, respectively. The E-test was evaluated as a reliable method for the

Table 1. E-test and microdilution results of synergistic activity.

		E TEST			MICRODILUTION		
	Strains	AP-CS	AP-VO	AP-FL	AP-CS	AP-VO	AP-FL
RESISTANT TO AMP B	*C. albicans*	Synergistic	Indifferent	Indifferent	Indifferent	Indifferent	Indifferent
	C.parapsilosis	Indifferent	Additive	Synergistic	Indifferent	Antagonistic	Indifferent
	C.parapsilosis	Indifferent	Indifferent	Indifferent	Indifferent	Indifferent	Indifferent
	C.parapsilosis	Indifferent	Antagonistic	Indifferent	Additive	Indifferent	Indifferent
	C.parapsilosis	Indifferent	Indifferent	Indifferent	Antagonistic	Antagonistic	Antagonistic
RESISTANT TO AZOLES	*C. albicans*	Indifferent	Antagonistic	Antagonistic	Indifferent	Antagonistic	Antagonistic
	C. albicans	Synergistic	Indifferent	Antagonistic	Additive	Indifferent	Antagonistic
	C. albicans	Indifferent	Antagonistic	Antagonistic	Additive	Antagonistic	Indifferent
	C. albicans	Antagonistic	Antagonistic	Antagonistic	Indifferent	Antagonistic	Indifferent
	C. albicans	Synergistic	Antagonistic	Antagonistic	Indifferent	Antagonistic	Antagonistic
C. parapsilosis ATCC 22019		Indifferent	Indifferent	Antagonistic	Antagonistic	Indifferent	Antagonistic
C. krusei ATCC 6258		Indifferent	Indifferent	Indifferent	Indifferent	Indifferent	Indifferent

AP; amphotericin B, CS; caspofungin, VO; voriconazole, FL; flukonazole, S; synergistic (FIC≤0.50), AD; additive (FIC >0.50 and ≤1.0), ID; indifferent (FIC >1.0 and ≤4.0), and AG; antagonistic.

evaluation of the interaction between the antifungals. There was no statistical difference observed between azole- and AP-resistant groups (p>0.05).

4. Discussion

In this study, we analyzed the *in vitro* interactions between AP and VO, FL or CS. These were applied against clinical isolates of *Candida spp*. which were resistant to azoles or AP. To establish the possible beneficial effect of these combination therapies *in vitro*, we selected strains of *C. parapsilosis* and *C. albicans* with reduced susceptibility to AP and azoles. The procedures used in the present study were a checkerboard microdilution and E-test based-method, according to the CLSI recommendations.

The total rate of agreement between the checkerboard and E-test methods was approximately 72%, while the best results were obtained with AP-VO combination treatment (approximately 75% agreement). Even though these methods use different conditions and endpoints, there was frequent agreement between the results of the two methods [7]. There is no consensus on which definition to use in synergy studies. These definitions, as well as a lack of a statistical criterion to define these interactions, contribute to these varying results [1]. We established that the E-test was a reliable method for synergy testing of antifungals. The checkerboard method is difficult and time-consuming for routine antimicrobial synergy testing, but we suggest that the E-test can easily be applied to susceptibility testing of *Candida* strains as it is less labor-intensive and less time-consuming.

The standardisation of these techniques for routine laboratory testing is needed because of the common use of combination therapies against the growing numbers of multiple-drug-resistant strains.

Although antifungal drugs may interact differently under different conditions in *in vitro* systems, variable results can be obtained even when the same *in vitro* methodology is used depending on the nature and the intensity of drug interactions [8]. The standard approach in the field of medical microbiology is the calculation of the FIC index. Despite the fact that this method has some important disadvantages, it is widely used. The first disadvantage is that one FIC index is used for many results; the result of every well in the checkerboard is confined in one index. There may be more refinement necessary since at some concentrations there may be synergism, but there may be indifference or even antagonism for others. Another disadvantage is that it is not clear at which MIC endpoint the combination should be read and to ultimately determine the FIC index for some antifungal combinations [1]. For AP, endpoints are easily defined (one dilution) and the MIC is read as the lowest drug concentration that showed 100% growth inhibition (MIC-0).

Recently, AP has been tested in combination with many other drugs to determine whether it has possibly enhanced activity when it is used in combinations [9]. *In vitro* and *in vivo* studies have shown wide variations in effects when the polyene is combined with FL or VO [10]. An experimental trial comparing FL alone or combined

with AP for the treatment of candidemia showed that the latter regimen tended to improve the treatment success rate and achieved a more rapid clearance of the organism from the bloodstream [11].

Our investigations showed that synergism between AP and other antifungals, as measured by both the classical checkerboard microdilution and E-test method, occurred rarely.

Since the mechanism of action of CS differs from the mechanism of action of AP, antagonism might not be expected. FL must penetrate the cell wall to reach its site of activity; therefore, it has been speculated that AP may enhance the activity of FL and VO by opening pores in the cell wall and increasing the access of azoles to the cell membrane. However, our study found study drug combinations of FL and CS or VO with AP to result in indifferent activity for most of the strains (19 of results with E-test and 20 of results with checkerboard microdilution). Synergism was demontrated between AP and CS in three strains. Antagonism was demonstrated between AP and azoles in 11 strains.

Although the exact mechanism of the interaction between the agents is unknown, one theoretical explanation for such an effect would be the simultaneous disruption of the fungal cell membrane by VO and FL as well as disruption of the cell wall by CS, thereby ultimately decreasing cell stability and leading to fungal cell death [1].

No significant differences were found in synergy or antagonism rates between CS, FL and VO with respect to AP. Synergy was demonstrated *in vitro* for AP-CS combination, while antagonism was observed in response to both AP-VO and AP-FL combinations.

In conclusion, based on clinically achievable drug concentrations with commonly used dosage regimens, synergy would be the most suitable for routine empirical clinical use.

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