In Vitro Antifungal Combination of Flucytosine with Amphotericin B, Voriconazole, or Micafungin against Candida auris Shows No Antagonism

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SUSCEPTIBILITY

In Vitro Antifungal Combination of Flucytosine with Amphotericin B, Voriconazole, or Micafungin against *Candida auris* Shows No Antagonism

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ABSTRACT *Candida auris* is an emerging, multidrug-resistant pathogen responsible for invasive hospital-acquired infections. Flucytosine is an effective anti-*Candida* species drug, but which cannot be used as a monotherapy because of the risk of development of resistant mutants during treatment. It is, therefore, noteworthy to test possible combinations with flucytosine that may have a synergistic interaction. In this study, we determined the *in vitro* interaction between flucytosine and amphotericin B, micafungin, or voriconazole. These combinations have been tested against 15 *C. auris* isolates. The MIC ranges (geometric mean [Gmean]) of flucytosine, amphotericin B, micafungin, and voriconazole were 0.125 to 1 μg/ml (0.42 μg/ml), 0.25 to 1 μg/ml (0.66 μg/ml), 0.125 to 0.5 μg/ml (0.3 μg/ml), and 0.03 to 4 μg/ml (1.05 μg/ml), respectively. When tested in combination, indifferent interactions were mostly observed with fractional inhibitory concentration index values from 0.5 to 1, 0.31 to 1.01, and 0.5 to 1.06 for the combinations of flucytosine with amphotericin B, micafungin, and voriconazole, respectively. A synergy was observed for the strain CBS 10913 from Japan. No antagonism was observed for any combination. The combination of flucytosine with amphotericin B or micafungin may be relevant for the treatment of *C. auris* infections.

KEYWORDS *Candida auris*, antifungal, combination, amphotericin B, flucytosine, micafungin, voriconazole

Candida auris is an multidrug-resistant emerging fungal pathogen responsible for invasive infection (1–3). *C. auris* was first described from an external ear canal drainage specimen from a Japanese patient in 2009 (4). After 2009, *C. auris* was highlighted worldwide and has been the subject of many publications (5–9). But the cases of *C. auris* have been mainly reported from India, Central and North America, Spain, United Kingdom, Kuwait, South Africa, Israel, and Oman (10–16). Recently, an isolate of *C. auris* has been found in China (17), and the first case of *C. auris* infection has been described in France (18).

C. auris has a close phylogenetic relationship with *Candida haemulonii*, *Candida duobushaemulonii*, and *Clavispora lusitaniae* which can lead to erroneous identifications using commercial biochemical identification methods (19, 20). Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) or molecular identification based on sequencing the D1-D2 region of the 28S ribosomal DNA allows the correct identification of *C. auris* (21).

The emergence of *C. auris* is alarming especially because this fungal pathogen has the potential to harbor or develop multidrug resistance. Only 4 drug classes are available for the systemic treatment of *Candida* infections, including the echinocandins (caspofungin, micafungin, and anidulafungin), azoles (fluconazole, itraconazole, vori-

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TABLE 1 *In vitro* interactions of amphotericin B with flucytosine against *Candida auris*[a]

Isolate	MIC (μg/ml) of drug alone		MIC (μg/ml) of the drug in combination		Lowest FICI for the combination	Interaction
	AMB	5-FC	AMB	5-FC		
CBS 12372	0.25	0.5	0.06	0.25	0.75	N
CBS 12373	0.25	0.5	0.125	0.06	0.63	N
CBS 12766	1	0.5	0.5	0.06	0.63	N
CBS 12767	1	0.5	0.5	0.06	0.63	N
CBS 12768	1	0.5	0.5	0.06	0.63	N
CBS 12769	1	0.5	0.5	0.06	0.63	N
CBS 12770	0.5	0.5	0.25	0.25	1	N
CBS 12771	1	0.5	0.5	0.06	0.63	N
CBS 12772	1	0.5	0.5	0.06	0.63	N
CBS 12773	1	0.5	0.5	0.03	0.56	N
CBS 12774	1	0.5	0.5	0.06	0.63	N
CBS 12775	1	0.5	0.5	0.06	0.63	N
CBS 12776	1	0.5	0.5	0.06	0.63	N
CBS 12777	0.25	0.5	0.125	0.06	0.63	N
CBS 10913	0.25	0.25	0.06	0.06	0.50	S

[a]AMB, amphotericin B; 5-FC, flucytosine; N, no interaction; S, synergy; FICI, fractional inhibitory concentration index.

conazole, posaconazole, and isavuconazole), polyenes (amphotericin B), and, finally, the pyrimidine analogue flucytosine (22). Resistance to each of these classes has been reported. Although uncommon, some *C. auris* strains exhibit elevated MICs for all three major classes of antifungal drugs, i.e., azoles, polyenes, and echinocandins (23, 24). There are currently recommendations for the treatment of *C. auris* infections with echinocandins as first-line therapy (25). Amphotericin B could be used for patients not responding to echinocandin therapy, depending on MIC results (2, 3). However, it has been shown that amphotericin B resistance is not uncommon (26).

Amphotericin B, flucytosine, echinocandins, and azoles have different cellular targets, and their combination might be of interest in the treatment of *C. auris* infections. The combination of flucytosine with amphotericin B has been shown *in vitro* to be synergistic against several fungal pathogens, such as *Cryptococcus neoformans* (27). Amphotericin B and flucytosine are used in several fungal infections, such as cryptococcosis, but also in *Candida* species infections (e.g., endocarditis, meningitis, and endophthalmitis) (28, 29). The hypothesis would be that amphotericin B, by forming pores of the fungal membrane, would facilitate the intracellular penetration of flucytosine. Furthermore, flucytosine also exhibits synergy with azoles against *C. neoformans* and *Candida albicans* both *in vitro* and *in vivo* (30–33).

The aim of the present study was to evaluate the *in vitro* interaction between flucytosine and either a polyene (amphotericin B), an echinocandin (micafungin), or an azole (voriconazole) against several *C. auris* isolates from different origins.

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RESULTS

The results for the tested drugs alone and in combination against *C. auris* isolates are summarized in Tables 1, 2, and 3. All experiments were run in duplicate with similar results. MICs of the drugs alone were within ± 2 $\log_2$ dilutions in 100% of the cases. Therefore, results from one replicate are shown. The MIC ranges of drugs alone against the strains were 0.125 to 1 μg/ml for flucytosine, 0.25 to 1 μg/ml for amphotericin B, 0.125 to 0.5 μg/ml for micafungin, and 0.03 to 4 μg/ml for voriconazole. With the checkerboard microdilution assay, when amphotericin was combined with flucytosine, the MIC ranges of amphotericin B and flucytosine decreased from 0.25 to 1 μg/ml to 0.06 to 0.5 μg/ml and from 0.125 to 1 μg/ml to 0.03 to 0.25 μg/ml, respectively. These values correspond to a median (range) decrease of 1- (1- to 2-) fold and 3- (1- to 4-) fold for amphotericin B and

TABLE 2 *In vitro* interactions of micafungin with flucytosine against *Candida auris*[a]

Isolate	MIC (μg/ml) of drug alone		MIC (μg/ml) of the drug in combination		Lowest FICI for the combination	Interaction
	MICA	5-FC	MICA	5-FC		
CBS 12372	0.25	0.5	0.125	0.008	0.52	N
CBS 12373	0.25	1	0.125	0.03	0.53	N
CBS 12766	0.5	0.5	0.25	0.008	0.52	N
CBS 12767	0.25	0.5	0.03	0.25	0.63	N
CBS 12768	0.5	0.5	0.25	0.008	0.52	N
CBS 12769	0.25	0.5	0.125	0.25	1	N
CBS 12770	0.25	0.25	0.125	0.125	1	N
CBS 12771	0.25	0.25	0.125	0.125	1	N
CBS 12772	0.5	0.25	0.25	0.015	0.56	N
CBS 12773	0.25	0.5	0.125	0.25	1	N
CBS 12774	0.25	0.5	0.25	0.008	1.02	N
CBS 12775	0.25	0.5	0.125	0.25	1	N
CBS 12776	0.5	0.5	0.25	0.008	0.52	N
CBS 12777	0.5	0.5	0.25	0.008	0.52	N
CBS 10913	0.125	0.125	0.03	0.008	0.31	S

[a]MICA, micafungin; 5-FC, flucytosine; N, no interaction; S, synergy; FICI, fractional inhibitory concentration index.

flucytosine, respectively. The fractional inhibitory concentration index (FICI) ranged from 0.5 to 1 which was indicative of no-interaction except for one isolate (CBS 10913) for which a synergy was observed. When micafungin was combined with flucytosine, the MIC ranges of micafungin and flucytosine decreased from 0.125 to 0.5 μg/ml to 0.03 to 0.25 μg/ml and from 0.125 to 1 μg/ml to 0.008 to 0.25 μg/ml, respectively. This corresponds to a median (range) decrease of 1- (0- to 3-) fold and 4- (1- to 6-) fold for micafungin and flucytosine, respectively. Synergistic effects of micafungin with flucytosine were shown against the *C. auris* isolate number CBS 10913 from Japan (FICI, 0.31). When voriconazole was combined with flucytosine, the MIC ranges of voriconazole and flucytosine decreased from 0.03 to 4 μg/ml to 0.008 to 2 μg/ml and from 0.125 to 1 μg/ml to 0.008 to 0.5 μg/ml, respectively. These results correspond to a median (range) decrease of 7- (0- to 8-) fold and 1- (0- to 5-) fold for voriconazole and flucytosine, respectively. FICI ranged from 0.504 to 1.06, which was indicative of no interaction. No antagonist effects were observed for any combination.

An example of analysis (strain CBS 10913) with the response surface approach based on the Bliss model for the three combinations is shown in Fig. 1. Synergy was observed

TABLE 3 *In vitro* interactions of voriconazole with flucytosine against *Candida auris*[a]

Isolate	MIC (μg/ml) of drug alone		MIC (μg/ml) of the drug in combination		Lowest FICI for the combination	Interaction
	VORI	5-FC	VORI	5-FC		
CBS 12372	1	0.25	0.5	0.125	1	N
CBS 12373	1	0.5	0.008	0.5	1	N
CBS 12766	1	0.5	0.03	0.25	0.53	N
CBS 12767	1	0.25	0.008	0.25	1	N
CBS 12768	1	0.5	0.008	0.25	0.51	N
CBS 12769	1	0.5	0.008	0.5	1	N
CBS 12770	4	0.25	2	0.008	0.53	N
CBS 12771	2	0.5	0.008	0.25	0.51	N
CBS 12772	1	0.25	0.008	0.25	1	N
CBS 12773	1	0.5	0.008	0.5	1	N
CBS 12774	2	0.5	0.008	0.5	1	N
CBS 12775	2	0.5	0.008	0.25	0.51	N
CBS 12776	1	0.5	0.008	0.25	0.51	N
CBS 12777	2	0.25	0.008	0.25	1	N
CBS 10913	0.03	0.125	0.03	0.008	1.06	N

[a]VORI, Voriconazole; 5-FC, flucytosine; N, no interaction; S, synergy; FICI, fractional inhibitory concentration index.

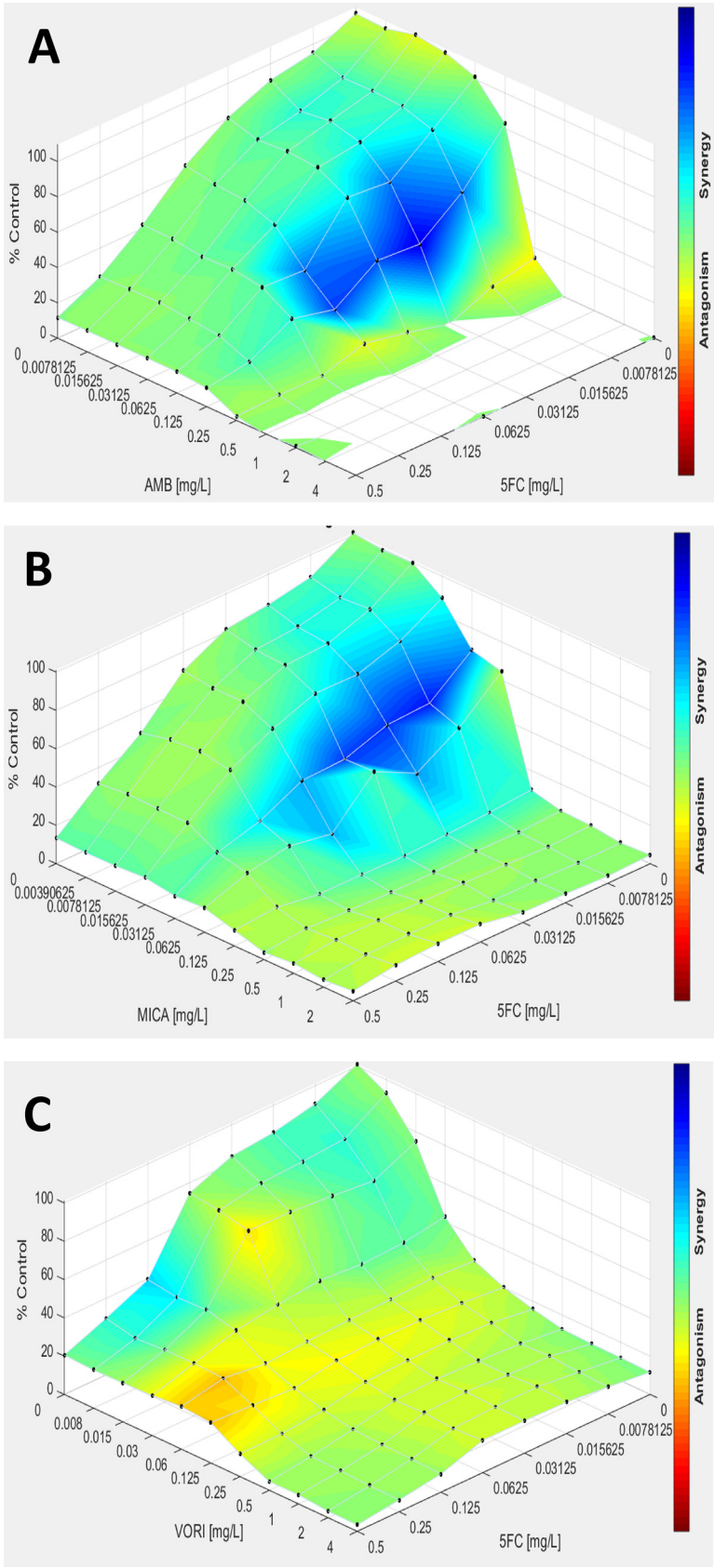

FIG 1 Combination of flucytosine with amphotericin B (A), micafungin (B), and voriconazole (C) against *C. auris* CBS 10913 analyzed by response surface modeling. Combined results from two independent experiments were used for analysis.

for the combinations of flucytosine with either amphotericin B or micafungin, while the interaction between flucytosine and voriconazole was indifferent.

DISCUSSION

So far, evidence to support combination therapy in infections with *C. auris* is lacking, and clinicians make decisions on a case-by-case basis. Nevertheless, as *C. auris* is often a multidrug-resistant organism, combination therapy could be interesting, as suggested by the few *in vitro* studies performed (34–36). In a recent study, interactions between azoles and echinocandins showed synergy for the combination of micafungin with voriconazole against all the isolates tested (34). Moreover, the same study also showed indifferent interactions when micafungin was combined with fluconazole and indifferent interactions when caspofungin was combined with fluconazole or voriconazole.

In the present study, we tested the combination of flucytosine with a polyene, an echinocandin, or an azole. Flucytosine is an interesting anti-*Candida* species drug because this nucleic base analogue penetrates the fungal cells through a transporter (cytosine permease) and blocks DNA synthesis and protein synthesis. Flucytosine has excellent bioavailability *per os*. Because this molecule is water-soluble and not linked to plasma proteins, its distribution in most tissues is homogeneous as well as its diffusion in cerebrospinal fluid. Against *C. auris*, flucytosine is very active, as shown in the present study as well as in previous studies (21, 23, 37). Nevertheless, flucytosine is never used as a monotherapy because of the rapid selection of resistant mutants during treatment. Therefore, it seems advisable to test *in vitro* combinations with this molecule. The major interests of using a combination treatment with flucytosine are to benefit from the good flucytosine pharmacokinetics and to potentially have a synergistic interaction with the partner drug. A third motivation for combination therapy might be to reduce the risk of further resistance development in *C. auris* during therapy, although this is not trivial, as this organism has a high potential for resistance acquisition. It is also important to ensure that these drug combinations do not lead to antagonism.

In the present study, we used the checkerboard microdilution method. Analyzing results for this technique is not straightforward, particularly for the choice of the most suitable inhibition endpoint which could be different for analyzing the activity of the drugs alone or in combination. Moreover, interactions are only calculated for few concentrations (corresponding to the MIC). For this reason, we also used an alternative method of response surface modeling (based on the Bliss independency model) to visualize the interaction when synergy was found by the FICI method. The main advantages of this approach are that it is independent of any endpoint and it takes in account all the concentrations tested in the microplate. The same results were obtained by the two mathematical models used (Loewe and Bliss).

Overall, although the three combinations showed mainly indifferent interactions, we observed that flucytosine can be used in combination with other drugs without risk of antagonism. Of note, the only isolate against which synergy was observed was the type strain CBS 10913, characterized by its lower MICs than more recent isolates from India or other countries (23, 38). The synergistic interactions could be linked to this particular susceptibility profile. Nevertheless, despite the high susceptibility of CBS 10913 to voriconazole compared with the other strains, synergy was not observed for the voriconazole-flucytosine combination. One limitation of our study is the test of isolates only belonging to the East Asian or South Asian clades. No isolates representing other clades of *C. auris* were included. There are currently 4 or 5 phylogeographic clades (3, 37, 39). The clades are genetically and phenotypically distinct, and there could be alternative results among the other clades.

Results of antifungal combinations are important because therapeutic options for *C. auris* are limited. Although the echinocandins appear to be the best choice for initial treatment, the development of new antifungal drugs with activity against *C. auris* will be essential to control multidrug-resistant isolates. Before new drugs are available, the

emergence of this species and its multidrug resistance encourages new therapeutic strategies, including the combination of antifungals.

In summary, we demonstrated that there is no antagonism when flucytosine is combined *in vitro* with amphotericin B, voriconazole, or micafungin.

MATERIALS AND METHODS

A total of 15 *C. auris* isolates were included, of which 12 were clinical isolates collected from Indian patients during the years 2009 to 2011 (10) and 3 were the control and type strains from Korea (CBS 12372 and CBS 12373) from 2004 to 2006 (40) and Japan (CBS 10913) from 2009 (4). All strains have been previously identified by molecular techniques (4, 10, 40, 41).

The interactions of flucytosine with either amphotericin B, micafungin, or voriconazole were investigated by using a microdilution checkerboard method based on the EUCAST reference technique with 96-well microtiter plates (38). Drug dilutions were prepared to obtain four times the final concentration. Final concentrations ranged from 0.008 to 0.5 μg/ml for flucytosine, 0.008 to 4 μg/ml for amphotericin B, 0.004 to 2 μg/ml for micafungin, and 0.008 to 4 μg/ml for voriconazole. For two-dimensional microplate preparation, 50 μl of each concentration of flucytosine was added into wells 1 to 11 of each column and then 50 μl of the partner drug was added into wells A to H of each line. Line H and column 11 contained the partner drug and flucytosine alone, respectively. Column 12 served as the growth control containing only 50 μl of double-strength RPMI-2% glucose with dimethyl sulfoxide (DMSO). Plates were incubated at 37°C for 24 h and read spectrophotometrically. A growth inhibition endpoint of 50% was used both for the drugs tested alone and in combination. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality controls. The experiments were performed in duplicate. Drug-free microplates were also incubated after inoculation with three strains, including the type strain, to ensure that possible heterogenous growth across the microplates (i.e., lower growth in the center area of the plate compared to outer wells) was not responsible for the observed effect (synergy or antagonism).

Two different methods were used to interpret the interactions of drugs. First, the fractional inhibitory concentration index (FICI) was calculated. The FICI was defined as $FIC_A + FIC_B = (C_A/MIC_A) + (C_B/MIC_B)$ where MIC_A and MIC_B are the MICs of drugs A and B alone and C_A and C_B are the concentrations of the drugs in combination, in all wells corresponding to an MIC. The interaction was considered synergistic when the FICI was $\leq$0.5, indifferent at $>$0.5 to $\leq$4.0, and antagonistic at $>$4 (42). An alternative approach, independent of an endpoint, was also used for visualization of the interaction between drugs. Briefly, data from combinations were processed for a response surface analysis by the Combenefit software, using the Bliss independence model (43, 44).

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