In Vitro Activities of Novel Azole Compounds ATTAF-1 and ATTAF-2 against Fluconazole-Susceptible and -Resistant Isolates of Candida Species

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ABSTRACT The *in vitro* activities of two novel azole compounds (aryl-1,2,4-triazol-3-ylthio analogues of fluconazole [ATTAFs]) and five comparator antifungal agents against 52 clinical *Candida* isolates from 5 different species were determined. The novel azole compounds had the lowest geometric mean MICs, followed by fluconazole. Moreover, combinations of these compounds with fluconazole exhibited synergistic effects against fluconazole-susceptible (22 of 23 isolates), fluconazole-susceptible dose-dependent (10 of 13 isolates), and fluconazole-resistant (1 of 16 isolates) *Candida* isolates.

KEYWORDS *In vitro* susceptibility, triazole derivatives, *Candida* species

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Candidiasis is a serious life-threatening infection that is associated with significant morbidity and mortality rates. The incidence of this infection has increased in recent years, especially among immunocompromised patients (1, 2). *Candida* species are the fourth most common agent of hospital-acquired candidemia (3–5). Guidelines for the management of candidiasis have recommended the use of azoles, polyenes, and echinocandins (6, 7). However, toxic effects of amphotericin B and resistance to azoles and echinocandins in *Candida* species have recently become serious clinical challenges (8–10). Fluconazole is the most commonly used agent for systemic candidiasis, given its low toxicity, high solubility, and wide tissue distribution (11). However, the use of fluconazole for prophylaxis and treatment is thought to be a potential risk factor, leading to the gradual development of azole-resistant species (12). Accordingly, there is an urgent need for the introduction of a novel class of antifungal agents with potent activities and new mechanisms of action, to improve the management of *Candida* infections (13).

Replacement of one triazole ring in the fluconazole structure with other heterocyclic moieties for the purpose of developing new antifungal agents has received particular attention in medicinal chemistry. We previously designed and synthesized numerous triazole alcohols by replacing the 1,2,4-triazol-1-yl group in the fluconazole structure with a 4-amino-5-aryl-3-mercapto-1,2,4-triazole motif (14, 15). Since this newly introduced motif represented a new type of side chain in triazole alcohol antifungals, we focused on structural refinement of the primary lead compound and removed the amino group from the structure to obtain new entities, namely, aryl-1,2,4-triazol-3-ylthio analogues of fluconazole (ATTAFs). In particular, the compounds ATTAF-1 and ATTAF-2,

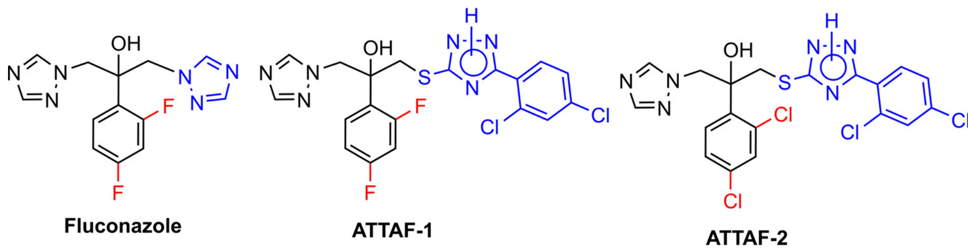

FIG 1 Chemical structures of fluconazole, ATTAF-1, and ATTAF-2.

containing a (2,4-dichlorophenyl)-1,2,4-triazol-3-ylthio moiety (Fig. 1), were found to be potential agents against *Candida* species, with no significant cytotoxicity against the HepG2 cell line (15). Although ATTAF-1 and ATTAF-2 are triazole alcohol-derived analogues, their increased antifungal activity, in comparison with fluconazole, might be attributable to the presence of the (2,4-dichlorophenyl)-1,2,4-triazol-3-ylthio scaffold as an additional pharmacophoric structure, with a mechanism of action distinct from that of fluconazole. Therefore, we aimed to determine the *in vitro* activity of ATTAF-1 and ATTAF-2, in comparison with five clinically important antifungal drugs, against fluconazole-susceptible and -resistant *Candida* isolates. Moreover, we investigated the combination of these compounds with fluconazole.

Compounds ATTAF-1 and ATTAF-2 were synthesized and characterized as in our previous study (15). Fluconazole (Pfizer, Groton, CT, USA), itraconazole (Janssen Research Foundation, Beerse, Belgium), voriconazole (Pfizer Central Research, Sandwich, United Kingdom), amphotericin B (Sigma, St. Louis, MO, USA), and anidulafungin (Pfizer) were obtained as reagent-grade powders from the respective manufacturers and were used for preparation of the CLSI microdilution trays.

Fifty-two *Candida* isolates from five different species, including fluconazole-susceptible isolates ($n = 23$), fluconazole-susceptible dose-dependent isolates ($n = 13$), and fluconazole-resistant isolates ($n = 16$) (according to the new CLSI species-specific clinical breakpoints for fluconazole against *Candida* species [16]), were obtained from the reference culture collection of the Invasive Fungi Research Center (Mazandaran University of Medical Sciences, Sari, Iran) (Table 1). Isolates had been identified previously through sequencing of the internal transcribed spacer (ITS) ribosomal DNA (rDNA) region. Antifungal susceptibility testing was performed according to CLSI guidelines (17, 18), and MICs were determined after 24 h of incubation at 35°C. The antifungal agents were prepared at final concentrations of 0.016 to 16 μg/ml for amphotericin B, itraconazole, and voriconazole, 0.063 to 64 μg/ml for fluconazole, ATTAF-1, and ATTAF-2, and 0.008 to 8 μg/ml for anidulafungin. The MIC endpoints were defined as 100% inhibition for amphotericin B and >50% inhibition for the other drugs. For calculations, high off-scale MICs were raised to the next $\log_2$ dilution step, while low off-scale MICs were left unchanged (19, 20). Differences in mean values were determined by using Kruskal-Wallis and Mann-Whitney tests, with the SPSS statistical package (version 7.0). *P* values of <0.05 were considered statistically significant. In addition, the interactions of ATTAF-1 and ATTAF-2 with fluconazole were investigated by using a microdilution checkerboard technique with 96-well microtiter plates (21). The concentration ranges used depended on the MIC results for each isolate, i.e., the maximum concentration was 2 times the MIC and then serial dilutions were performed. *In vitro* combinations of fluconazole with voriconazole were tested as controls against 11 *Candida* isolates from 5 different species (fluconazole-susceptible isolates [$n = 5$], fluconazole-susceptible dose-dependent isolates [$n = 3$], and fluconazole-resistant isolates [$n = 3$]) to compare the interactions of the newly synthesized azole compounds with fluconazole. To assess the interactions of combinations of drugs, further analysis was conducted using the fractional inhibitory concentration index (FICI). The interaction was defined as synergistic if the FICI was ≤0.5, indifferent if the FICI was >0.5 to ≤4.0, and antagonistic if the FICI was >4 (21).

TABLE 1 *In vitro* susceptibilities of five antifungal drugs and two novel azole compounds (ATTAF-1 and ATTAF-2) against 52 *Candida* isolates from five different species

Species and compound/agent	No. of isolates with MIC (μg/ml) of[a]:															MIC range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC mode (μg/ml)	MIC GM (μg/ml)
	≤0.008	0.016	0.031	0.063	0.125	0.25	0.5	1	2	4	8	16	32	64	>64					
C. albicans ($n = 21$)																				
ATTAF-1			1	**11**	2	1	1	1	1		2	1				0.031–16	0.063	8	0.063	0.21
ATTAF-2				**14**	1	1		1	1			1	2			0.063–32	0.063	16	0.063	0.22
Fluconazole							**8**	5	1	4					3	0.5–128	1	128	0.5	2
Itraconazole				2		7	**7**	2	1	1	1					0.063–8	0.5	2	0.5	0.46
Voriconazole				2	6	**8**	2	1	2							0.063–2	0.25	1	0.25	0.25
Anidulafungin	**11**	8	1	1												0.008–0.063	0.008	0.016	0.008	0.01
Amphotericin B						1	**11**	5	4							0.25–2	0.5	2	0.5	0.74
C. glabrata ($n = 10$)																				
ATTAF-1				1	3	**4**						1	1			0.063–32	0.25	32	0.25	0.5
ATTAF-2				**5**	1	1	1						1	1		0.063–64	0.125	64	0.063	0.35
Fluconazole									1	3	2				**4**	2–128	8	128	128	17.14
Itraconazole						1	**4**	2	1	2						0.25–4	1	4	0.5	0.93
Voriconazole					2	1	**5**		2							0.125–2	0.5	2	0.5	0.46
Anidulafungin	**6**	3	1													0.008–0.031	0.008	0.031	0.008	0.01
Amphotericin B			1	1	2	2	1	**2**	1							0.031–2	0.25	2	1	0.25
C. krusei ($n = 9$)																				
ATTAF-1				1	1	4			1		1	1				0.063–16	ND[b]	ND	ND	ND
ATTAF-2			1	1		1	3	1						2		0.031–64	ND	ND	ND	ND
Fluconazole								1	3	2	1				2	1–128	ND	ND	ND	ND
Itraconazole					1	2	3	1				2				0.125–16	ND	ND	ND	ND
Voriconazole					3	1	2	1		1	1					0.125–8	ND	ND	ND	ND
Anidulafungin	6	1		1	1											0.008–0.125	ND	ND	ND	ND
Amphotericin B				2	1	2	1	2	1							0.063–2	ND	ND	ND	ND
C. parapsilosis ($n = 8$)																				
ATTAF-1			3	2	2	1										0.031–0.25	ND	ND	ND	ND
ATTAF-2			3		4	1										0.031–0.25	ND	ND	ND	ND
Fluconazole							3	1	1	3						0.5–4	ND	ND	ND	ND
Itraconazole				4	1	2	1									0.063–0.5	ND	ND	ND	ND
Voriconazole			2	3	2		1									0.031–0.5	ND	ND	ND	ND
Anidulafungin			1	7												0.031–0.063	ND	ND	ND	ND
Amphotericin B	6	2														0.008–0.016	ND	ND	ND	ND
C. tropicalis ($n = 4$)																				
ATTAF-1				3	1											0.063–0.125	ND	ND	ND	ND
ATTAF-2				3	1											0.063–0.125	ND	ND	ND	ND
Fluconazole							2	1	1							0.5–2	ND	ND	ND	ND
Itraconazole				2		1	1									0.063–0.5	ND	ND	ND	ND
Voriconazole				2		1	1									0.063–0.5	ND	ND	ND	ND
Anidulafungin	3	1														0.008–0.016	ND	ND	ND	ND
Amphotericin B				2		1	1									0.063–0.5	ND	ND	ND	ND

[a]Numbers in bold are modal values.
[b]ND, not determined.

Table 1 summarizes the MIC range, mode, and geometric mean (GM), MIC_{50}, and MIC_{90} for ATTAF-1 and ATTAF-2 and five comparators against 52 clinical *Candida* isolates from 5 different species. In terms of GM MIC values, anidulafungin, followed by the newly synthesized azole compounds, exhibited potent activity against all *Candida* isolates ($n = 52$). Interestingly, the widest range (0.5 to 128 μg/ml) and highest MIC_{90} (128 μg/ml) value for fluconazole was observed against *Candida albicans*. The GM MIC values against *C. albicans* were 0.01, 0.21, 0.22, 0.25, 0.46, 0.74, and 2 μg/ml for anidulafungin, ATTAF-1, ATTAF-2, voriconazole, itraconazole, amphotericin B, and fluconazole, respectively. The GM MIC values of ATTAF-1 and ATTAF-2 were lower than that of fluconazole against *Candida glabrata*, and the MIC_{50} of ATTAF-1 (0.25 μg/ml) was 5 $\log_2$ dilution steps lower than that of fluconazole (8 μg/ml). The checkerboard analysis of the tested compounds is summarized in Table 2. The FICI results revealed synergistic effects against fluconazole-susceptible (22 of 23 isolates), fluconazole-susceptible dose-dependent (10 of 13 isolates), and fluconazole-resistant (1 of 16 isolates) *Candida* isolates when ATTAF-1 and ATTAF-2 were combined with fluconazole. Remarkably, ATTAF-1 and ATTAF-2 were more active than fluconazole against *C. albicans* isolates and showed synergistic activity against 16 isolates (76.1%) (Table 2).

TABLE 2 Interactions between fluconazole and the novel compounds (ATTAF-1 and ATTAF-2) against *Candida* isolates

Species and isolate	ATTAF-1 study[a] MIC (μg/ml) FLC	ATTAF-1	FLC/ATTAF-1	FICI/INT	ATTAF-2 study MIC (μg/ml) FLC	ATTAF-2	FLC/ATTAF-2	FICI/INT
C. albicans (*n* = 21)								
IFRC 25	0.5	0.063	0.031/0.016	0.31/SYN	0.5	0.125	0.125/0.031	0.5/SYN
IFRC 27	0.5	0.063	0.063/0.016	0.37/SYN	0.5	0.063	0.063/0.016	0.37/SYN
IFRC 37	0.5	0.063	0.063/0.016	0.37/SYN	0.5	0.25	0.125/0.031	0.37/SYN
IFRC 600	0.5	0.063	0.031/0.016	0.31/SYN	0.5	0.063	0.031/0.016	0.31/SYN
IFRC 604	0.5	0.063	0.031/0.016	0.25/SYN	0.5	0.063	0.016/0.016	0.28/SYN
IFRC 120	1	0.25	0.125/0.031	0.25/SYN	1	0.125	0.125/0.031	0.37/SYN
IFRC 614	1	0.063	0.031/0.016	0.28/SYN	1	0.125	0.063/0.016	0.19/SYN
IFRC 1055	1	0.063	0.25/0.016	0.5/SYN	1	0.125	0.125/0.016	0.25/SYN
IFRC 10	1	0.25	0.125/0.063	0.37/SYN	1	0.125	0.125/0.031	0.37/SYN
IFRC 13	1	0.125	0.125/0.031	0.37/SYN	1	0.125	0.063/0.031	0.31/SYN
IFRC 15	1	0.125	0.063/0.031	0.31/SYN	1	0.25	0.063/0.031	0.18/SYN
IFRC 24	2	0.063	0.063/0.016	0.28/SYN	2	0.25	0.125/0.063	0.31/SYN
IFRC 14	2	0.25	0.5/0.125	0.75/IND	2	0.125	0.25/0.063	0.63/IND
IFRC 18	2	0.125	0.125/0.031	0.31/SYN	2	0.125	0.125/0.031	0.31/SYN
IFRC 38	4	1	0.25/0.063	0.12/SYN	4	1	0.25/0.125	0.18/SYN
IFRC 26	4	0.5	0.5/0.063	0.25/SYN	4	0.125	0.25/0.031	0.31/SYN
IFRC 603	4	1	1/0.5	0.75/IND	4	4	2/2	1/IND
IFRC 616	4	0.25	0.063/0.063	0.26/SYN	4	1	0.25/0.125	0.18/SYN
IFRC 1260	≥64	8	16/4	0.62/IND	≥64	32	16/16	0.62/IND
IFRC 1261	≥64	16	16/16	1.12/IND	≥64	32	16/16	0.62/IND
IFRC 1262	≥64	8	16/4	0.62/IND	≥64	16	32/8	0.75/IND
C. glabrata (*n* = 10)								
IFRC 1276	2	0.125	0.125/0.031	0.31/SYN	2	0.125	0.5/0.031	0.5/SYN
IFRC 1274	4	0.25	1/0.031	0.37/SYN	4	0.5	1/0.063	0.37/SYN
IFRC 1275	4	0.125	0.5/0.031	0.37/SYN	4	0.25	0.5/0.031	0.25/SYN
IFRC 671	4	0.25	0.5/0.063	0.25/SYN	4	0.063	0.25/0.016	0.31/SYN
IFRC 680	8	0.25	2/0.125	1.25/IND	8	0.063	2/0.063	1.25/IND
IFRC 339	8	0.125	4/0.063	1/IND	8	0.063	4/0.063	1.25/IND
IFRC 648	≥64	32	32/8	0.5/SYN	≥64	64	32/16	0.5/SYN
IFRC 1063	≥64	16	64/16	1.5/IND	≥64	16	64/16	1.5/IND
IFRC 1065	≥64	32	64/8	0.72/IND	≥64	32	32/16	0.72/IND
IFRC 704	≥64	16	64/16	1.5/IND	≥64	16	64/16	1.5/IND
C. krusei (*n* = 9)								
IFRC 1251	4	0.125	1/0.031	0.5/SYN	4	0.25	1/0.031	0.37/SYN
IFRC 1052	4	0.25	1/0.031	0.37/SYN	4	0.5	1/0.063	0.37/SYN
IFRC 1058	4	1	1/0.125	0.37/SYN	4	1	1/0.063	0.31/SYN
IFRC 85	4	4	1/1	0.5/SYN	4	2	0.5/0.125	0.18/SYN
IFRC 1013	4	4	1/2	0.75/IND	4	4	1/2	0.75/IND
IFRC 1012	4	1	1/0.5	0.75/IND	4	2	1/1	0.75/IND
IFRC 1014	16	4	4/2	0.75/IND	16	2	4/1	0.75/IND
IFRC 1280	≥64	8	32/4	0.72/IND	≥64	64	64/64	1.5/IND
IFRC 1281	≥64	16	32/16	1.25/IND	≥64	64	64/64	1.5/IND
C. parapsilosis (*n* = 8)								
IFRC 1015	0.5	0.125	0.031/0.031	0.31/SYN	0.5	0.125	0.125/0.031	0.5/SYN
IFRC 1269	0.5	0.125	0.031/0.031	0.31/SYN	0.5	0.125	0.063/0.031	0.37/SYN
IFRC 1270	0.5	0.125	0.031/0.031	0.31/SYN	0.5	0.125	0.125/0.031	0.5/SYN
IFRC 1271	1	0. 25	0.125/0.031	0.25/SYN	1	0.25	0.25/0.031	0.37/SYN
IFRC 1059	2	0.125	0.25/0.031	0.37/SYN	2	0.25	0.5/0.063	0.5/SYN
IFRC 261	4	0.5	2/0.25	1/IND	4	0.5	2/0.125	0.75/IND
IFRC 1017	4	0.125	4/0.125	2/IND	4	0.25	4/0.25	2/IND
IFRC 1016	4	0.25	2/0.125	1/IND	4	0.5	4/0.5	2/IND
C. tropicalis (*n* = 4)								
IFRC 32	0.5	0.125	0.063/0.031	0.37/SYN	0.5	0.125	0.063/0.031	0.37/SYN
IFRC 1060	1	0.125	0.125/0.031	0.37/SYN	1	0.125	0.25/0.031	0.5/SYN
IFRC 1057	2	0.25	0.5/0.063	0.37/SYN	2	0.25	0.5/0.063	0.5/SYN
IFRC 1058	2	0.5	0.25/0.063	0.25/SYN	2	0.125	0.25/0.031	0.37/SYN

[a]FLC, fluconazole; FICI, fractional inhibitory concentration index; INT, interpretation; IND, indifference; SYN, synergy.

Moreover, synergistic activity against *C. glabrata*, *Candida parapsilosis*, *Candida krusei*, and *Candida tropicalis* was observed with 5 strains (50%), 5 strains (62.5%), 4 strains (44.4%), and 4 strains (100%), respectively. Overall, no antagonistic effects were observed against *Candida* isolates with these combinations. Remarkably combinations of fluconazole with voriconazole (used as controls) revealed unfavorable antifungal effects against 11 *Candida* isolates, with a high FICI range of 1.5 to 4, in comparison with FICI ranges of 0.25 to 2 and 0.31 to 2 for ATTAF-1 and ATTAF-2, respectively. Based on the findings, there were no significant differences in the activities of ATTAF-1 and ATTAF-2 against specific *Candida* isolates ($P > 0.05$).

With advances in modern medicine, leading to the availability and indiscriminate use of chemotherapeutic, immunosuppressive, and broad-spectrum antifungal agents, the increased incidence of severe candidiasis has been recently attributed to the large population of high-risk individuals (1, 2). Although fluconazole is the drug of choice for prophylaxis and treatment of candidiasis, prolonged use of this agent has contributed to the development of drug resistance in *Candida* isolates (20). Accordingly, novel therapeutic strategies, such as combination therapy, are essential for increasing the efficacy and reducing the toxicity of antifungal agents. Major attempts have been made to develop potent and safe antifungal agents with unique mechanisms of action (20). Fluconazole analogues with a triazole-modified scaffold display enhanced activity against *Candida* and *Cryptococcus* species, compared to filamentous fungi (15, 22). In the current study, ATTAF-1 and ATTAF-2, two promising novel azole compounds, could show potent activity against all *Candida* species when used alone or in combination with fluconazole. In line with the present results, Shi et al. (23) and Ramírez et al. (24) showed that the newly synthesized azole-based compounds were more active than fluconazole and the combination of these compounds with fluconazole could exert synergistic effects. Moreover, Ji et al. (25) synthesized triazole derivatives based on the structure of lanosterol 14α-demethylase (CYP51) and revealed that these compounds have better activity against *C. albicans* than does fluconazole. ATTAF-1 and ATTAF-2 share general structural features with the triazole alcohol class of antifungal agents, while exhibiting novel and distinct characteristics. The increased antifungal potency of these compounds might be due to secondary activities or actions within *Candida* isolates not shared by fluconazole. In previous studies, the mechanisms of azole resistance in different *Candida* isolates, including decreased intracellular concentrations of the target enzyme, changes in the drug target, and increased production of lanosterol 14α-demethylase, have been identified (26). The mechanisms of action of azole compounds and their derivatives have been precisely determined and established. Although our newly synthesized azole compounds showed more potent antifungal activities than did fluconazole, the mechanism of action involved might differ from that of fluconazole; moreover, synergistic activities apparently did not have major potential significance, since these interactions were observed mostly for isolates that were not resistant to fluconazole, and the synergistic mechanisms remained unclear. Therefore, we need to determine which subsets of events and mechanisms are primarily responsible for the observed growth inhibition with the synergistic use of azole compounds. Further analysis of the differences between different compounds and fluconazole could elucidate the underlying mechanisms of action. In conclusion, although ATTAF-1 and ATTAF-2 exhibited potent activities against clinical *Candida* isolates, their effectiveness, alone or in combination with fluconazole, for the treatment of *Candida* infections needs to be determined; in addition, the underlying mechanisms of action should be investigated.

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We declare no potential conflicts of interest.

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