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Differences in Interactions between Azole Drugs Related to Modifications in the 14-α Sterol Demethylase Gene (*cyp51A*) of *Aspergillus fumigatus*

G. Garcia-Effron, E. Mellado, A. Gomez-Lopez, L. Alcazar-Fuoli, M. Cuenca-Estrella, and Juan L. Rodriguez-Tudela*

Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain

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The combined activity of different azole drugs was investigated. Thirty-one *Aspergillus fumigatus* strains were tested, including two *cyp51A*$^-$ and one *cyp51B*$^-$ gene-knockout strain and azole-susceptible and -resistant strains with different resistance mechanisms. The combination of itraconazole and voriconazole was synergistic for all strains except for those with gene knockouts.

Invasive aspergillosis (IA) treatment is complicated due to several factors such as problems associated with the diagnosis of IA, the seriousness of the underlying diseases, and the limited number of therapeutic options (3, 15). In addition, there has been an increase in reported cases of antifungal drug resistance (2, 4, 5, 10). Azole-resistant clinical strains have shown two distinct patterns of in vitro resistance associated with amino acid substitutions in the Cyp51A protein (G54 and M220) (7, 17, 20, 22). Spontaneous azole-resistant laboratory mutants with some Cyp51A substitutions have been isolated as well (16, 17, 22). These data suggest that azole resistance among clinical strains may become more common in the future associated with the spread of prophylaxis, preemptive treatments, specific therapies with azole agents, and the use of demethylase inhibitors as pesticides in agriculture (8, 11, 12, 27).

It is necessary to increase the number of therapeutic options for treating IA. In this sense, some reports have documented in vivo and in vitro encouraging results with drug combinations. The description of two different 14-α sterol demethylase-like genes (*cyp51A* and *cyp51B*) in *Aspergillus* spp. (19) opens the possibility that each enzyme could interact differently with azole drugs. The aim of this study was to explore the in vitro activity of azole drug combinations against a collection of *Aspergillus fumigatus* strains.

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Strains. Thirty-one *Aspergillus fumigatus* strains from the Mycelial Collection of the Spanish National Center for Microbiology were used throughout this work. These strains were divided in four groups according to their azole antifungal drug MIC patterns and the Cyp51A amino acid substitutions: group 1, itraconazole (ITC)-susceptible group (S group), including eight azole-susceptible strains, without substitutions on Cyp51A; group 2, the knockout (KO) group, including two strains with *cyp51A*-targeted gene disruption and one strain with *cyp51B*-targeted gene disruption (data not published); group 3, the G54 group, 12 azole-resistant strains (itraconazole MICs of ≥8 μg/ml) with a G54 substitution in Cyp51A (7); and group 4, the M220 group, eight azole-resistant strains that show an M220 substitution in Cyp51A (20). *A. fumigatus* ATCC 204305 and *Aspergillus flavus* ATCC 204304 were included as control isolates in each set of MIC determinations. See Table 1 for details.

Data analysis. The significance of the differences in MICs and summation of the fractional inhibitory concentration (ΣFIC) were determined by Student's *t* test (unpaired, unequal variance). A *P* value of <0.01 was considered significant. Statistical analysis was done with the Statistical Package for the Social Sciences (version 12.0; SPSS S.L., Madrid, Spain).

Antifungal susceptibility testing. Antifungal agents utilized were ITC (Janssen Pharmaceutical S.A., Madrid, Spain), voriconazole (VRC) (Pfizer S.A., Madrid, Spain), and fluconazole (FLC) (Pfizer). The individual MICs were determined following the microdilution method recommended by NCCLS, document M-38A (23), with modifications (5, 25, 26). The concentrations assayed ranged from 0.015 to 8 μg/ml for ITC and VRC and from 1.25 to 640 μg/ml for FLC. Experiments were repeated at least three times on three separate days.

Drug interaction evaluation. In vitro drug interaction was evaluated as described earlier (1, 14, 18) with a two-dimensional, two-agent broth microdilution checkerboard technique, using 96-well flat-bottomed microtitration plates. The combinations assayed were ITC-FLC, VRC-FLC, and ITC-VRC. Different concentration intervals were used if the strains were ITC susceptible or ITC resistant. To determine the nature of the in vitro interaction between drugs, the data obtained were analyzed using methods described before (1, 14, 18). The ΣFIC interpretations were as follows: (i) a value of ≤0.5 was considered synergy, (ii) indifference was defined as a value between >0.5 and ≤4, and (iii) values of >4 were considered as antagonistic (14, 24).

All strains' individual MICs and ΣFIC are shown in Table 1. The in vitro drug interaction for the S group showed a ΣFIC

* Corresponding author. Mailing address: Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Carretera Majadahonda-Pozuelo Km 2, 28220 Madrid, Spain. Phone: 34-91-8223919. Fax: 34-91-5097966. E-mail: juanl.rodriguez-tudela@isciii.es.

TABLE 1. Antifungal susceptibility results of individual azole agents for *A. fumigatus* strains used in this work

Strain[c]	CYP51A amino acid			MIC (μg/ml)[a]			ΣFIC (interpretation)[b]		
	Group	54	220	ITC	VRC	FLC	ITC-FLC	ITC-VRC	VRC-FLC
CM-237	S	G	M	0.25	0.50	640	0.917 (I)	0.430 (S)	0.750 (I)
ATCC 9197	S	G	M	0.50	0.50	640	0.708 (I)	0.522 (I)	0.750 (I)
CM-2121	S	G	M	0.25	0.50	640	0.750 (I)	0.503 (I)	0.880 (I)
CM-2127	S	G	M	0.50	0.50	1,280	0.917 (I)	0.511 (I)	1.000 (I)
CM-2198	S	G	M	0.25	0.50	640	0.875 (I)	0.461 (S)	0.750 (I)
CM-2120	S	G	M	1.00	0.50	640	0.917 (I)	0.386 (S)	0.880 (I)
ATCC 204305	S	G	M	0.25	0.25	640	0.750 (I)	0.490 (S)	0.630 (I)
CM-2194	S	G	M	0.50	1.00	1,280	0.710 (I)	0.610 (I)	0.610 (I)
CM-2705[d]	A-KO			0.06	0.50	80	0.940 (I)	0.730 (I)	0.660 (I)
CM-2707[e]	A-KO			0.06	0.25	160	0.880 (I)	0.750 (I)	0.870 (I)
CM-2706[f]	B-KO	G	M	0.25	0.50	640	1.000 (I)	1.000 (I)	0.500 (I)
CM-2162	G54	V	M	16	0.25	160	0.240 (S)	0.190 (S)	0.830 (I)
CM-1244	G54	E	M	16	0.25	160	0.177 (S)	0.258 (S)	1.190 (I)
CM-2160	G54	E	M	16	0.50	320	0.273 (S)	0.227 (S)	1.340 (I)
CM-2161	G54	E	M	16	0.50	160	0.266 (S)	0.227 (S)	0.920 (I)
CM-2697[g]	G54	E	M	16	0.50	160	0.177 (S)	0.190 (S)	0.520 (I)
CM-2698[g]	G54	E	M	16	0.25	80	0.216 (S)	0.263 (S)	0.520 (I)
CM-2699[h]	G54	E	M	16	0.50	320	0.193 (S)	0.170 (S)	0.520 (I)
CM-2097	G54	R	M	16	0.50	160	0.156 (S)	0.177 (S)	1.250 (I)
CM-2700[h]	G54	R	M	16	0.25	160	0.096 (S)	0.193 (S)	0.590 (I)
CM-2701[h]	G54	R	M	16	0.25	160	0.085 (S)	0.115 (S)	0.590 (I)
CM-2702[h]	G54	R	M	16	0.50	160	0.188 (S)	0.146 (S)	0.560 (I)
CM-2266	G54	W	M	16	0.50	1,280	0.526 (I)	0.172 (S)	1.170 (I)
CM-1245	M220	G	V	16	1.00	1,280	2.000 (I)	0.295 (S)	2.000 (I)
CM-1252	M220	G	V	16	1.00	1,280	2.000 (I)	0.219 (S)	2.000 (I)
CM-2158	M220	G	V	16	2.00	1,280	2.000 (I)	0.219 (S)	2.000 (I)
CM-2159	M220	G	K	16	2.00	1,280	2.000 (I)	0.341 (S)	2.150 (I)
CM-2164	M220	G	T	16	1.00	1,280	2.000 (I)	0.263 (S)	2.000 (I)
CM-2709[g]	M220	G	K	16	2.00	1,280	2.000 (I)	0.439 (S)	1.750 (I)
CM-2713[g]	M220	G	V	16	1.00	1,280	2.000 (I)	0.219 (S)	2.000 (I)
CM-2735[g]	M220	G	T	16	1.00	1,280	2.000 (I)	0.169 (S)	1.750 (I)

[a] Geometric mean (three repetitions on three separate days).
[b] Arithmetic mean (three repetitions on three separate days). S, synergistic; I, indifferent.
[c] Strains are clinical isolates except as noted otherwise, with the exception of the ATCC strains.
[d] CM-2705 is a *cyp51A* gene-knockout strain with CM-237 as the parental strain.
[e] CM-2707 is a *cyp51A* gene-disrupted strain with CM-1252 as the parental strain.
[f] CM-2706 is a *cyp51B* gene-knockout strain with CM-237 as the parental strain.
[g] Gene replacement of the CM-237 wild-type *cyp51A* gene with the mutated strain CM-1244 (CM-2697 and CM-2698), CM-2159 (CM-2709), CM-1252 (CM-2713), and CM-2164 (CM-2735) *cyp51A* gene copy.
[h] Spontaneous mutants isolated using CM-237 as parental strain and ITC (8 μg/ml) as a selection drug.

interpreted as indifferent for all the combinations tested except for four strains, where ITC-VRC showed a synergistic pattern (arithmetic mean [AM], 0.440; range, 0.386 to 0.461). All combinations exhibited indifferent patterns against knockout strains (ITC-FLC: AM, 0.940; range, 0.88 to 1.00; ITC-VRC: AM, 0.827; range, 0.73 to 1.00; VRC-FLC: AM, 0.677; range, 0.5 to 0.87). Notably, in comparison with the rest of the isolates analyzed (Table 1) FLC MICs were significantly lower ($P < 0.01$) in both *cyp51A*-knockout strains.

On the other hand, the combination ITC-VRC exhibited a synergistic pattern against all strains included in the G54 group (AM, 0.194; range, 0.115 to 0.263). For ITC-FLC combination, synergy was observed for all strains except one (CM-2266) (Table 1) (AM, 0.194; range, 0.085 to 0.526). This group presented a uniform behavior for the combination VRC-FLC with an indifferent pattern (AM, 0.777; range, 0.510 to 1.250). Finally, the combinations ITC-FLC and VRC-FLC showed indifferent values of ΣFIC for all strains in the M220 group (Table 2).

Overall, the combination ITC-VRC showed synergy (AM, 0.271; range, 0.169 to 0.439, excluding KO group) (Table 2) for 24 out of 28 strains (85.71%). Notably, all strains with ΣFIC of >0.5 belonged to ITC-susceptible or to KO groups. In general, except for the G54 group, the combinations that had FLC as one of the components presented an indifferent pattern (for ITC-FLC combination: AM, 0.898; range, 0.085 to 2.000; for VRC-FLC combination: AM, 1.139; range, 0.520 to 2.150).

Some reports have documented successful treatments of *Scedosporium prolificans* infections with terbinafine plus azole compounds (13). Both agents inhibit ergosterol biosynthesis by blocking different steps in the ergosterol biosynthesis pathway: the azole drugs target lanosterol 14-α sterol demethylase, while terbinafine inhibits squalene epoxidase (9). Our results, obtained with the combination VRC-ITC, suggest that a similar effect could occur with Cyp51A and Cyp51B inhibition, indicating that these enzymes could act in the same pathway but on different substrata or that they might be aiming at different steps in the ergosterol biosynthesis pathway. This hypothesis was reinforced by the fact that all combinations show indiffer-

TABLE 2. Summary of azole drug individual MICs and interaction interpretation using the ΣFIC

Group	*n*	MIC (μg/ml)[a]			ΣFIC (interpretation)[b]		
		ITC	VRC	FLC	ITC-FLC	ITC-VRC	VRC-FLC
S	8	0.438	0.531	800.0	0.818 (I)	0.489 (S)	0.781 (I)
G54	12	16.0	0.375	201.6	0.194 (S)	0.189 (S)	0.777 (I)
M220	8	16.0	1.375	1,280.0	2.000 (I)	0.271 (S)	1.956 (I)
A-KO	2	0.060	0.350	113.4	0.910 (I)	0.740 (I)	0.758 (I)
B-KO	1	0.250	0.500	640.0	1.000 (I)	1.000 (I)	0.500 (I)

[a] Geometric mean (three repetitions on three separate days).
[b] Arithmetic mean (three repetitions on three separate days). S, synergistic; I, indifferent.

ent patterns for the KO group due to the lack of one of the enzymes and the consequent blockade of one of the possible pathways (Table 2).

Therapeutic options to treat IA are scarce, and *A. fumigatus* azole drug resistance is emerging (6, 21). In addition, therapeutic options are narrowing since azole antifungal drugs exhibit structural resemblances and cross-resistance is expected (17). An alternative to face this problem is the use of drug combinations as a potential therapeutic option, which could be exploited clinically. Despite the results obtained in this work, further in vitro and in vivo studies are needed before proposing an azole combination as a possible IA treatment. Functional analysis of both Cyp51s and in vivo models to test the results obtained here are needed to confirm this indirect evidence. These experiments are under way in our laboratory.

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