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The Strength of Synergistic Interaction between Posaconazole and Caspofungin Depends on the Underlying Azole Resistance Mechanism of *Aspergillus fumigatus*

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The majority of azole resistance mechanisms in *Aspergillus fumigatus* correspond to mutations in the *cyp51A* gene. As azoles are less effective against infections caused by multiply azole-resistant *A. fumigatus* isolates, new therapeutic options are warranted for treating these infections. We therefore investigated the *in vitro* combination of posaconazole (POSA) and caspofungin (CAS) against 20 wild-type and resistant *A. fumigatus* isolates with 10 different resistance mechanisms. Fungal growth was assessed with the XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt] method. Pharmacodynamic interactions were assessed with the fractional inhibitory concentration (FIC) index (FICi) on the basis of 10% (FICi-0), 25% (FICi-1), or 53 0% (FICi-2) growth, and FICs were correlated with POSA and CAS concentrations. Synergy and antagonism were concluded when the FICi values were statistically significantly (*t* test, $P < 0.05$) lower than 1 and higher than 1.25, respectively. Significant synergy was found for all isolates with mean FICi-0 values ranging from 0.28 to 0.75 (median, 0.46). Stronger synergistic interactions were found with FICi-1 (median, 0.18; range, 0.07 to 0.47) and FICi-2 (0.31; 0.07 to 0.6). The FICi-2 values of isolates with tandem-repeat-containing mutations or codon M220 were lower than those seen with the other isolates ($P < 0.01$). FIC-2 values were inversely correlated with POSA MICs ($r_s = -0.52$, $P = 0.0006$) and linearly with the ratio of drug concentrations in combination over the MIC of POSA ($r_s = 0.76$, $P < 0.0001$) and CAS ($r_s = 0.52$, $P = 0.0004$). The synergistic effect of the combination of POSA and CAS (POSA/CAS) against *A. fumigatus* isolates depended on the underlying azole resistance mechanism. Moreover, the drug combination synergy was found to be increased against isolates with elevated POSA MICs compared to wild-type isolates.

The opportunistic fungal pathogen *Aspergillus fumigatus* has been associated with several life-threatening infections in immunocompromised patients. Although azoles are the mainstay of antifungal therapy, treatment of aspergillosis is a difficult task which is further complicated by the lack of therapeutic efficacy in infections due to multiply azole-resistant *A. fumigatus* (1–3).

Azoles are inhibitors of the 14-α sterol demethylase in *A. fumigatus*, which is a product of *cyp51A* and *cyp51B*. Since the discovery of these orthologs to the *Candida albicans egr11* gene, a number of single nucleotide polymorphisms (SNPs) have been found, several of which have been associated with elevated azole MICs *in vitro*, corresponding to treatment failure *in vivo* (1, 4–7). It is believed that SNPs may develop through azole treatment or through exposure to azole fungicides in the environment (8–10). Treatment-induced SNPs in *cyp51A* are mainly allocated at codon 38, 54, or 220, while fungicide-induced SNPs are mostly located at codon 98, usually combined with tandem repeats within the promoter (8–10). Very recently, a new environmental azole resistance mechanism consisting of TR_{46}/Y121F/T289A was reported to be associated with voriconazole treatment failure in patients with invasive aspergillosis (11, 12).

Regardless of the factor that triggers the development of SNPs in *cyp51A*, no tradeoff between resistance and loss of virulence has been observed (13). Indeed, animal experiments performed with isolates harboring *cyp51A*-mediated resistance mechanisms and the numerous reported cases of acute pulmonary aspergillosis, central nervous system aspergillosis, and disseminated disease indicate that resistant mutants retain their virulence properties (1–3, 14, 15).

The increased emergence of resistance has reduced the already limited repertoire of available antifungal agents against aspergillosis, thereby necessitating the need for developing new treatment alternatives. In recent years, combination therapy has been gaining interest and popularity, as this can enhance therapeutic efficacy and broaden the antifungal spectrum (16).

Although posaconazole is mainly proposed as a prophylactic against fungal infections, in a recent monocentric and retrospective study, the combination of caspofungin and POSA was suggested as a therapeutic regimen for effective and tolerable treatment of invasive aspergillosis in immunocompromised patients with disease refractory to primary treatment (17). Moreover, *in vitro* and *in vivo* studies showed synergy between POSA and CAS against *A. fumigatus* wild-type strains (18, 19). Lepak and colleagues, however, showed that therapy using a combination of

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TABLE 1 MICs of posaconazole and MECs of caspofungin based on the CLSI-M38A2 methodology[a]

Isolate ID	Disease	Mutation(s)[c]	Visual reading	
			POSA MIC (mg/liter)	CAS MEC (mg/liter)
v52-35	Proven invasive aspergillosis	TR_{34}/L98H	0.5	1
v45-07	Proven invasive aspergillosis	TR_{34}/L98H	1	1
v61-76	Proven invasive aspergillosis	TR_{34}/L98H	0.5	1
v094-10	Proven invasive aspergillosis	46/TR	0.25	0.5
v107-65	Proven CNS aspergillosis	46/TR	1	1
v049-29	Proven Aspergillus osteomyelitis	53/TR	0.5	1
v059-73	Clinical isolate (unknown entity)	G54W	>8	1
v079-25	Clinical isolate (unknown entity)	G54W	>8	1
3038	Proven invasive aspergillosis	M220R	>8	0.25
v28-77	Aspergilloma	M220I	1	1
v59-27	Allergic pulmonary aspergillosis	M220K	16	1
v13-09	Probable invasive aspergillosis	M220V	1	1
v59-72	Aspergilloma	G138C	>8	1
AZN 8196	Proven invasive aspergillosis	—	0.06	0.5
v52-76	Proven invasive aspergillosis	—	0.06	1
v28-29	Proven invasive aspergillosis	—	0.06	1
v67-38 (S1)[b]	Chronic pulmonary aspergillosis	—	0.06	1
v67-37 (S2)[b]	Chronic pulmonary aspergillosis	—	0.125	1
v67-36 (R1)[b]	Chronic pulmonary aspergillosis	HapE/P88L	0.5	1
v67-35 (R2)[b]	Chronic pulmonary aspergillosis	HapE/P88L	0.5	1

[a] ID, identifier; MEC, minimal effective concentration.
[b] These four isolates were cultured consecutively from a single patient (21). Two isolates exhibited a wild-type (WT) susceptibility profile (S1 and S2), while two were azole resistant (R1 and R2). Microsatellite genotyping of all four isolates showed identical genotypes. No *cyp51A* mutations were found, but a P88L substitution in the CCAAT-binding transcription factor complex subunit HapE seemed to be responsible for the azole-resistant profile (22).
[c] —, no mutations in *cyp51A* and/or *hapE*.

POSA and CAS (POSA/CAS) *in vivo* did not enhance efficacy for POSA-susceptible isolates but produced synergistic activity against two POSA-resistant isolates (20).

Whether the interaction of POSA/CAS activity against azole-resistant isolates is specific and hence is dependent on the underlying mechanisms of resistance or on the MIC factor is unknown. Therefore, the goal of the present study was to investigate the *in vitro* interactions of this drug using clinical *A. fumigatus* isolates with a wide range of azole MICs and, most importantly, with different resistance mechanisms.

MATERIALS AND METHODS

Clinical isolates. A total of 20 clinical *A. fumigatus* isolates were selected based on the azole resistance mechanisms (Table 1). Three isolates were defined as wild type (isolates AZN 8196, v52-76, and v28-29) on the basis of the *in vitro* susceptibility profile and the lack of mutations in *cyp51A*. Thirteen isolates were defined as non-wild type on the basis of the *in vitro* susceptibility profile and the presence of mutations in *cyp51A* that have been shown to be associated with azole resistance. Three isolates (v52-35, v45-07, and v61-76) harbored the TR_{34}/L98H resistance mechanism, two isolates (v94-10 and v107-65) harbored TR_{46}/Y121F/T289A, and one isolate (v49-29) harbored TR_{53}. Four isolates harbored substitutions at codon M220 (M220I, isolate v28-77; M220V, v13-09; M220K, v59-07; and M220R, 3038), two isolates a substitution at codon G54 (G54W, v59-73 and v79-25), and one isolate a substitution at codon G138 (G138C, isolate v59-72).

In addition, four isogenic *A. fumigatus* isolates (isolates S1, S2, R1, and R2) were used that were cultured serially from a single patient with chronic granulomatous disease (21). The patient failed azole-echinocandin therapy for treatment of chronic pulmonary *Aspergillus* infection. At the outset of treatment, the first two recovered isolates (S1 and S2) showed a wild-type profile; however, after 2 years of therapy, the next two isolates (R1 and R2) gained properties of resistance to all azoles. Despite elevated expression of *cyp51A* in isolates R1 and R2 compared to the S1 and S2 isolates, no SNPs were found in *cyp51A*, indicating regulation of *cyp51A* by the HapE resistance mechanism (21, 22). In fact, the novel resistance mechanism was caused by a P88L substitution in CCAAT-binding transcription factor complex subunit HapE (22).

As previously described, all isolates were stored at −80°C and subcultured (23, 24). All *A. fumigatus* isolates were identified based on the morphological characteristics and sequencing of the β-tubulin and calmodulin genes, as described previously (7). The *cyp51A* coding region and its promoter were sequenced as previously described (5, 25). *Candida krusei* ATCC 63058, *C. parapsilosis* ATCC 22019, and *A. fumigatus* MYA3561 were used as quality controls.

Susceptibility testing. Antifungal susceptibility testing was performed based on the M38-A2 method of the Clinical and Laboratory Standards Institute (CLSI) (26). The drug interaction assay was performed using an 8-by-12-square checkerboard design, as previously described (27). POSA and CAS concentrations ranged from 8 to 0.002 mg/liter and from 4 to 0.06 mg/liter, respectively. Fungal growth was assessed using spectrophotometry with the modified XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt] method after incubation for 48 h, as described previously (28, 29). The MIC of POSA and the minimal effective concentration (MEC) of CAS were determined as the lowest concentration showing complete inhibition (>90%) and partial inhibition (>50%; MIC-2) of growth (27). All experiments were performed in three independent replicates.

Pharmacodynamic interaction analysis. The synergistic, additive, or antagonistic effect of paired (or more) combinations of drugs was captured by the fractional inhibitory concentration (FIC) index (FICi). This numerical value is calculated by the summation of the FIC for each drug (drugs A and B), where the FIC is determined by dividing the MIC of the

TABLE 2 Results of FIC index analysis based on 50%, 25%, and 10% growth endpoints for each group of *A. fumigatus* azole-resistant and azole-susceptible isolates harboring different or no mutations in *cyp51A*

Growth endpoint (%)	Isolates	FIC index (range)				
		Drugs alone			Drugs in combinations	
		MIC_{POSA}	MIC_{CAS}	$FICi_{min}$[a]	C_{POSA}	C_{CAS}
50	Wild types	0.03 (0.02–0.03)	0.5 (0.5–1)	0.44 (0.19–1.06)*	0.01 (0.01–0.02)	0.13 (0.06–0.5)
	TR group	0.13 (0.01–0.5)	0.5 (0.25–1)	0.27 (0.08–0.76)*	0.01 (0.01–0.06)	0.06 (0.06–0.5)
	M220 group	4 (0.02–4)	1 (0.5–8)	0.13 (0.05–0.5)*	0.01 (0.01–1)	0.13 (0.06–1)
	G54/G138	4 (0.01–4)	0.5 (0.13–2)	0.5 (0.13–0.56)*	0.01 (0.01–0.25)	0.06 (0.06–0.5)
	HapE	0.03 (0.01–0.13)	1 (0.5–8)	0.25 (0.13–0.62)*	0.01 (0.01–0.03)	0.13 (0.06–1)
25	Wild types	0.05 (0.03–0.06)	8 (1–8)	0.22 (0.09–0.76)*	0.01 (0.01–0.02)	0.5 (0.13–2)
	TR group	0.5 (0–1)	8 (1–8)	0.13 (0.06–0.52)*	0.01 (0.01–0.13)	0.5 (0.06–1)
	M220 group	4 (0.25–4)	8 (1–8)	0.25 (0.07–0.5)*	0.02 (0.01–0.25)	1 (0.5–2)
	G54/G138	4 (0.06–4)	5 (0.5–8)	0.2 (0.06–1)*	0.01 (0.01–0.13)	0.5 (0.06–1)
	HapE	0.13 (0.03–0.5)	8 (2–8)	0.16 (0.06–0.62)*	0.01 (0.01–0.03)	0.5 (0.13–2)
10	Wild type	0.06 (0.03–0.06)	8 (8–8)	0.53 (0.38–0.64)*	0.02 (0.01–0.03)	1 (0.06–2)
	TR group	0.5 (0.25–1)	8 (8–8)	0.52 (0.25–0.75)*	0.13 (0.06–0.5)	1 (0.13–2)
	M220 group	1 (0.5–4)	8 (8–8)	0.63 (0.31–0.75)*	0.25 (0.25–0.5)	2 (1–2)
	G54/G138	0.25 (0.06–4)	8 (8–8)	0.25 (0.19–1.02)*	0.06 (0.03–1)	1 (0.06–2)
	HapE	0.13 (0.06–0.5)	8 (8–8)	0.5 (0.28–0.56)*	0.06 (0.02–0.13)	0.5 (0.25–2)

[a] *, $P < 0.05$.

combination of two drugs (drug A plus drug B) by the MIC of each drug alone as follows:

$$\text{a)}\ FIC_A = \frac{MIC_{(A+B)}}{MIC_{(A)}} \quad \text{b)}\ FIC_B = \frac{MIC_{(A+B)}}{MIC_{(B)}} \quad \text{c)}\ FICi = FIC_A + FIC_B$$

If the FICi $\sum FIC_{min}$ value is lower than 1, this indicates synergistic interaction between two drugs; if the FICi $\sum FIC_{max}$ value is higher than 1.25, then an antagonistic interaction exists, while the additivity range is within $\sum FIC_{min}$ values and $\sum FIC_{max}$ values of 1 to 1.25. In the current study, the fractional inhibitory concentration index was assessed as $C_{POSA}/MIC_{POSA} + C_{CAS}/MIC_{CAS}$, where MIC and C are the concentrations of POSA and CAS alone and in combination, respectively, corresponding to at least 10% (FICi-0), 25% (FICi-1), or 50% (FICi-2) of growth. The different endpoints of fungal growth were used in order to assess pharmacodynamic interactions at low (FICi-2), intermediate (FICi-1), and higher (FICi-0) drug concentrations. To capture both synergistic and antagonistic interactions, the FIC_{min} and FIC_{max} were calculated as the minimum and maximum FICi for each isolate and replicate. Synergy and antagonism were concluded when the $\log_2$ FICi values of the three independent replicates were statistically significantly ($P < 0.05$) lower than 1 and higher than 1.25, respectively, by Student's *t* test as proposed previously based on *in vitro-in vivo* correlation studies (30). In any other case, an additive effect was claimed.

Isolates were categorized into 5 groups based on their genetic and phenotypic characteristics as follows: (i) a wild-type group harboring neither *cyp51A* nor *hapE* mutations and demonstrating low MIC values; (ii) A TR group of isolates containing tandem repeats in the promoter (the TR group can be also referred to as the environmental resistance group because of its reported relationship with development of azole resistance due to pesticides [see introduction]); (iii) a M220 group which contained diverse substitutions at codon M220 and which were found in patients only after prolonged treatment; (iv) a G54W and G138C group (G/W) recovered from patients after prolonged azole treatment; and (v) a HapE group to be used as a control given the identical genetic backgrounds as well as the previously reported very low growth rate. Using these groups, we aimed to determine whether the FIC can be affected by a slow-growth phenotype.

Analysis. To associate *in vitro* interactions with azole resistance mechanisms, the FICis of the isolates from each group were compared using analysis of variance followed by Bonferroni's multiple-comparison test. To explore whether *in vitro* interactions were dependent on *in vitro* susceptibility to POSA and CAS, FICis were correlated with POSA and CAS MICs by Spearman correlation analysis. Similarly, *in vitro* interactions were also correlated with drug concentrations in synergistic combinations corresponding to the FIC_{min} as absolute concentrations and as multiples of MICs after calculation of the drug concentration in combination/MIC ratio. All replicates were analyzed individually. Approximated FICs calculated from off-scale MICs were excluded from the analysis.

RESULTS

The results of FICi analysis for three growth levels are shown in Table 1, where the median and range of the MICs of the drugs alone, the FIC_{min}, and the FIC_{max} and the drug concentrations in combination are shown. The MIC (<10% growth endpoint) of POSA was 0.06 (range, 0.03 to 0.06) mg/liter for the wild-type isolates, whereas higher MICs were found for isolates harboring the tandem repeats (TR group), namely, the M220, G54W, and G138C mutations, for which the MICs were 0.5 (range, 0.25 to 1) mg/liter, 1 (0.5 to 4) mg/liter, and 0.25 (0.06 to 4) mg/liter, respectively, and the S1 and S2 isolates without mutations in *cyp51A*, for which the MIC was 0.13 (0.06 to 0.5) mg/liter. The median CAS MEC (<50% growth endpoint) ranged from 0.5 to 1 mg/liter for all isolates.

Pharmacodynamic interactions. The $FICi_{min}$s for all isolates and growth endpoints were significantly lower than 1, indicating synergy ($P < 0.05$) (Table 2). None of the $FICi_{max}$s were significantly higher than 1.25, indicating that no antagonism was observed (data not shown). The lowest $FICi_{min}$s were found at the 25% growth endpoint for all three groups of isolates, with $FICi_{min}$s ranging from 0.06 to 0.76. Statistically significant differences between the four groups of isolates were found at the 50% growth endpoint, where the lowest $FICi_{min}$s were found for isolates of the M220 group (0.13 [range, 0.05 to 0.5] mg/liter) followed by the TR group (0.27 [0.08 to 0.76] mg/liter) and the HapE isolates (0.25 [0.13 to 0.62] mg/liter) (Fig. 1).

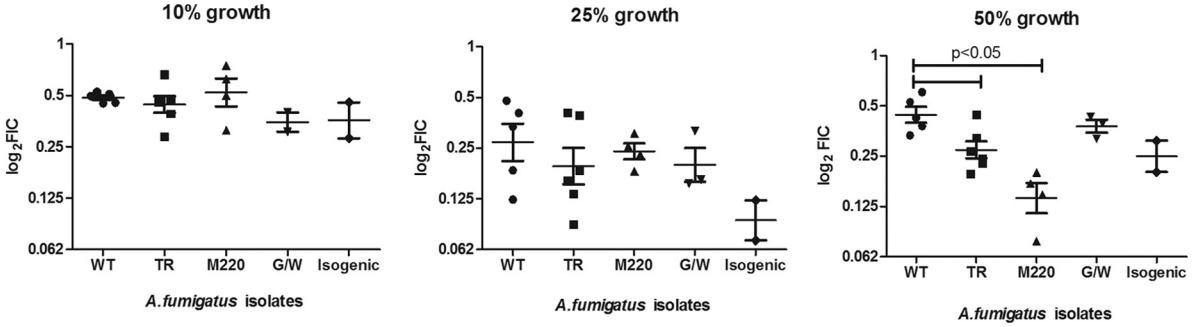

FIG 1 Graphical representation of $FICi_{min}$s determined at 10%, 25%, and 50% of growth. Five groups are presented: (i) the azole-susceptible group (wild type [WT]) with mutations neither in *cyp51A* nor in *hapE*, (ii) the azole-resistant TR group (TR_{34}/L98H, TR_{46}/Y121F/T289A, TR_{53}/L98), (iii) the azole-resistant M220 group (M220V, M220K, M220I, M220R), (iv) the azole-resistant G/W group (G138C and G54W), and (v) the isogenic group consisting of the two azole-resistant R1 and R2 strains with substitutions in *hpaE*. The parental S1 and S2 azole-susceptible strains from R1 and R2 were added to the WT group. Significant differences were found at the 50% growth endpoint, with the strongest synergy found for isolates harboring the M220 mutation.

Drug concentrations. The synergistic interactions at the 10% growth endpoint (MIC effect level) were found at median POSA concentrations of 0.02 mg/liter for wild-type isolates, 0.13 and 0.25 mg/liter for the TR group and M220 group, respectively, and 0.06 mg/liter for the G54W and G138C group and HapE isolates (Table 1), which corresponded to 0.25× to 0.5× MIC for all isolates irrespective of the MICs. At higher growth endpoints (sub-MIC effect levels), lower drug concentrations (<0.25× MIC) were required to show a synergistic effect. The median CAS concentrations of the synergistic interactions at the 50% growth endpoint (MEC effect level) were 0.06 to 0.13 mg/liter and corresponded to 0.125× to 0.25× MEC. At lower growth endpoints (supra-MEC effect level), the synergistic interactions were found at concentrations of 0.5 to 2 mg/liter, which corresponded to the MEC of CAS (Table 1). In Fig. 2, three checkerboard data are shown for three *A. fumigatus* isolates, 1 wild type and 2 with different azole resistance mechanisms (TR_{34}/L98H and M220), demonstrating similar FIC-0_{min}s but different FIC-2_{min}s. The drug concentrations where these synergistic interactions occur can be visualized for each drug and growth endpoint.

Correlation of FICs with MICs and drug concentrations in combinations. The FICi-2 values but not FICi-0 values and FICi-1 values were significantly correlated with POSA MICs ($r_s = -0.52$, $P = 0.0004$), as shown in Fig. 3. No significant correlation was found with the 50% growth CAS MIC (MIC-2) values ($r_s = -0.17$, $P = 0.25$). When drug concentrations in synergistic combination were analyzed as multiples of MIC but not as absolute concentrations, significant correlations between FICi-2s and POSA ($r_s = 0.76$, $P = <0.0001$) and CAS ($r_s = 0.52$, $P = 0.0004$) concentration/MIC-2 ratios were found (Fig. 4). In general, FICs lower than 0.25 corresponded to drug concentration/MIC ratios lower than 0.5.

DISCUSSION

The present study investigated the potential synergism of the combination of POSA and CAS against strains with different azole-resistant mechanisms and different POSA MICs. We used strains with single mutations in *cyp51A* or *hapE* which were isolated from patients with proven aspergillosis subjected to azole therapy (1, 21, 31, 32). In addition, we used a set of azole-resistant strains isolated from patients who were potentially infected through inhalation from the environment (33–35). These environmental az-

Wild-type (AZN 8196)

Caspofungin \ Posaconazole mg/L	2	1	0.5	0.25	0.12	0.06	0.031	0.016	0.008	0.004	0.002	0
4	1%	0%	0%	0%	0%	0%	0%	9%	18%	20%	19%	25%
2	1%	0%	0%	0%	0%	0%	0%	9%	18%	20%	19%	25%
1	2%	0%	0%	0%	0%	0%	0%	20%	29%	19%	21%	26%
0.5	1%	0%	0%	0%	0%	0%	0%	50%	40%	36%	29%	41%
0.25	1%	0%	0%	0%	0%	0%	1%	55%	64%	57%	61%	72%
0.125	1%	0%	0%	0%	0%	0%	4%	57%	57%	63%	69%	80%
0.0625	0%	0%	0%	0%	0%	0%	1%	51%	76%	65%	76%	81%
0	0%	0%	0%	0%	0%	0%	27%	64%	60%	75%	82%	100

Azole-resistant isolate harboring the TR_{34}/L98H (v5235)

Caspofungin \ Posaconazole mg/L	2	1	0.5	0.25	0.12	0.06	0.031	0.016	0.008	0.004	0.002	0
4	0%	1%	1%	1%	10%	13%	15%	14%	14%	15%	19%	30%
2	1%	0%	0%	3%	9%	8%	14%	11%	12%	12%	13%	30%
1	1%	0%	0%	9%	17%	12%	11%	18%	16%	17%	15%	36%
0.5	1%	0%	0%	8%	19%	32%	32%	38%	42%	59%	45%	77%
0.25	1%	0%	0%	11%	30%	45%	40%	46%	49%	60%	54%	88%
0.125	1%	0%	0%	13%	33%	42%	45%	59%	53%	63%	65%	85%
0.0625	0%	0%	0%	20%	34%	39%	49%	53%	57%	59%	64%	89%
0	1%	0%	0%	17%	37%	53%	60%	63%	63%	78%	83%	100

Azole-resistant isolate harboring the M220 mutation (3038)

Caspofungin \ Posaconazole mg/L	8	4	2	1	0.5	0.25	0.12	0.06	0.03	0.016	0.008	0
4	-3%	-2%	-2%	-2%	1%	22%	25%	34%	41%	45%	30%	35%
2	0%	-2%	-2%	-1%	12%	21%	30%	34%	28%	27%	31%	41%
1	32%	14%	10%	16%	17%	19%	27%	27%	27%	27%	27%	30%
0.5	50%	37%	30%	38%	45%	40%	53%	58%	58%	58%	58%	58%
0.25	49%	41%	26%	45%	45%	46%	66%	59%	53%	53%	53%	53%
0.125	48%	37%	41%	40%	44%	49%	52%	60%	57%	57%	57%	57%
0.0625	38%	39%	39%	35%	42%	50%	47%	59%	52%	52%	52%	52%
0	55%	57%	55%	52%	51%	60%	70%	59%	61%	64%	63%	100%

FIG 2 Checkerboard of the POSA/CAS combination with an azole-susceptible *A. fumigatus* wild-type isolate (top checkerboard) and two azole-resistant *A. fumigatus* isolates harboring TR_{34}/L98H (middle checkerboard) and M220 (bottom checkerboard) mutations in *cyp51A*. Note that the FICi-0_{min} values (0.504, 0.376, and 0.5) were not significantly different, whereas significant differences were found for FICi-2_{min} (1.064, 0.311, and 0.125) for the three isolates, respectively. Numbers inside the checkerboard cells represent percentages of fungal growth assessed with the modified XTT methodology, whereas the intensities of background color represent the three growth endpoints (<10%, white; <25%, light gray; <50%, dark gray).

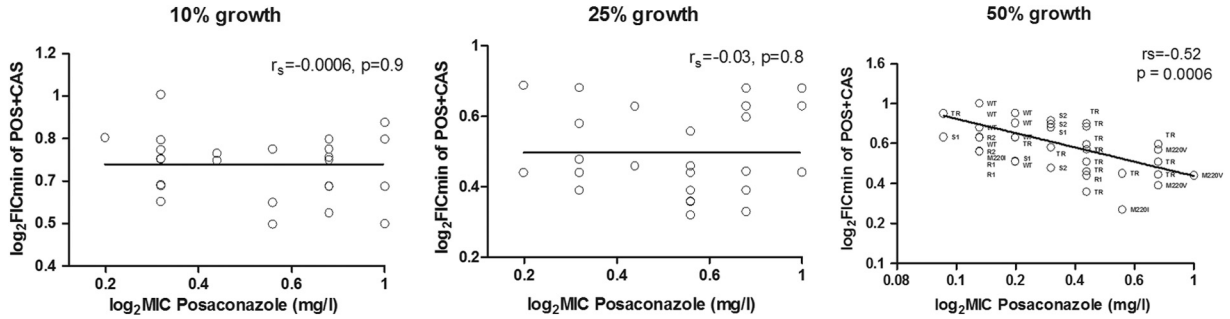

FIG 3 Correlation between FIC indices and posaconazole MICs. r_s = Spearman correlation coefficient.

ole-resistant strains carried different numbers of tandem repeats in the promoter of *cyp51A* (10, 33).

We found that the combination of POSA/CAS was synergistic against all *A. fumigatus* isolates. Note that synergism between POSA and CAS was identified not only in *A. fumigatus* but also in *Zygomycetes* and *Candida* spp. (36–38). The previously reported FICi of 0.32 against azole-susceptible *A. fumigatus* was similar to the FICi found in the present study.

In the current study, however, POSA/CAS synergy in isolates with *cyp51A*-mediated azole resistance revealed unexpected differences in drug interactions between the different resistance mechanisms and MICs. Interestingly, the strongest synergy was found for the group of isolates harboring the tandem repeats at the promoter region of *cyp51A* and for the group with substitutions at the M220 hot spot region. Our results are supported by a murine model of pulmonary aspergillosis in which POSA monotherapy and CAS monotherapy demonstrated suboptimal outcomes (40% to 50% survival); however, the drug combination led to enhanced efficacy (70% to 80% survival), mostly for the groups infected with POSA-resistant isolates with drug MICs of 2 and 8 mg/liter, respectively (20). These two resistant isolates were also reported to contain G138C and TR_{34}/L98H mutations in *cyp51A*.

To the best of our knowledge, drug synergy that is dependent on the resistance mechanism has not yet been reported for fungi. Although a marked synergistic effect between POSA and CAS was demonstrated in previous investigations, the mechanism of this remains unclear (18, 37). Our data show that the POSA MIC is a major determinant of the strength of the synergistic interaction and that this synergistic effect is concentration dependent, with strong interactions observed at concentrations of <0.5× MIC. In order to explain the synergistic interaction between POSA and CAS, Guembe et al. suggested that the inhibition of ergosterol biosynthesis by POSA may change the membrane, making the FKS1 enzyme more accessible or sensitive to inhibition by CAS (37). They also suggested that cell wall alterations by CAS may facilitate the penetration of POSA into the cell (37). Although these hypotheses may explain the synergistic azole-echinocandin interaction, they do not explain the differential synergistic interactions of the POSA/CAS combination that depend on the azole resistance mechanism in *A. fumigatus*. This phenomenon could be explained by specific qualitative or quantitative modifications of membrane sterols directly altering the membrane and indirectly altering the cell wall function. Recently, Alcazar-Fuoli et al. investigated the sterol composition of azole-susceptible and azole-resistant *A. fumigatus* strains (39). Resistance in these mutants developed due to deficiencies in different enzymatic steps of the ergosterol biosynthesis pathway (Cyp51A, Cyp51B, Erg3A, Erg3B, and Erg3C). The analysis showed that, although membrane sterols of azole-resistant *A. fumigatus* strains were qualitatively and quantitatively similar to those of the susceptible strains, the relative compositions differed, depending on the deficient enzyme (39). This indicates that alterations in the fungal membrane sterol composition may lead to differential penetration characteristics of azoles, thereby affecting POSA/CAS interaction. Yet further studies of membrane and cell wall changes performed using isolates carrying the substitutions presented in this paper will shed further light on the mechanism of azole resistance and POSA/CAS drug synergy. Earlier reports proposed that azole resistance developed

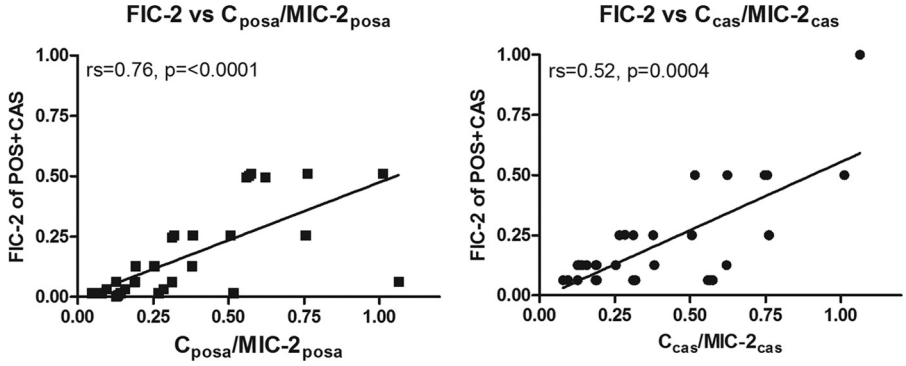

FIG 4 Correlation between FICi-2s of POS/CAS combination and POSA (left graph) and CAS (right graph) concentrations over MIC-2 ratio. r_s = Spearman correlation coefficient. posa, posaconazole; cas, caspofungin.

by *cyp51A* substitutions was associated with a lack of or a reduced drug binding affinity to the 14a-demethylases due to altered amino acids close to the heme factor (40). However, the authors reported that the enzyme activity of azole-resistant isolates was not affected despite the low affinity to azoles. Thus, ergosterol is still produced in *cyp51A* mutants, most likely by the same or other enzymes activated in order to overcome stress situations and to preserve survival. These changes of fungal membrane sterol composition caused by azoles may also affect the 1,3-β-D-glucan synthetase function and thereby the CAS activity.

Furthermore, in a murine model of disseminated aspergillosis, although increased area under the concentration-time curve (AUC) plasma concentration values were associated with increased survival in the groups infected with the TR_{34}/L98H and M220I isolates (MIC = 0.5 mg/liter), the G54W-infected groups (MIC > 16 mg/liter) showed no improved response (23). Apparently, this occurs because the heme factor in G54W mutants is completely blocked, thereby preventing access for POSA (41). Interestingly, the current *in vitro* study showed that even this obstinate POSA-resistant isolate was significantly inhibited with the combination of POSA/CAS, reaching an FIC similar to that observed in wild-type isolates.

Additionally, we recently found that acquisition of azole resistance mechanisms by *A. fumigatus* is sometimes associated with a fitness penalty in terms of slow growth (13). To determine the relationship between virulence and fungal growth, 15 of the 20 strains involved in the current study were used (13). That investigation revealed a strong relationship between *in vivo* virulence and *in vitro* fungal-growth-curve parameters, leading to the development of a novel mathematical model which is able to predict virulence based on *in vitro* growth characteristics. One of three TR_{34}/L98H strains and isolates harboring M220K were slower growers than other isolates with either identical or nonidentical mutations and were consequently less virulent. However, the slowest growers *in vitro* which also demonstrated the lowest virulence *in vivo* were the two HapE isolates. Intriguingly, although the HapE strains were the slowest growers of the 20 isolates, we did not find a POSA/CAS synergy similar to that of the M220I or TR groups. In fact, the POSA/CAS effect on the HapE isolates revealed no significant synergistic difference from the wild-type group, indicating that the growth rate did not play a role in this unique drug interaction phenomenon.

Understanding of the acquired azole resistance mechanisms may be important to increase treatment efficacy. Overall, the current study demonstrated that POSA at concentrations ranging from 0.002 mg/liter to 0.1 mg/liter combined with CAS at concentrations from 0.06 to 0.13 mg/liter resulted in 50% reduction of growth. What is of great interest is that these levels of both drugs are clinically achievable and that the combination may therefore be clinically useful for treatment of azole-resistant aspergillus diseases (42, 43).

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