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IN-VITRO SENSITIVITY OF AMPHOTERICIN B, ITRACONAZOLE AND FLUCONAZOLE - RESISTANT AGAINST *CANDIDA ALBICANS*

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ABSTRACT

Aim: To study *In-Vitro* sensitivity of Amphotericin B, Itraconazole and Fluconazole -Resistant against *Candida albicans.*

Methods and Results: A panel of 14 clinical isolates of *Candida albicans* was tested. Strains were labeled and given a unique identification number. *Candida albicans* ITCC 4718 were included as quality control organisms in each set of experiments. Interactions in vitro between Amphotericin B, Itraconazole, Voriconazole, and Fluconazole against Itraconazole-resistant *Candida albicans* clinical strains were determined. Fluconazole and Voriconazole exhibited the most potent interactions with synergy against at least 50% of isolates, and the average fractional concentration index was 0.38

Conclusion: Fluconazole and Voriconazole exhibited the most potent interactions with synergy against isolates and Antagonism was not found for any combination.

Significance and Impact of the Study: Fungal infections are frequently tricky to manage prudence has to be exercised in the use of antifungal drugs to capture any additional increase in the resistance.

INTRODUCTION: Fungal infections are a challenge, particularly in the growing number of immunosuppressed patients seen in modern medical facilities as a result of increases in transplantation, infection (especially HIV), premature births, and aggressive antibiotic and anticancer therapies [1, 2, 3].

Invasive fungal infections are infections of the bloodstream and organs within the body (e.g. meningitis, pneumonia, peritonitis) and are important causes of morbidity and mortality in liver, pancreas, heart, kidney and lung (i.e. solid organ) transplant recipients [4, 5].

Fungi are eukaryotes and, despite the presence of a cell wall, fungi are more similar to mammalian cells on a cellular level, making the treatment of mycotic infections difficult. Additionally, fungi replicate more slowly than bacteria and are often difficult to quantify, particularly for moulds, which complicate efficacy assessments [5, 6]. The significant clinical implication of resistance has led to heightened interest in the study of antifungal resistance from different angles [7] .The development of Antifungal drug resistance is not a new phenomenon, micro-organisms have been responding to toxic environmental stresses for millennia [8].

Indeed it is likely that the mechanisms utilized to confer resistance to 'novel' synthetic drugs have been selected from an extensive repertoire that has enabled microorganisms to survive for so long in changing environments [9, 10]. The efficacy of commonly used antifungal drug amphotericin B has been limited more by toxicity than by lack of efficacy, although resistance has been increasing [11]**.** Other drugs like ketoconazole, fluconazole and clotrimazole are limited in their spectrum and their use may produce strain resistance [12].

In contrast to clinical isolates, *Candida albicans* mutants that are highly resistant to itraconazole are easily selected in vitro [13, 14]. Several resistance mechanisms have been described, and azole cross-resistance has been observed [15]. These data suggest that itraconazole resistance among clinical strains may become more common in the future, associated with the spread of antifungal therapies [16]. Combination therapy could be an alternative to mono-therapy for patients with invasive infections due to resistant organisms and for some patients who failed to respond to standard treatment [14]. The increase in available antifungal compounds has raised the number of potential combinations, a therapeutic resource which could be exploited clinically [14, 16].

For these reasons, there is an urgent need for new active molecules that can serve as lead for further development in antifungal chemotherapy [17]. We have analyzed the combined activity in vitro of several antifungal agents against a collection of 14 itraconazole-resistant (MICs of >8.0 µg ml^{-1}) clinical isolates of *Candida albicans*. Antifungal resistance is particularly problematic as initial diagnosis of systemic fungal infection can be delayed and there are few antifungal drugs available [19, 20].

MATERIAL AND METHODS:

Collection of *Candida albicans* strains: The *Candida albicans* of clinical isolates were tested in this study represents a collection of 14 Strains were collected from hospitals, Each isolates was labeled and given a identification number AF-72, Br109, F/919, F/69, AF-786, F/699, AF1237, AF-1422, F/6919, F/7075, Br130, Br181, SO/3827, SO/3829, F/6919 obtained from different patients. Original strain *Candida albicans* ITCC 4718 were included as quality control organisms in each set of experiments.

Antifungal **Susceptibilit**y **testing**: The individual MICs were determined by following the National Committee for Clinical Laboratory Standards (NCCLS) reference method [20] with slight alterations. The alteration included the use of RPMI 1640 with L-glutamine buffered to pH 7 with 0.165 M MOPS (Morpholinepropanesulfonic acid) and 1 M NaOH complemented with 18 g of glucose per liter (RPMI–2% glucose, Sigma, USA) and inoculums preparation by microscopic enumeration with a cell-counting hemocytometer (Neubauer chamber) [21,22]. All inoculums suspensions were quantified by plating on Sabouraud Dextrose Agar (S.D.A) plates.

Sterilized 96 wells plastic plates were used in the study. The plates were inoculated with 100 µl of the inoculums suspensions in each well. The plates were incubated at 35°C for 48 hrs in a humid atmosphere [23], for amphotericin B, itraconazole, and voriconazole, MICs were defined as the lowest concentration of the antifungal agent that entirely inhibited fungal growth, for caspofungin, two dissimilar illustration determinations of the endpoint were observed:

(i) Absolute inhibition of growth (MIC) and;

(ii) The lowest drug concentration consequential in unusual hyphen development by assessment with an inverted microscope or the minimum effective concentration (MEC) [24, 25, 26].

RESULTS: The combined effects were analyzed by the summation of the fractional concentration index (FICi). For combinations including caspofungin, the FICi was also calculated by taking into account both the MIC and the MEC of the echinocandin. The interactions were defined as synergistic when the FICi was ≤0.6 and as antagonistic if FICi was and indifference or no interaction was defined by a FICi that was >0.6 but ≤4. Duplicate testing on three separate days was performed.

Analysis of results: 14 clinical isolates were used against the drugs, in which out of 12 strains, the MIC of voriconazole was ≤ 2.0 µg ml^{-1}, and for two strains, the MIC of voriconazole was ≥4.0 µg ml^{-1}. MICs of Fluconazole were repeatedly more than 16.0 µg ml^{-1}.

In addition, Fluconazole displayed an excellent activity in vitro when MECs were dogged. The arithmetical mean of the Fluconazole MEC was 1.66 µg ml^{-1}, and MECs ranged from 0.50 to 4.0 µg ml^{-1}. The MIC of amphotericin B was ≤ 0.6 µg ml^{-1}; all samples were resistant against itraconazole *in vitro* and MICs were observed >8.0 µg ml^{-1}.

The collective effect of antifungal agents *in vitro*, in **Table 1** demonstrates arithmetic means of FICi values following six recurrences per combination of compounds and per sample. The amphotericin B-voriconazole combination demonstrated an in dissimilar result, with FICi values averaging 0.77. The combinations of antifungal activities of compounds have demonstrated a synergistic effect against 6 out of 14 strains (42.8%), particularly, synergy observed for the two strains that treated voriconazole MICs of ≥4 µg/ml (F/699and F/6919). The average FICi of the amphotericin B-itraconazole grouping for the 14 clinical strains was 1.46.

When analyzing combinations with caspofungin, significant differences were found between FICi's obtained by using MICs and those calculated with MECs. Indifference was found for the amphotericin B-caspofungin combination against the majority of clinical isolates. Average FICi's with MICs and MECs were 0.81 and 0.67, respectively. However, synergy was described for 1 of 14 isolates (7.1%) with MICs and for 5 of 14 strains (35.7%) if the FICi was calculated by using MECs.

Antagonism was not observed. The combined effect of the itraconazole- Fluconazole combination was classified as indifference regardless of the values used for FICi calculation. However, the average FICi with MECs was 0.55, an index close to synergy. In addition, a synergistic effect was observed in 10 of 14 (64.3%) strains, and antagonism was not found. Regarding the voriconazole-Fluconazole combination, synergistic interaction was noticed, with the average FICi's with MICs and MECs being 0.50 and 0.38, respectively.

Antagonism was absent, and synergy was described for 7 of 14 (50%) isolates if the FICi included MICs and for 10 of 14 (64.3%) organisms if the MEC was used for FICi calculation. Unlike the amphotericin B-oriconazole combination, voriconazole-Fluconazole did not exhibit synergy against the two strains with voriconazole MICs of ≥4 µg ml^{-1}, and the combination showed an indifferent interaction for the two isolates.

TABLE 1: FiCi's OF 14 CLINICAL ISOLATES PER ANTIFUNGAL COMBINATION

Strain	FiCi's for combination							
	AMB-ITC	AMB-VRC	AMB-FLU		ITC-FLU		VRC-FLU	
	MIC (in ug/ml)	MIC (in ug/ml)	MIC (in ug/ml)	MIC (in ug/ml)	MIC (in ug/ml)	MEC (in ug/ml)	MIC (in ug/ml)	MEC (in ug/ml)
F/6919	1.0	1.0	1.0	1.0	2.0	1.5	0.55	0.50
F/919	2.0	1.0	0.75	0.50	2.0	0.28	0.26	0.19
AF1237	2.0	2.0	1.0	1.0	2.0	0.26	0.55	0.55
AF-1422	0.75	1.0	0.55	0.41	2.0	0.31	0.55	0.37
AF-786	1.0	0.50	0.55	0.55	2.0	0.37	0.62	0.62
Br181	0.75	0.75	1.0	1.0	2.0	0.19	0.18	0.18
SO/3827	0.55	1.0	1.0	0.55	1.5	1.5	0.50	0.50
SO/3829	0.75	0.50	1.0	0.55	0.55	0.37	0.26	0.14
Br109	2.0	1.0	0.75	0.56	2.0	1.5	0.18	0.14
F/699	2.0	0.37	0.50	0.50	2.0	0.75	1.50	0.62
F/7075	3.0	0.25	0.75	0.26	2.0	0.19	0.28	0.25
Br130	3.0	0.25	0.56	0.50	2.0	0.19	0.18	0.18
F/6919	1.0	0.50	1.0	1.0	2.0	0.28	0.62	0.62
F/69	0.75	0.75	1.0	1.0	2.0	0.04	0.50	0.19

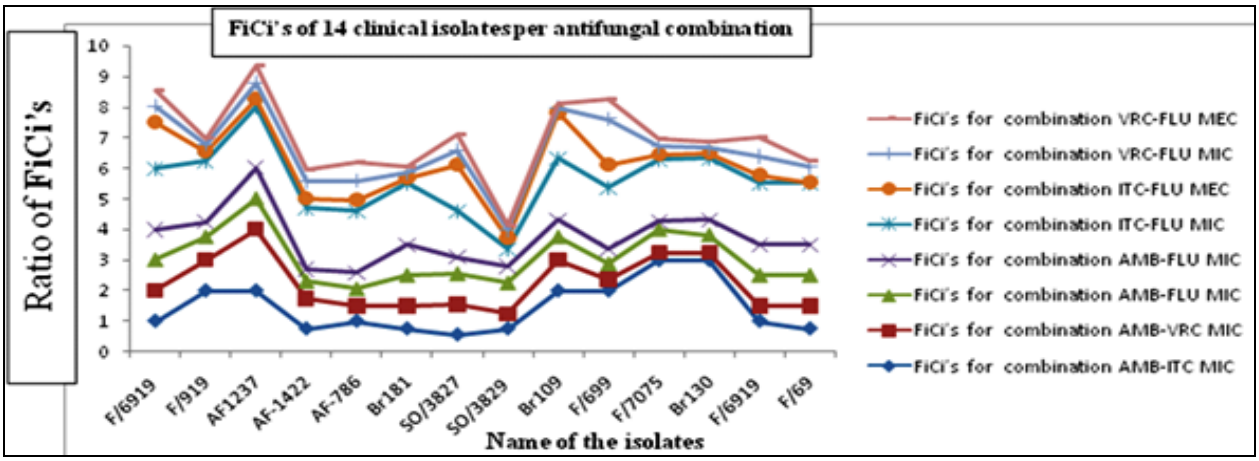

FIG. 1: FiCi's OF 14 CLINICAL ISOLATES PER ANTIFUNGAL COMBINATION

DISCUSSION: Clinical in formations have explained cases of invasive *Candidiasis* that reacted to this grouping [27]. Concerning combinations of Fluconazole and azole agents, lessons in vitro have displayed synergy against *Candida species*, variable from 38 to 100% of isolates, depending on the grouping and crossing point definitions [28, 29, 30]. Particularly, synergy was predicted for the mainstream of isolates when susceptibility testing finish points were distinct as considerable inhibition of growth.

Lesser ratio of synergy was found if the endpoint was distinct as the lowest concentration of the antifungal agent that entirely introverted fungal growth or when the MEC was selected for evaluating relations. Fluconazole in grouping with moreover itraconazole or voriconazole has been revealed to be competent in animal models of *Candidiasis* and in caring for some complicated treatment of human illness caused by species of *Candida* [31, 32, 33, 34].

CONCLUSION: An unresponsive result was examined for groupings of amphotericin B and azole drugs. Grouping with caspofungin endowed with a dissimilar effect, depending on the antifungal drugs and MIC or MEC mark of completion determination, but antagonism was not present. Amphotericin B-Fluconazole and itraconazole- Fluconazole combinations proved a dissimilar consequence at what time the MIC was used; even if the combinations were synergistic adjacent to a numeral of strains if the MEC was used as the illustration endpoint [35, 36]. The contradictory results could be elucidated mainly by the standard used for evaluating antifungal interaction. Fluconazole plus voriconazole exhibited a synergistic result in spite of the conclusion point used.

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REFERENCES:

1. Hajjeh RA, Sofair AN, Harrison L H. Incidence of bloodstream infections due to Candida species and in vitro susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. *J Clin Microbiol*.2004; 42:1519-1527
2. Barberino MG, Silva N, Reboucas C, Barreiro K, Alcantara AP, Netto E M, Albuquerque L, Brites C. Evaluation of Blood Stream Infections By *Candida* In Three Tertiary Hospitals In Salvador, Brazil: A Case-Control Study. *Braz. J. Infect Dis.* 2006; 1:36-40.
3. Lorenz MC, Fink G R. The glyoxylate cycle is required for fungal virulence. *Nature.* 2001; 412:83–86.
4. Geiger A M, Foxman B, Gillespie BW. The epidemiology of vulvovaginal candidiasis among university students. *Am J Public Health*. 1995; 85: 1146–8.
5. Trick W E, Fridkin S K, Edwards J R. National Nosocomial Infections Surveillance System Hospitals. Secular trend of hospital-acquired candidemia among intensive care unit patients in the United States during 1989-1999. *Clin Infect Dis.* 2002; 35: 627-630.
6. Ellis M. Invasive fungal infections: evolving challenges for diagnosis and therapeutics. Mol Immunol. 2002; 38:947–57.
7. Lipsett P A .Surgical critical care: fungal infections in *surgical patients Critical Care Medicine.* 2006;34: S215–224.
8. Magee B B, Magee PT. Induction of mating in *Candida albicans* by construction of MTLa and MTLalpha strains. *Science.* 2000; 289:310–13.
9. Graybill J R. The future of antifungal therapy. *Clin. Infect. Dis.*, Suppl. 1996; 2: S166-78.
10. Singh N. Changing spectrum of invasive candidiasis and its therapeutic implications. *Clinical Microbiology and Infection.* 2001; 2:1-7.
11. Georgopapadakou N and Walsh T. Antifungal agents: chemotherapeutic targets and immunologic stratagies. Antimicrob Agents Chemother. 1996; 40: 279–291.
12. Kontoyiannis D P. Lewis RE: Antifungal drug resistance of pathogenic fungi. *Lancet.* 2002; 359:1135-1144.
13. Pfaller M A, Messer S A, Boyken L, Hollis R J, Rice C, Tendolkar S and Diekema D J . In vitro activities of voriconazole, posaconazole, and fluconazole against 4,169 clinical isolates of *Candida* spp. and *Cryptococcus neoformans* collected during 2001 and 2002 in the

ARTEMIS global antifungal surveillance program. Diagn. *Microbiol. Infect. Dis* 2004;48: 201–205

14. Sanglard D, Ischer F, Monod M, Bille J. Susceptibilities of Candida albicans multidrug transporter mutants to various antifungal agents and other metabolic inhibitors. *Antimicrob Agents Chemother*. 1996;40: 2300-2305.
15. Dodgson A R, Dodgson K J, Pujol C, Pfaller M A& Soll D R . Cladespecific flucytosine resistance is due to a single nucleotide change in the *FUR1*gene of *Candida albicans*. *Antimicrob Agents Chemother.* 2004;48: 2223–2227.
16. Cohen L E, Anderson J B & Kohn L M. Evolution of drug resistance in *Candida albicans*. *Ann Rev. Microbiol.* 2002; 56:139–165.
17. Cowen L E, Sanglard D, Calabrese D, Sirjusingh C, Anderson J B, Kohn LM . Evolution of drug resistance in experimental populations of *Candida albicans*. *J. Bacterial.* 2000; 182:1515–22.
18. Borst P . Genetic mechanisms of drug resistance. A review. *Acta Oncol*. 1991; 30: 87–105.
19. Chryssanthou E and Cuenca-Estrella M. Comparison of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing proposed standard and the E-test with NCCLS broth microdilution method for voriconazole and caspofungin susceptibility testing of yeast species. *J. Clin. Microbiol* 2002; 40: 3841-4.
20. National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard, 2nd ed. M27-A2. National Committee for Clinical Laboratory Standards, WAYNE, PA. 2002.
21. Vermitsky J P, Liu T, Edlind T D and Rogers P D. *PDR1* regulates multidrug resistance in *Candida glabrata*: gene disruption and genomewide expression studies. *Mol. Microbiol*. 2006; 61:704–722.
22. Vermitsky J-P and Edlind T D. Azole resistance in *Candida glabrata*: coordinate upregulation of multidrug transporters and evidence for a Pdr1-like transcription factor. *Antimicrob. Agents Chemother*. 2004;48: 3773– 3781
23. Herbrecht R, Denning D W, Patterson T F, Bennett J E, Greene R E, Oestmann J W. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. *N. Engl. J. Med*. 2002; 347:408–415.
24. Hoffman H L and Rathbun R C. Review of the safety and efficacy of voriconazole. *Expert Opin. Investig*. *Drugs* .2002; 11:409–429.
25. Swinne D, Watelle M, Van der Flaes M, Nolard N. *In vitro* activities of voriconazole (UK-109, 496), fluconazole, itraconazole, and amphotericin B against 132 non-*albicans* bloodstream yeast isolates (CANARIstudy). *Mycoses* 2004; 47: 77–183.
26. Walsh T J, Pappas P, Winston D J, Lazarus H M, Petersen F, Raffalli J . Voriconazole compared with liposomal amphotericin B for empirical antifungal therapy in patients with neutropenia and persistent fever. *N. Engl. J. Med*. 2002; 346: 225–234.
27. Purkins L, Wood N, Greenhalgh K, Eve M D, Oliver S D . The pharmacokinetics and safety of intravenous voriconazole-a novel wide spectrum antifungal agent. *Br. J. Clin. Pharmacol*. 2003; 56: 2–9.
28. Perfect J D, Marr K A, Walsh T J, Greenberg R N, DuPoint B, Torre-Cisneros J de la, Just-Nubling G, Schlamm H T, Lutsar I, Espinel-Ingroff A, Johnson E . Voriconazole treatment for less-common, emerging, or refractory fungal infections. *Clin. Infect. Dis*. 2003; 36:1122–1131.
29. Pfaller M A, Rhine-Chalberg R, Redding S W, Smith J, Farinacci G, Fothergill A W. Variations in fluconazole susceptibility and electrophoretic karyotype among oral isolates of *Candida albicans* from patients with AIDS and oral candidiasis. *Journal of Clinical Microbiology* 1994; 32:59-64.
30. Kuhlberg B J, Sobel J D, Ruhnke M, Pappas P G, Viscoli C, Rex J H, Cleary J D, Rubinstein E, Church L W, Brown J M, Schlamm H T, Oborsha I T, Hilton F, Hodges M R. Voriconazole versus a regimen of amphotericin B followed by fluconazole for candidemia in nonneutropenic patients: a randomized non-inferiority trial. *Lancet* 2005; 366: 1435– 1442.
31. Garcia-Effron G, Park S, Perlin D S. Correlating echinocandin MIC and kinetic inhibition of fks1 mutant glucan synthases for Candida albicans: implications for interpretive breakpoints. *Antimicrob Agents Chemother* 2009; 53: 112– 122.
32. Garcia-Effron G, Kontoyiannis DP, Lewis R E, Perlin D S. Caspofungin-resistant Candida tropicalis strains causing breakthrough fungemia in patients at high risk for hematologic malignancies. *Antimicrob Agents Chemother* 2008; 52: 4181–4183.
33. Goldman GH, Ferreira da Silva, dos Reis Marques M E, Savoldi E, Perlin M, Park D et al.. Evaluation of fluconazole resistance mechanisms in Candida albicans clinical isolates from HIV-infected patients *Brazil. Diagn Microbiol Infect. Dis*. 2004; 50: 25-32.
34. Arendrup M C, Garcia-Effron G, Buzina W, Mortensen K L, Reiter N, Lundin C, Jensen EH, Lass-Flörl C, Perlin DS, Bruun B . Breakthrough Aspergillus fumigatus and Candida albicans double infection during caspofungin treatment: laboratory characteristics and implication for susceptibility testing. *Antimicrob Agents Chemother* 2008; 53:1185–1193.
35. Miller C D, Lomaestro B W, Park S, Perlin D S. Progressive esophagitis caused by Candida albicans with reduced susceptibility to caspofungin. *Pharmacotherapy* 2006; 26: 877–80.
36. Lengeler K B, Davidson RC, DSouza C, Harashima T, Shen W C . Signal transduction cascades regulating fungal development and virulence. *Microbiol. Mol. Biol. Rev* 2000; *64:746*–85.

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