









Heightened Efficacy of Anidulafungin When Used in Combination with Manogepix or 5-Flucytosine against *Candida auris* In Vitro

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ABSTRACT *Candida auris* is an emerging, multidrug-resistant fungal pathogen that causes refractory colonization and life-threatening, invasive nosocomial infections. The high proportion of *C. auris* isolates that display antifungal resistance severely limits treatment options. Combination therapies provide a possible strategy by which to enhance antifungal efficacy and prevent the emergence of further resistance. Therefore, we examined drug combinations using antifungals that are already in clinical use or are undergoing clinical trials. Using checkerboard assays, we screened combinations of 5-flucytosine and manogepix (the active form of the novel antifungal drug fosmanogepix) with anidulafungin, amphotericin B, or voriconazole against drug resistant and susceptible *C. auris* isolates from clades I and III. Fractional inhibitory concentration indices (FICI values) of 0.28 to 0.75 and 0.36 to 1.02 were observed for combinations of anidulafungin with manogepix or 5-flucytosine, respectively, indicating synergistic activity. The high potency of these anidulafungin combinations was confirmed using live-cell microfluidics-assisted imaging of the fungal growth. In summary, combinations of anidulafungin with manogepix or 5-flucytosine show great potential against both resistant and susceptible *C. auris* isolates.

KEYWORDS *Candida auris*, antifungal combination, anidulafungin, flucytosine, manogepix, synergy

Candida auris is an emerging fungal pathogen that causes nosocomial invasive infections and that is difficult to eradicate, following the colonization of hospitalized patients (1). *C. auris* was first identified in 2009 in Japan, but, since then, outbreaks have been observed on most continents (1, 2). *C. auris* strains have been subdivided into four genetic clades, namely, the South Asian (I), East Asian (II), South African (III) and South American (IV) clades (3), with a potential fifth Iranian clade having been identified more recently (4). The organism colonizes the skin and can lead to mucosal or bloodstream infections, predominately in immunocompromised hosts (1). Invasive *C. auris* infections are associated with mortality rates between 28% and 60%, and treatment failure due to antifungal resistance is often observed (1, 3, 5–11).

To date, only four classes of antifungal drugs are available for the treatment of invasive fungal infections: azoles, polyenes, echinocandins and the nucleoside analogue 5-flucytosine. 5-flucytosine has high oral bioavailability with high activity against *C. auris*, but it is not generally used in monotherapy due to the rapid emergence of resistance (12). Current guidelines recommend echinocandin treatment as a first line therapy for

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invasive candidiasis and for *C. auris* infections, in particular (13, 14). However, echinocandin resistance can develop during treatment (15, 16). Resistance to all four existing classes of antifungals has been reported in *C. auris*, with various drug susceptibilities and resistance mechanisms between clades (17). Around 90% of *C. auris* isolates show resistance to fluconazole, with various susceptibilities to other azoles (3, 6, 9, 18). Resistance to amphotericin B and the echinocandins appears to be less common, having been reported in 13 to 35% and 2 to 7% of tested isolates, respectively (3, 9, 18). Alarmingly, up to 41% of the isolates exhibit resistance to two or more antifungal classes (3, 18). Consequently, the Centers for Disease Control and Prevention (CDC) recently added *C. auris* to its list of urgent antibiotic resistance threats (19), and the World Health Organization (WHO) declared it a critical threat in its fungal priority pathogens list (14).

The limited number of antifungal drugs as well as the increased threat of antifungal resistance in *C. auris* means that novel treatment strategies are urgently needed. Combinations of antifungals with different mechanisms of action provide one proposed therapeutic strategy. Previous *in vitro* studies investigated combinations of echinocandins with azoles or the polyene amphotericin B (20–24) as well as combinations of 5-flucytosine with the other three antifungal classes in *C. auris* (25–27). These studies observed either synergy or indifference and no antagonism for all of the tested combinations, with variability between *C. auris* isolates. The most promising combinations were azoles combined with echinocandins which, in two studies, resulted in synergy against all tested isolates (20, 23).

Combinations with 5-flucytosine are of particular interest, as its combinations with amphotericin B and fluconazole have been shown to be superior to monotherapy in phase III clinical trials against cryptococcal meningitis (28). As a result of these trials, 5-flucytosine is now more widely available globally, including in countries such as South Africa, which suffers a high burden of *C. auris* candidemia (28, 29). Echinocandin combinations with 5-flucytosine have been reported to be indifferent in most cases, but these combinations have shown 100% growth inhibition and fungicidal activity against multidrug-resistant isolates (25–27).

None of these studies included the new antifungal fosmanogepix, which has recently completed phase 1 and 2 clinical trials and is one of several new antifungals in the pipeline that may also exhibit activity against *C. auris* (30). Fosmanogepix is a prodrug that is converted into the active compound manogepix by systemic phosphatases (31). Manogepix inhibits a novel antifungal target, namely, Gwt1, which is involved in the GPI-anchor biosynthetic pathway, thereby leading to a decrease in cell wall-anchored mannoproteins (31). In the present study, we examined combinations of manogepix or 5-flucytosine with anidulafungin, amphotericin B, or voriconazole against a range of resistant and susceptible *C. auris* isolates *in vitro*.

RESULTS

Antifungal activity against *C. auris* isolates. The antifungal susceptibility profiles of 25 *C. auris* isolates were determined in order to select a subset of isolates with different drug susceptibilities for antifungal combination testing. The ranges of minimal inhibitory concentrations (MICs) for the *C. auris* isolates against the tested antifungals are summarized in Table 1 and Table S1. The MIC values for amphotericin B clustered around the breakpoint of 2 mg/L, which is a known problem for the broth microdilution susceptibility testing of amphotericin B in RPMI medium, making it difficult to distinguish resistant and susceptible isolates (32). Fluconazole showed a large percentage of resistant *C. auris* isolates (96%; breakpoint ≥ 32 mg/L) with high MIC values that ranged from 4 to ≥ 128 mg/L, whereas the other triazole that was tested (voriconazole) displayed more potent antifungal activity, with the MIC values ranging from 0.06 to 16 mg/L and with 40% resistant isolates (breakpoint ≥ 2 mg/L). Of all of the antifungals tested with an available breakpoint, anidulafungin produced the lowest percentage of resistant isolates (32%; ≥ 4 mg/L). The most potent antifungal activity against *C. auris* was observed for manogepix (MIC₅₀/MIC₉₀, 0.008/0.03 mg/L; range, 0.004 to 0.03), and this was followed by 5-flucytosine (MIC₅₀/MIC₉₀, 0.25/0.25 mg/L; range, 0.125 to 0.25).

TABLE 1 Antifungal MIC distribution for 25 *C. auris* isolates

Drug	MIC (mg/L)															MIC ₅₀ ^a	MIC ₉₀ ^b	%R ^c		
	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32				64	128
AMB					0	0	0	0	0	1	<u>24</u> ^d	0	0	0				2	2	96.0
FLC							0	0	0	0	0	1	0	0	5	5	<u>14</u>	≥128	≥128	96.0
VRC				0	1	2	6	1	5	<u>9</u>	0	0	1				1	2	40.0	
AFG				0	3	3	5	3	2	0	1	<u>0</u>	8				0.25	≥8	32.0	
5FC			0	0	0	0	11	<u>14</u>	0	0	0	0					0.25	0.25	No BP	
MGX	0	<u>11</u>	5	1	8	0	0	0	0	0							0.008	0.03	No BP	

^aMIC at which 50% of isolates were inhibited.^bMIC at which 90% of isolates were inhibited.^cPercentage of resistant isolates.^dModal MICs are indicated with underlined numbers. A gray background indicates a tentative *C. auris* breakpoint, according to the CDC. 5FC, 5-flucytosine; AFG, anidulafungin; AMB, amphotericin B; BP, breakpoint; FLC, fluconazole; MGX, manogepix; VRC, voriconazole. Empty cells indicate drug concentrations that were not tested.

Interaction of antifungal drug combinations against *C. auris* isolates. Based on their MIC values, 11 *C. auris* isolates with different drug susceptibility profiles were selected to investigate the interactions of anidulafungin, amphotericin B, and voriconazole with 5-flucytosine or manogepix. The FICI values for these combinations, as determined by the checkerboard assays, are presented in Table 2 and Fig. 1. The FICI values of separate repeats can be found in Tables S2 and S3). The combination of anidulafungin with 5-flucytosine resulted in synergistic interactions for 10/11 isolates (synergy, 2/11 isolates; partial synergy, 8/11 isolates). Meanwhile the combination of anidulafungin with manogepix led to synergy in all 11 isolates (synergy, 5/11 isolates; partial synergy, 6/11 isolates). These FICI values corresponded to a median (range) decrease in the MIC of 2 log₂-fold (1 to 4 log₂-fold) for anidulafungin and 2 log₂-fold (0 to 4 log₂-fold) for 5-flucytosine (Fig. 2A) or 3 log₂-fold (1 to 9 log₂-fold) for anidulafungin and 2 log₂-fold (1 to 3 log₂-fold) for manogepix (Fig. 2B). Additionally, both anidulafungin combinations achieved fungistatic activity with log₁₀-fold reductions in the CFU/mL values of 2.2 and 0.8, compared to the starting inoculum for the combination with manogepix and 5-flucytosine, respectively, whereas the corresponding monotherapies only had a negligible antifungal effect (Fig. S7).

The combination of amphotericin B with 5-flucytosine did not show full synergy for any of the tested isolates, though partial synergy was observed in 4/11 isolates (median FICIs, 0.63 to 0.75). The other isolates showed either additive (5/11 isolates) or indifferent (2/11 isolates; median FICIs, 1.01) interactions for amphotericin B with 5-flucytosine. For the combination of manogepix and 5-flucytosine, 3/11 isolates displayed partial synergy (median FICIs, 0.54 to 0.58), and 4/11 isolates showed additive or indifferent interactions (median FICIs, 1.01). The combination of manogepix and 5-flucytosine led to large reductions in the MIC by a median (range) of 7 log₂-fold

TABLE 2 FICI values for 5 antifungal combinations against 11 *C. auris* isolates^a

Isolate	AFG + 5FC Median (range)	AFG+mGX Median (range)	AMB + 5FC Median (range)	VRC + 5FC Median (range)	MGX + 5FC Median (range)
B19460	0.49 (0.48 to 0.50)	0.50 (0.50 to 0.52)	1.01 (1.00 to 1.01)	4.48 (1.01 to 8.00)	1.01 (1.00 to 1.01)
B19618	<u>0.56</u> (0.49 to 1.01)	<u>0.52</u> (0.51 to 1.00)	1.00 (0.52 to 1.02)	4.50 (1.02 to 5.00)	1.00 (1.00 to 1.02)
B17040	0.56 (0.15 to 0.56)	0.51 (0.33 to 0.55)	1.00 (1.00 to 1.01)	<u>1.01</u> (1.00 to 1.01)	1.01 (0.76 to 1.02)
B17041	0.74 (0.69 to 0.98)	0.65 (0.37 to 0.77)	1.00 (0.75 to 1.00)	<u>1.00</u> (0.56 to 1.02)	1.00 (0.63 to 1.01)
B18560	0.60 (0.30 to 0.61)	<u>0.28</u> (0.19 to 0.75)	1.01 (1.01)	1.00 (1.00 to 4.48)	0.56 (0.53 to 1.00)
B18843	0.98 (0.49 to 1.00)	0.63 (0.53 to 0.75)	1.00 (0.63 to 1.01)	4.50 (1.01 to 4.50)	1.00 (0.63 to 4.41)
B12694	<u>0.36</u> (0.24 to 0.37)	0.52 (0.20 to 0.62)	0.63 (0.53 to 1.00)	<u>1.01</u> (1.01)	0.54 (0.50 to 1.05)
B12663	<u>0.74</u> (0.38 to 1.00)	<u>0.33</u> (0.33 to 1.01)	0.75 (0.51 to 1.01)	<u>1.00</u> (1.00 to 1.01)	1.01 (0.51 to 1.05)
B12664	0.75 (0.62 to 0.98)	0.39 (0.29 to 0.51)	0.75 (0.62 to 0.75)	<u>1.01</u> (0.63 to 1.01)	0.58 (0.57 to 1.01)
B20931	0.53 (0.18 to 0.60)	0.49 (0.30 to 0.56)	1.00 (0.53 to 1.00)	1.01 (0.63 to 1.01)	1.01 (0.56 to 1.01)
B21040	1.02 (0.56 to 1.03)	0.75 (0.56 to 1.00)	0.75 (0.63 to 1.00)	1.01 (1.01)	1.00 (0.53 to 1.00)

^aSynergy, dark gray; partial synergy, medium gray; indifference/additivity, white; antagonism, light gray. Underlined values indicate resistance to either AFG or VRC. 5FC, 5-flucytosine; AFG, anidulafungin; AMB, amphotericin B; MGX, manogepix; VRC, voriconazole.

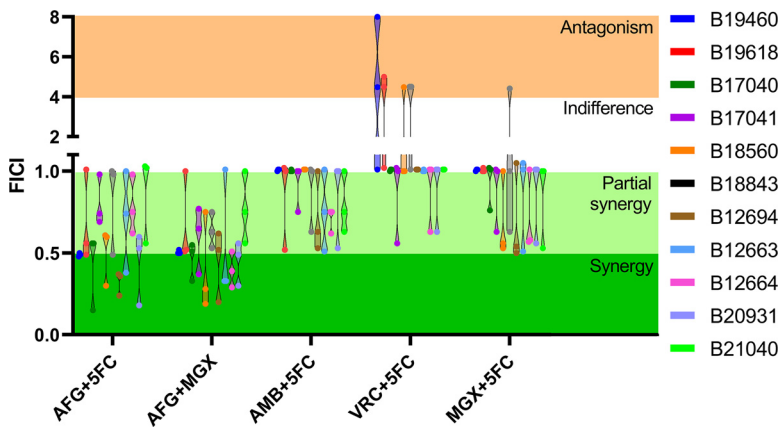


FIG 1 *In vitro* interactions of AFG, MGX, AMB, VRC, and 5FC, according to the FICI values for 11 *C. auris* isolates. Minimum FICI values are shown in the absence of antagonism. Otherwise, maximum FICI values are reported. Drug interaction ranges are indicated by background color: Synergy, dark green; partial synergy, light green; indifference, white; antagonism, red. The symbols represent the FICI values of three independent experiments. 5FC, 5-flucytosine; AFG, anidulafungin; AMB, amphotericin B; MGX, manogepix; VRC, voriconazole.

(1 to 8 log₂-fold) for 5-flucytosine, whereas the manogepix MIC values were only decreased by a median (range) of 0 log₂-fold (0 to 2 log₂-fold) (Fig. S1C). The drug combination resulting in the least favorable interactions was voriconazole with 5-flucytosine, with 3/11 isolates displaying antagonistic interactions (median FICIs, 4.48 to 4.50) and the remaining isolates displaying additive (3/11 isolates) or indifferent (5/11 isolates; median FICIs, 1.01) interactions.

Response surface analyses were also used to examine the drug combinations, and an example is shown in Fig. 3 for the multidrug-resistant isolate B12663 (see Fig. S2–S6 for the other isolates). Consistent with the FICI scores, the synergy maps indicate synergy

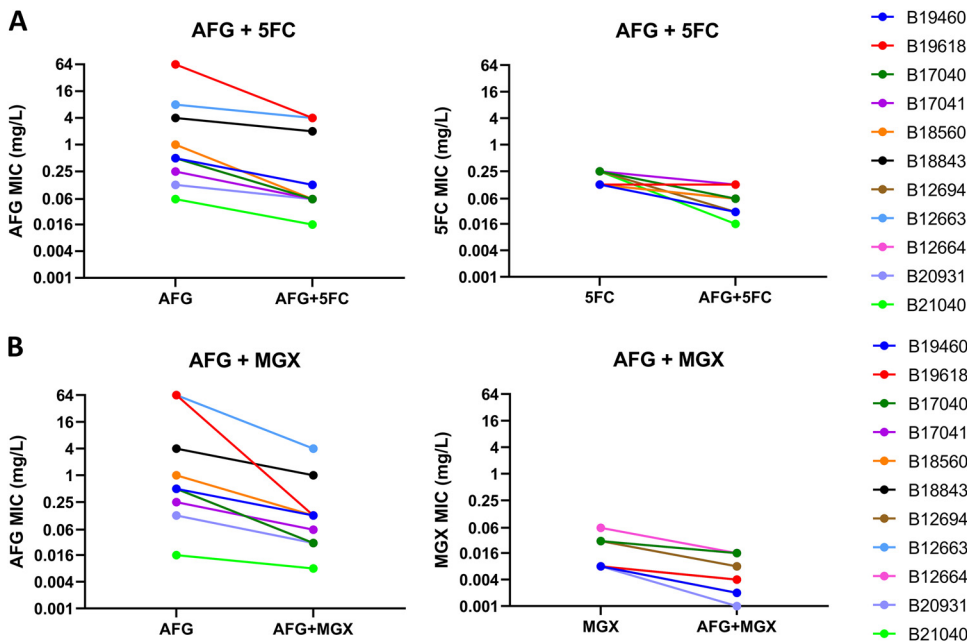


FIG 2 Changes in MIC values due to antifungal combinations for 11 *C. auris* isolates in combinations of anidulafungin with 5-flucytosine (A) and manogepix (B), compared to the antifungals in monotherapy, as determined via checkerboard assays. The symbols represent the median values of three independent experiments. 5FC, 5-flucytosine; AFG, anidulafungin; MGX, manogepix.

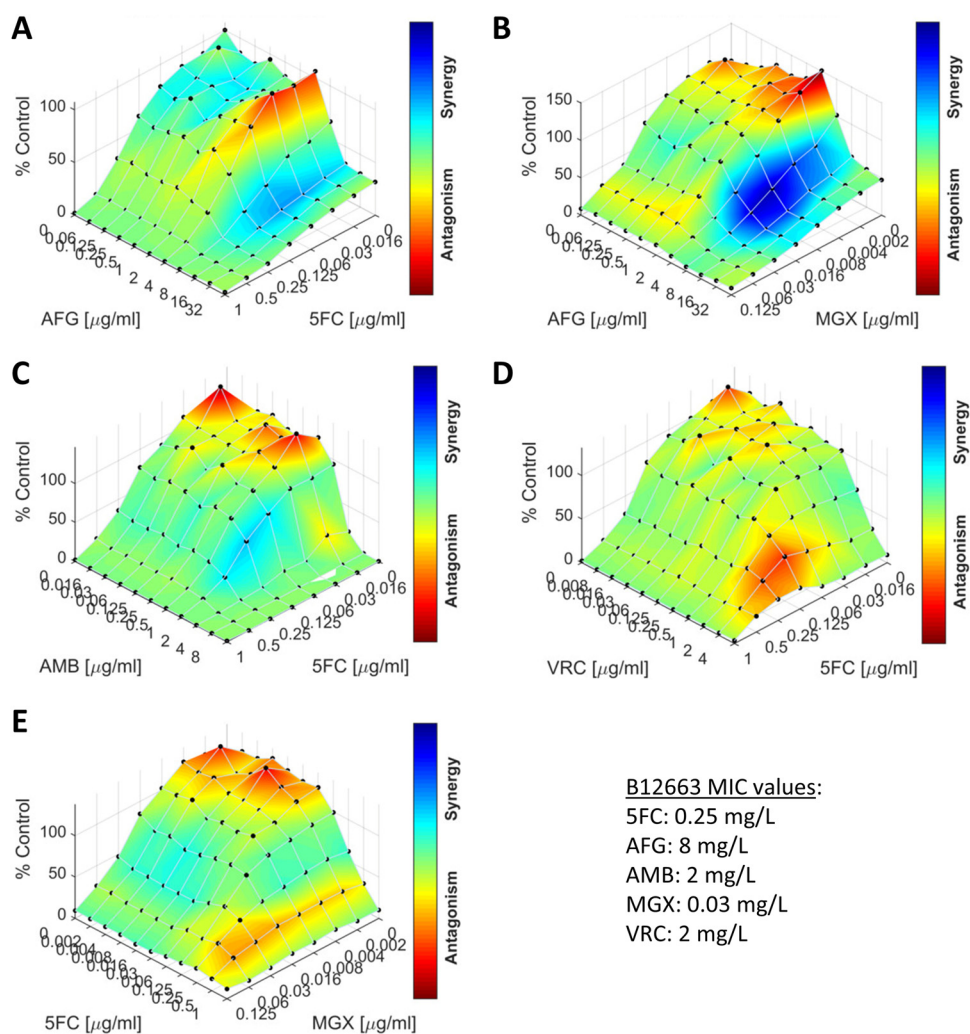


FIG 3 Synergy maps for 5 antifungal combinations against the multidrug-resistant *C. auris* isolate B12663. The interactions of 5-flucytosine with anidulafungin (A), amphotericin B (C), or voriconazole (D), as well as the interactions of manogepix with anidulafungin (B) or 5-flucytosine (E) were analyzed with Combeneft ($n = 3$). The graphs show the growth percentage, relative to the drug-free control, with the color scale representing the drug interaction. 5FC, 5-flucytosine; AFG, anidulafungin; AMB, amphotericin B; MGX, manogepix; VRC, voriconazole.

for the combination of anidulafungin and manogepix (median FICI, 0.33) and weak synergy for combinations of 5-flucytosine with anidulafungin (median FICI, 0.74) or amphotericin B (median FICI 0.75). In contrast to the FICI calculation, which only focuses on the drug concentrations that correspond to the MIC values, the response surface analysis permits the examination of drug interactions over a wide range of tested concentrations. This revealed antagonism at the lower end of some concentration ranges that was missed by the FICI approach, highlighting the concentration-dependence of the interactions.

Real time imaging of anidulafungin combinations against a multidrug-resistant *C. auris* isolate using microfluidics. A microfluidics imaging approach was employed to further investigate the effects, at a single-cell level, of the two most promising drug combinations: anidulafungin with manogepix and anidulafungin with 5-flucytosine. This system is less static than the traditional broth microdilution method, as the cells are constantly perfused with fresh medium containing different antifungal drugs. Again, the multidrug-resistant *C. auris* isolate B12663 was chosen for analysis. Both drug combinations showed dramatic effects upon cell growth, markedly reducing the sizes of colonies, compared to the relevant monotherapies and media-only controls (Fig. 4A; Movies S1 and S2). The doubling times, measured by the two-dimensional colony area changes, increased significantly in the presence of the drug combinations,

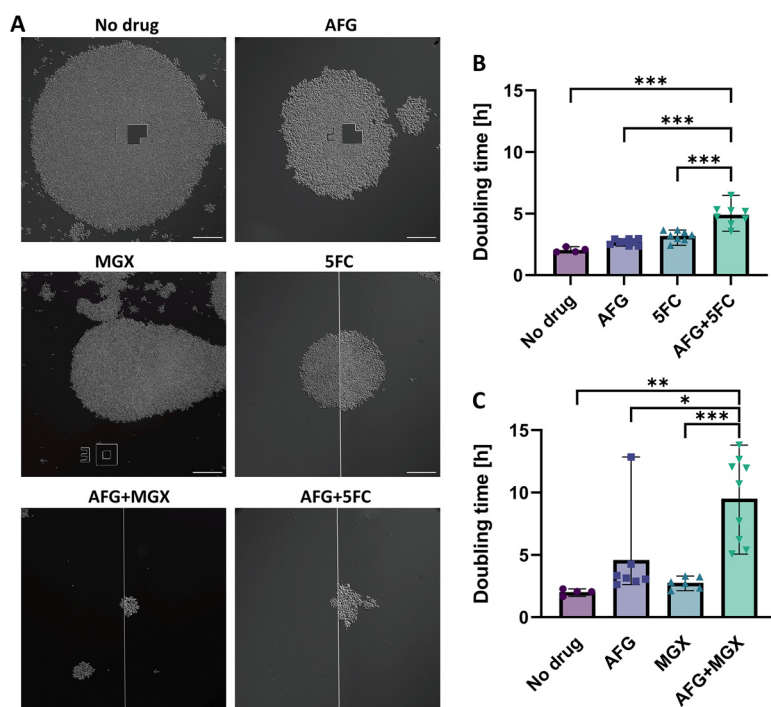


FIG 4 Microfluidics imaging of *C. auris* under antifungal combination exposure. DIC images from two representative experiments (A) and doubling times (B and C) of *C. auris* B12663 cells grown in the presence of RPMI 2% G-MOPS for 4 h. These were followed by further RPMI 2% G-MOPS or treatment with anidulafungin, 5-flucytosine, and manogepix, alone or in combination, at their MICs for 16 h. The doubling times were calculated via the two-dimensional colony area changes for several colonies from two independent experiments. Mean \pm range. Scale bars: 100 μ m. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P < 0.001$ (one-way analysis of variance (ANOVA) with Bonferroni's correction). 5FC, 5-flucytosine; AFG, anidulafungin; MGX, manogepix.

compared to the individual antifungals. An increase from 3.19 h (5-flucytosine alone) to 4.90 h ($P < 0.001$) was observed for anidulafungin combined with 5-flucytosine (Fig. 4B). Similarly, an increase from 2.75 h (manogepix alone) to 9.50 h ($P < 0.001$) was seen for the anidulafungin-manogepix combination (Fig. 4C). These changes in doubling time correspond to 63.5% (anidulafungin-5-flucytosine) and 96.5% (anidulafungin-manogepix) decreases in the colony area after 24 h, compared to 5-flucytosine and manogepix, respectively (data not shown). These findings were again consistent with those of the checkerboard and response surface analysis experiments in that the combination of anidulafungin and manogepix showed the most potent impacts on cell growth and in that this was followed by the combination of anidulafungin plus 5-flucytosine.

The cellular morphology was further examined at higher magnification after exposing the *C. auris* cells to the antifungals in monotherapy or combination for 24 h (Fig. S8). In drug-free medium, the cells had a well-defined, oval morphology. Under exposure to anidulafungin, manogepix, and both anidulafungin combinations, the cells displayed a rounder morphology with the formation of aggregates, whereas the 5-flucytosine treatment resulted in a more elongated phenotype. Additionally, enlarged, round cells were observed in the presence of manogepix and both combinations.

DISCUSSION

The emergence and global spread of multidrug-resistant *C. auris* strains poses a serious health threat. The high prevalence of antifungal resistance reported for *C. auris* isolates (3, 6–9, 11, 18, 24) was also observed in the isolates that were used in this study, with the majority of the isolates being resistant to fluconazole, 40% being resistant to voriconazole, and 32% being resistant to anidulafungin. The ability of *C. auris* to develop

resistance to all of the available classes of antifungal drugs severely limits treatment options.

New antifungal drugs, such as fosmanogepix, are currently under development (reviewed in [30]). *C. auris* currently appears susceptible to the active version of this new class of drugs (manogepix), but there is a high risk of resistance developing after its introduction into the clinic, unless precautionary measures are taken. Combination therapies provide a proven strategy by which to prevent the emergence of resistance to a single drug, and they have already been employed in the treatment of viral and bacterial infections (33). Additionally, combination therapies have the potential to improve efficacy through additive or synergistic interactions, which allows for lower drug doses to be used, thereby reducing dose-related toxicity.

Thus far, nine studies have examined antifungal drug combinations against *C. auris*. The majority of these studies focused on combinations of azoles with echinocandins (20, 23, 24, 34), while a smaller number evaluated polyene-echinocandin interactions (21, 22) or combinations with 5-flucytosine (25–27). These studies reported mainly synergistic (including partial synergy) or indifferent interactions, with interstrain variability being observed for some combinations. None of these studies included manogepix. Both manogepix and 5-flucytosine have potent antifungal activity against *C. auris*, as is shown here and as was observed by others (35–40). Therefore, we examined interactions of the echinocandin anidulafungin, the azole voriconazole, and the polyene amphotericin B with either 5-flucytosine or manogepix via checkerboard assays, response surface analyses, and microfluidics imaging.

According to the FICI values and response-surface analyses, the most potent combination (with respect to the number of *C. auris* isolates that displayed synergy) was anidulafungin plus manogepix, and this was followed by the combination of anidulafungin with 5-flucytosine. The high efficacy of these combinations was also confirmed by microfluidics imaging, which revealed dramatic reductions in fungal growth, compared to the relevant monotherapies. The interactions between 5-flucytosine with either amphotericin B or manogepix were additive or indifferent for the majority of the isolates, whereas the combination of voriconazole with 5-flucytosine was indifferent or antagonistic.

Applying our FICI thresholds, Bidaud and coworkers also reported mainly partially synergistic or additive interactions for combinations of amphotericin B, voriconazole, or micafungin with 5-flucytosine (25). However, they did not observe the antagonism for the combination of voriconazole with 5-flucytosine that we observed here. Another study reported the 100% growth inhibition of amphotericin B or anidulafungin-resistant *C. auris* isolates for amphotericin B-5-flucytosine combinations (0.25/1 mg/L) or anidulafungin-5-flucytosine combinations (0.008/1 mg/L) (26). Based on our OD₅₃₀ measurements, more than 90% growth inhibition was also achieved for the majority of the susceptible and resistant isolates that we analyzed, and this growth inhibition could be reached at lower concentrations for some isolates. To the best of our knowledge, antifungal combinations with fosmanogepix/manogepix have not been studied previously against *Candida* species. One recent study compared amphotericin B monotherapy with the combination therapy of fosmanogepix and amphotericin B in invasive mouse infection models of *Aspergillus fumigatus*, *Rhizopus arrhizus* var. *delemar*, and *Fusarium solani* (41). In all three models, mortality and fungal burden were significantly reduced in the mice that were treated with the combination therapy, compared to those that were treated with amphotericin B or fosmanogepix alone (41).

For the majority of the combinations and isolates that we examined, the interactions were partially synergistic or additive. However, even these interactions could be of interest clinically, as the ultimate goal is to reduce the fungal burden with a view to supporting the immune system in clearing the infection. This reduction in fungal growth could be clearly observed in the microfluidics imaging for the combination of anidulafungin with 5-flucytosine, which only displayed a partially synergistic interaction for the imaged isolate in the checkerboard assays. Furthermore, partially synergistic or additive interactions can lead to reductions in the MICs, potentially allowing for a lowering of antifungal doses,

thereby reducing toxicity. Reductions in MICs for partially synergistic, additive, and indifferent combinations have also been observed by others (20, 24), and Caballero and colleagues reported that additive combinations of isavuconazole–echinocandin combinations against *C. auris* can result in fungistatic effects that were absent for single agents in time-kill assays (23). This finding is similar to our results showing negligible antifungal activity for anidulafungin, manogepix, and 5-flucytosine in monotherapy, whereas the combinations of these two antifungals with anidulafungin showed heightened efficacy, with the reductions in CFU/mL approaching the cidal threshold. The lack of fungicidal activity of the echinocandins against *C. auris* in time-kill assays has also been observed by others who reported either a fungistatic effect or the complete absence of antifungal activity (22, 23, 42, 43). In comparison to anidulafungin monotherapy, the anidulafungin combinations resulted in 2.1 and 3.6 log₁₀-fold reductions in CFU/mL for the 5-flucytosine and manogepix combinations, respectively, highlighting their advantage over monotherapy.

Cost and additional toxicities are potential barriers to the implementation of antifungal combinations, and, to date, the routine use of antifungal combinations has been largely confined to cryptococcal infections. However, affordable generic echinocandins and 5-flucytosine are now available, and short courses of 5-flucytosine are known to be safe, giving feasible current options with which to try to prevent the inevitable increase in *C. auris* resistance that is a consequence of the continued use of monotherapies. Furthermore, early studies of combination approaches with new agents, such as fosmanogepix, could expand the options for clinical evaluation and prolong their clinical efficacy.

In most cases, the synergistic interactions that we observed for anidulafungin combined with manogepix or 5-flucytosine were within clinically relevant concentrations. Serum anidulafungin concentrations of up to 7 mg/L are achievable in patients (44, 45), which is above the anidulafungin concentrations corresponding to synergistic interactions for most isolates. For 5-flucytosine, all of the concentrations that we tested fell well below the achievable serum concentrations (44). In the case of fosmanogepix, no clinical pharmacokinetics data are publicly available, to our knowledge. Several safety and pharmacokinetics clinical studies for fosmanogepix have been completed, but no results are available yet (NCT02956499, NCT02957929, NCT03333005). However, the manogepix concentrations at which synergy was observed were relatively low, ranging between 0.002 and 0.03 mg/L.

It should be noted that the current study employed a relatively small number of isolates and that there was an unequal representation of *C. auris* clades. Additionally, the clustering of the amphotericin B MICs around the breakpoint made it difficult to categorize the isolates according to their amphotericin B susceptibility. Hence, other susceptibility testing methods, such as the Etest, are recommended (17).

In summary, combinations of anidulafungin with manogepix or 5-flucytosine show the highest potential against the tested *C. auris* isolates. Further studies are needed to determine the mechanisms that underlie these drug interactions and to evaluate their efficacy and safety in a murine model as well as whether these combinations also protect against the development of resistance.

MATERIALS AND METHODS

Fungal isolates. 25 clinical *C. auris* isolates belonging to clades I, III, and IV that were isolated from 6 patients from a range of sites (blood, urine, respiratory tract, skin) were obtained from the CDC (Table 3). The clade designations were based on whole-genome sequencing (Gifford et al., in preparation). The isolates were maintained at -80°C in 25% glycerol broth and subcultured on Sabouraud dextrose agar (SDA) at 37°C for up to 48 h.

Antifungal susceptibility testing. Antifungal susceptibility testing was performed using the broth microdilution method, according to the EUCAST guidelines (46). Flat-bottom, tissue-treated 96-well plates were used. Anidulafungin (MedChem Express), amphotericin B (Merck), fluconazole (Thermo Scientific), 5-flucytosine (Thermo Scientific), fosmanogepix (MedChem Express), manogepix (MedChem Express), and voriconazole (Sigma-Aldrich) were dissolved in 100% dimethyl sulfoxide (DMSO). The range of antifungal concentrations tested were 0.016 to 8 mg/L for anidulafungin, 0.03 to 16 mg/L for amphotericin B and voriconazole, 0.25 to 128 mg/L for fluconazole, 0.008 to 4 mg/L for 5-flucytosine, 0.004 to 2 mg/L for fosmanogepix, and 0.002 to 1 mg/L for manogepix. Antifungal dilution series were

TABLE 3 *Candida auris* isolates

Isolate no.	Clade ^a	Origin	Isolation day ^b	Isolated from	Reference
B12406	South American	USA	Day 0	Patient A, urine	50
B15223	South American	USA	Day 294	Patient A, blood	
B19460	South Asian	USA	Day 0	Patient B, sputum	
B19547	South Asian	USA	Day 16	Patient B, unknown	
B19617	South Asian	USA	Day 46	Patient B, urine	
B19837	South Asian	USA	Day 79	Patient B, urine	
B19618	South Asian	USA	Day 62	Patient B, urine	
B17040	South Asian	USA	Day 0	Patient C, urine	
B17041	South Asian	USA	Day 15	Patient C, sputum	
B17073	South Asian	USA	Day 44	Patient C, urine	
B17201	South Asian	USA	Day 67	Patient C, urine	
B18560	South Asian	USA	Day 0	Patient D, blood	
B18845	South Asian	USA	Day 72	Patient D, blood	
B18841	South Asian	USA	Day 103	Patient D, blood	
B18843	South Asian	USA	Day 96	Patient D, blood	
B12692	South Asian	USA	Day 11	Patient E, rectal	51
B12694	South Asian	USA	Day 0	Patient E, groin swab	51
B12663	South Asian	USA	Day 11	Patient E, urine	51
B12664	South Asian	USA	Day 11	Patient E, respiratory	51
B12688	South Asian	USA	Day 11	Patient E, groin swab	51
B20931	South African	USA	Day 0	Patient F, blood	
B21040	South African	USA	Day 3	Patient F, trachea aspirate	
B21041	South African	USA	Day 3	Patient F, groin swab	
B21042	South African	USA	Day 3	Patient F, blood	
B21043	South African	USA	Day 3	Patient F, blood	

^aClade designation is based on whole-genome sequencing (Gifford et al., in preparation).

^bIn reference to the isolation date of the first isolate from the respective patient. Empty cells indicate isolates without known references.

prepared in RPMI supplemented with glucose to 2% and buffered at pH 7 using 3-(N-morpholino) propanesulfonic acid (MOPS) at a final concentration of 0.165 mol/L (RPMI 2% G-MOPS). Spectrophotometer readings at 530 nm were taken after incubation at 37°C for 24 h. The MIC endpoint for amphotericin B was defined as the lowest concentration leading to a 90% reduction in growth, compared to the drug-free control, whereas 50% reductions in growth, compared to the drug-free control, were used for all of the other antifungal agents. The tentative CDC breakpoints for *C. auris* were used to define resistance to anidulafungin (≥ 4 mg/L), amphotericin B (≥ 2 mg/L), fluconazole (≥ 32 mg/L), and voriconazole (≥ 2 mg/L) (<https://www.cdc.gov/fungal/candida-auris/c-auris-antifungal.html>). A known issue for the broth microdilution susceptibility testing of amphotericin B in RPMI medium is the clustering of the MICs around the breakpoint of 2 mg/L, which makes it difficult to distinguish resistant and susceptible isolates (32). There are no breakpoints available for 5-flucytosine and fosmanogepix. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as quality control strains, as recommended by the EUCAST guidelines (46). All experiments were performed in triplicate.

Antifungal combination testing. The interactions of the antifungal drugs were tested using checkerboard assays that were based on the EUCAST guidelines (46). The range of antifungal concentrations tested was dependent on the MIC of each isolate, with the highest concentration at $4 \times \text{MIC}$. Columns 3 to 12 of a 96-well microtiter plate were filled with 50 μL of drug A, and rows B to H were filled with 50 μL of drug B. Column 1 served as the drug-free growth and sterility control. The inoculum was prepared by suspending five distinct colonies from 40 to 48-hour-old cultures in distilled water, counting the cell number using a haemocytometer, and adjusting the inocula to 5×10^5 cells/mL. The plates were inoculated with 100 μL and incubated at 37°C for 24 h. OD readings were taken after 24 h by using a spectrophotometer at 530 nm. All experiments were performed in triplicate.

Two different approaches were applied in the analysis of the drug interactions. The fractional inhibitory concentration index (FICI) was calculated as follows:

$$\text{FICI} = \frac{C_A}{\text{MIC}_A} + \frac{C_B}{\text{MIC}_B}$$

C_A and C_B are the concentrations of the drugs A and B in combination, and MIC_A and MIC_B are the MICs of the drugs alone. The MIC values were rounded to the next highest 2-fold concentration if the endpoint was not reached within the tested concentration range. The interaction was considered synergistic for $\text{FICI} \leq 0.5$, partially synergistic between >0.5 and <1.0 , additive at 1.0, indifferent between >1.0 and <4 and antagonistic >4 (24). In the following, the term “any synergy” refers to FICI values of <1 , thereby including complete and partial synergy. In the presence of antagonism, the maximum median FICI values were reported. Otherwise, the minimum median FICI values were given. Additionally,

the drug interactions were visualized using a response surface analysis approach with the Combenefit software package (version 2.021) under the application of the Bliss independence model (47).

Microfluidics imaging. *C. auris* B12663 cells were grown and prepared as described above. Inocula were adjusted to 2×10^5 cells/mL. Antifungal monotherapy and combination treatments were prepared in RPMI 2% G-MOPS at the MIC. CellASIC ONIX Y04C microfluidic plates (Millipore Merck) were washed with RPMI 2% G-MOPS by applying 5 lb/in² perfusion for 5 min, using a CellASIC ONIX2 microfluidic system (version 1.0.4, Millipore Merck). Yeasts were loaded into the CellASIC culture chambers by applying 8 lb/in² for 5 s twice (Thomson et al., in preparation). The adhered cells were then perfused with RPMI 2% G-MOPS for 4 h at 1 lb/in². After 4 h, the cells were exposed to either the antifungal(s) or RPMI 2% G-MOPS for the drug-free control by applying 5 lb/in² for 5 min, and following this with perfusion at 1 lb/in² for 20 h at 37°C, during which the microfluidic plates were subjected to multipoint 4D imaging on an inverted AxioObserver Z1 microscope (Carl Zeiss). Differential interference contrast (DIC) images were captured using a 20×/0.8 NA PlanApoChromatic DIC objective and a 16-bit ORCA-Fusion sCMOS camera (Hamamatsu). The area of colonies over time was measured in Fiji 1.53t (48), using an adapted method for migration analysis from Venter and Niesler (49). Briefly, during the time series, the colony edges were found (Process → Find Edges). The image was blurred 15 times (Process → Smooth) and inverted (Edit → Invert) before thresholding (Image → Adjust → Threshold: Default) to quantify the total fungal area (Analyze → Analyze Particles). The increases in the 2-dimensional colony area were used to calculate the doubling times.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 4 MB.

SUPPLEMENTAL FILE 2, AVI file, 2.4 MB.

SUPPLEMENTAL FILE 3, AVI file, 2.4 MB.

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