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Antifungal activities of tacrolimus in combination with antifungal agents against fluconazole-susceptible and fluconazole-resistant *Trichosporon asahii* isolates

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ABSTRACT

The antifungal activity of tacrolimus in combination with antifungal agents against different fungal species has been previously reported. Here we report the *in vitro* interactions between tacrolimus and amphotericin B, fluconazole, itraconazole, and caspofungin against 30 clinical isolates of both fluconazole-susceptible and fluconazole-resistant *Trichosporon asahii*. For these analyses, we used the broth microdilution method based on the M27-A3 technique and checkerboard microdilution method. Tacrolimus showed no activity against *T. asahii* strains (minimal inhibitory concentrations, MICs > 64.0 $\mu g\,mL^{-1}$). However, a larger synergistic interaction was observed by the combinations tacrolimus + amphotericin B (96.67%) and tacrolimus + caspofungin (73.33%) against fluconazole-susceptible isolates. Combinations with azole antifungal agents resulted in low rates of synergism for this group (fluconazole + tacrolimus = 40% and itraconazole + tacrolimus = 10%). Antagonistic interactions were not observed. For the fluconazole-resistant *T. asahii* group, all tested combinations showed indifferent interactions. The synergism showed against fluconazole-susceptible *T. asahii* isolates suggests that the potential antifungal activity of tacrolimus deserves *in vivo* experimental investigation, notably, the combination of tacrolimus with amphotericin B or caspofungin.

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Introduction

The incidence of invasive mycoses caused by emergent fungal pathogens has proportionally grown with the increased number of immunocompromised hosts, such as AIDS patients, transplant recipients treated with immunosuppressive drugs, and those on cancer therapy.[1–3] *Trichosporon asahii* is the most frequently involved species in disseminated and deep-seated trichosporonosis including systemic infections due to therapeutic failure in transplanted patients.[2,3]

The first-line treatment for trichosporonosis includes the use of azole antifungal agents, since *Trichosporon* spp. is resistant to amphotericin B and echinocandins.[4–6] However, the frequent exposure to azoles can lead to development of secondary resistance to azoles and sometimes to multidrug resistance, resulting in therapeutic failures[7–9] and increasing mortality rates.[1,10] Combination therapy is a rational alternative that has been studied to improve the efficacy of antimicrobial therapy for difficult-to-treat infections, including overcoming concerns of antimicrobial resistance.[11–13]

Recent studies have shown that using fungal calcineurin pathways holds great promise for the future development of novel agents, including combination therapy with antifungals against fungal pathogens.[14–17] The antifungal activity obtained by the combination of the calcineurin inhibitor tacrolimus (FK506) plus antifungal agents has not yet been evaluated against *Trichosporon* species. In this context, the aim of this study was to evaluate the *in vitro* activity of the combination of FK506 with amphotericin B, fluconazole, itraconazole, and caspofungin against fluconazole-susceptible and fluconazole-resistant *T. asahii* strains.

Material and methods

Clinical isolates and molecular identification

One group of 30 fluconazole-susceptible (FS) strains of clinical isolates *T. asahii* maintained in the collection of the Department of Microbiology and Parasitology at the Federal University of Santa Maria, Santa Maria, RS, Brazil were studied. A second group of fluconazole-resistant strains (FR) ($n = 30$) was obtained from the FS group after sequential exposure to growing concentrations of fluconazole, as previously described by Fekete-Forgacs et al.,[18] with the following modifications: the final concentration of fluconazole was 128 $\mu g\,mL^{-1}$, and the incubation temperature was 35 °C with shaking for 48 h. Cells from this culture were plated on SDA plates, and a single colony was designated isolated FR. The standard strain *T. asahii* CBS 2479 was also included in the susceptibility tests.

The identity of these isolates was confirmed using standard microbiological and molecular methods. Total DNA was extracted according to the protocol described by Moller et al.[19] and Klassen et al.[20] with modifications. Amplification of the IGS1 region (rDNA intergenic) was performed by PCR using the primers 26F (5′ATCCTTTGCAGACGACTTGA-3′) and 5SR (5′AGCTTGACTTCGCAGATCGG-3′).[5] The PCR products were purified and sequenced. These sequences have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) with the following accession numbers: KR233249, KT438844, KR872655, KR872656, KR872657, KR872660, KR872661, KR233246, KR233247, KR233248, KT365854, KT365855, KR872662, KT438839, KT438840, KR872663, KR912056, KT438842, KT365856, KT438847, KR912058, KR912064, KT365858, KR912065, KT365859, KT438846, KT365860, KR912059, KT365861, KR912066.

Chemicals

Amphotericin B (AMB) (Sigma Chemical Co. – St. Louis, MO, USA), fluconazole (FCZ) (Sigma Chemical Co. – St. Louis, MO, USA), itraconazole (ITZ) (Frangon of Brazil, Pharmaceutical, Ltd., São Paulo, Brazil), caspofungin (CAS) (Merck, Darmstadt, Germany), and tacrolimus (FK506) (Janssen-Cilag Pharmaceutica, Beerse, Belgium) were employed.

The stock solutions of the drugs were prepared in dimethyl sulfoxide (DMSO, Sigma Chemical Co.), except for FCZ, which was diluted in sterile distilled water. FK506 was dissolved in methanol. Working solutions were prepared according to the document M27-A3 of the Clinical and Laboratory Standards Institute.[21]

In vitro susceptibility and drug interaction tests

Susceptibility assays were performed using the broth microdilution method, as described by the document M27-A3 of the Clinical and Laboratory Standards Institute.[21] The strains *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were included in the tests as controls. The minimum inhibitory concentrations (MICs) were defined as the lowest drug concentration able to inhibit 50% (for FCZ, ITZ, and CAS) or 100% (for AMB and FK506) of fungal growth when compared to the growth of the control. The highest concentrations used were 16 $\mu g\,mL^{-1}$ for AMB, 128 $\mu g\,mL^{-1}$ for FCZ, 16 $\mu g\,mL^{-1}$ for ITZ and 64 $\mu g\,mL^{-1}$ for CAS and FK506. The MICs employed as indicators for resistance were: $\geq 2\,\mu g\,mL^{-1}$ (AMB)[5,22,23]; $\geq 64\,\mu g\,mL^{-1}$ (FCZ)[6,24]; $\geq 1\,\mu g\,mL^{-1}$ (ITZ),[23] and $\geq 2\,\mu g\,mL^{-1}$ (CAS).[5,23] After exposing the strains to growing concentrations of FLZ, the strains were separated in two groups: (a) FS group (fluconazole-susceptible group), formed by the strains not exposed to FLZ; and (b) FR group (fluconazole-resistant group) formed by the same strains after exposure to FLZ, and now showing MICs ranging from 64 to 128 $\mu g\,mL^{-1}$.

In vitro combinations of antifungal agents with FK506 against the FS and FR groups of *T. asahii* were evaluated by the microdilution checkerboard method.[25] For the calculation of the fractional inhibitory concentration index (FICI), MIC values related to 100% inhibition of growth were used. Synergism was defined as the FICI ≤ 0.5, indifference was $0.5 < \text{FICI} \leq 4.0$, and antagonism FICI > 4.0. The FICIs were calculated for all wells along the turbidity/non-turbidity interface where the lowest FICI as the final point was determined.

Statistical analysis

The statistical analysis used to test the Susceptibility of the two groups (susceptible strains *vs.* resistant strains) to treatment with individual antifungal agents was analyzed with the *t*-test. Statistical significance was set at $p < 0.05$. Results

were analyzed using the software Graph Pad Prism5 (Graph Pad Software version 6.01, CA).

Results

The *in vitro* susceptibilities of 30 *T. asahii* isolates (FS) against the antifungal agents and FK506 are described in Table 1. FS strains showed low MICs range for FCZ (1.0–16.0 $\mu g\,mL^{-1}$) and ITZ (0.13–1.0 $\mu g\,mL^{-1}$) compared to FR strains: FCZ (64.0 to >128.0 $\mu g\,mL^{-1}$) and ITZ (0.5–16.0 $\mu g\,mL^{-1}$). Interestingly, for AMB, the number of resistant isolates decreased (90–3.33%) in the FR group (MIC range = 0.13–4.0 $\mu g\,mL^{-1}$); and for CAS the MICs remained similar in the two groups (MIC range for FS or FR = 4.0–16.0 $\mu g\,mL^{-1}$). FK506 did not show antifungal activity against FS and FR isolates at the highest concentration tested (MICs > 64.0 $\mu g\,mL^{-1}$). The statistical analysis showed significant differences between the susceptibility of FS *vs.* FR groups for azole antifungal agents (FLZ: $p < 0.0001$, ITZ: $p < 0.007$), as well as for AMB ($p < 0.0001$) and CAS ($p < 0.0001$).

The MICs and combinations results of antifungal agents and tacrolimus against *T. asahii* before and after resistance induction are presented in Tables 2 and 3. FK506 combined with AMB against the FS *T. asahii* isolates showed the highest percentage of synergism (96.67%), followed by the combination with CAS (73.33%). In the synergistic interactions between AMB + FK506 or CAS + FK506, the MIC values of FK506 decreased from 64 $\mu g\,mL^{-1}$ to 0.5 $\mu g\,mL^{-1}$. In contrast, in the FR group the majority of combinations with FK506 showed interactions classified as indifferent: AMB (76.67%), CAS (73.33%), FCZ (63.33%) and ITZ (50%). Antagonisms were not detected for combinations with FCZ and CAS but were showed with AMB (13.33%) and ITZ (10%).

Discussion

T. asahii is the most frequently involved species in disseminated fungal infections including cases of systemic infection in transplanted patients related to therapeutic failures.[2,3] The prognosis of trichosporonosis is very poor, showing mortality rates as high as 80%.[1,10] The lack of response to therapy is related to the relative resistance of *T. asahii* to many different antifungals.[3,4,7,9]

Moreover, the correct identification of *Trichosporon* species is important because the susceptibility is species dependent. In agreement with previous reports,[10,26,27] our results confirm that *T. asahii* clinical isolates seem to be more resistant *in vitro* to amphotericin B than to triazole compounds (Table 1).

Interestingly, our findings demonstrated that after sequential exposure to growing concentrations of fluconazole, the number of resistant isolates to AMB (MIC values $\geq 2\,\mu g\,mL^{-1}$) decreased from 90% to 3.33% (Table 1). Although this inverse relationship has been previously reported,[5,7,27,28] targeted deletion of *ERG3* or *ERG11* in *Candida albicans* and *Candida glabrata* seems to be a potential mechanism that may lead to increased susceptibility to AMB with increased resistance to azoles.[29,30]

Similarly, clinical failure and breakthrough infections with *Trichosoporon* have been reported with the use of echinocandins.[3,31–34] As expected, we found MICs $\geq 4\,\mu g\,mL^{-1}$ for caspofungin in all our isolates (Table 1). The cases of intrinsic resistance to echinocandins already described for *Cryptococcus* spp., *Trichosporon* spp., *Fusarium* spp., and zygomycetes are associated with insufficient sensitivity of the target enzyme, beta 1,3-D-glucan synthase, to the drug or a mutated form of the enzyme that precludes echinocandin binding.[35]

Regarding the azole antifungal agents, our results demonstrated that prolonged exposure of the clinical isolates to fluconazole showed an increased MIC for itraconazole characterizing cross-resistance among azoles (Table 1). Multidrug resistance to antifungal agents has been reported for *T. asahii* in previous studies.[4,7,9] *T. asahii* clinical isolates from nongranulocytopenic patients showed reduced susceptibility *in vitro* to AMB, flucytosine, fluconazole, itraconazole and ketoconazole.[7] Kushima et al.[9] described that long term use of fluconazole *in vivo* may lead to replacement of the amino acid *ERG11p* and thus trigger *T. asahii* resistance to multiple drugs. This study demonstrated that the MICs for other azole antifungal agents against *T. asahii* strains increased in parallel with the MIC for fluconazole, while the AMB MICs did not significantly change.[9]

Tacrolimus (previously known as FK506) is an effective immunosuppressant, obtained from *Streptomyces tsukubaensis*, and is widely used for prevention of transplant rejection.[36]

Table 1 – *In vitro* susceptibility of *Trichosporon asahii* isolates to antifungal agents and tacrolimus.

Agents	Group of isolates	Geometric mean	MIC range	MIC_{50}	MIC_{90}	Number (%) of resistant isolates
AMB	FS	2.24	1.0–8.0	2.0	4.0	27 (90)
	FR	0.41	0.13–4.0	0.25	1.0	1 (3.33)
FCZ	FS	2.41	1.0–16.0	2.0	8.0	0 (0)
	FR	64.00	64.0 to >128.0	64.0	64.0	30 (100)
ITZ	FS	0.27	0.13–1.0	0.25	0.50	1 (3.33)
	FR	1.52	0.5–16.0	1.0	4.0	27 (90)
CAS	FS	7.82	4.0–16.0	8.0	8.0	30 (100)
	FR	7.29	4.0–16.0	8.0	8.0	30 (100)
FK506	FS	ND	>64.0	ND	ND	ND
	FR	ND	>64.0	ND	ND	ND

MIC range, minimal inhibitory concentration range ($\mu g\,mL^{-1}$); MIC50, MIC at which 50% of isolates tested were inhibited; MIC90, MIC at which 90% of isolates tested were inhibited; AMB, amphotericin B; FCZ, fluconazole; ITZ, itraconazole; CAS, caspofungin; FK506, tacrolimus; FS e FR, fluconazole-susceptible and fluconazole-resistant *Trichosporon asahii* isolates; ND, not determined.

Table 2 – Minimal inhibitory concentrations (MICs) and combinations results of antifungal agents and tacrolimus against *Trichosporon asahii* before resistance induction.

Isolates	MICs ($\mu g\,mL^{-1}$) and combinations results before resistance induction											
	AMB/FK506			FCZ/FK506			ITZ/FK506			CAS/FK506		
	On its own	In the combination		On its own	In the combination		On its own	In the combination		On its own	In the combination	
06	2.00	0.50	S	8.00	1.00	I	1.00	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
11	2.00	0.25	S	2.00	1.00	I	1.00	2.00	I	16.00	8.00	I
	>64.00	0.50		>64.00	8.00		>64.00	0.50		>64.00	0.50	
13	2.00	0.50	S	4.00	1.00	S	1.00	1.00	I	16.00	8.00	I
	>64.00	0.50		>64.00	4.00		>64.00	0.50		>64.00	0.50	
19	2.00	0.25	S	4.00	1.00	S	1.00	1.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	16.00		>64.00	0.50		>64.00	0.50	
21	2.00	0.50	S	32.00	1.00	I	2.00	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	64.00		>64.00	0.50		>64.00	0.50	
29	4.00	1.00	S	64.00	1.00	I	2.00	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	64.00		>64.00	0.50		>64.00	0.50	
31	2.00	0.25	S	4.00	1.00	S	1.00	1.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	16.00		>64.00	0.5		>64.00	0.50	
32	4.00	1.00	S	16.00	1.00	I	2.00	1.00	I	32.00	16.00	I
	>64.00	0.50		>64.00	64.00		>64.00	0.50		>64.00	0.50	
36	1.00	0.13	S	8.00	1.00	I	1.00	1.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
43	2.00	0.25	S	64.00	1.00	S	1.00	0.25	S	32.00	4.00	S
	>64.00	0.50		>64.00	4.00		>64.00	0.50		>64.00	0.50	
44	4.00	0.25	S	8.00	1.00	I	1.00	2.00	I	32.00	16.00	I
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
46	2.00	0.50	S	4.00	1.00	I	1.00	1.00	I	32.00	16.00	I
	.>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
47	8.00	0.50	S	32.00	1.00	I	2.00	1.00	I	64.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
48	2.00	0.50	S	8.00	1.00	I	1.00	1.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
49	2.00	0.25	S	16.00	1.00	S	1.00	1.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	16.00		>64.00	0.50		>64.00	0.50	
50	2.00	0.25	S	8.00	1.00	S	1.00	1.00	I	32.00	16.00	I
	>64.00	0.50		>64.00	16.00		>64.00	0.50		>64.00	0.50	
51	4.00	0.25	S	2.00	1.00	I	1.00	1.00	I	32.00	16.00	I
	>64.00	0.50		>64.00	8.00		>64.00	0.50		>64.00	0.50	
53	4.00	0.25	S	4.00	1.00	S	2.00	0.50	S	16.00	16.00	I
	>64.00	0.50		>64.00	16.00		>64.00	0.50		>64.00	0.50	
54	2.00	0.50	S	8.00	1.00	I	1.00	1.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
56	2.00	0.50	S	8.00	1.00	I	1.00	0.50	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
58	2.00	0.50	S	16.00	1.00	I	1.00	1.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
59	1.00	0.50	I	16.00	1.00	S	1.00	0.25	S	64.00	8.00	S
	>64.00	0.50		>64.00	8.00		>64.00	0.50		>64.00	0.50	
60	2.00	0.25	S	8.00	1.00	S	1.00	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	16.00		>64.00	0.50		>64.00	0.50	
61	8.00	0.50	S	8.00	1.00	I	1.00	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
62	2.00	0.25	S	8.00	1.00	I	1.00	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
63	2.00	0.50	S	8.00	1.00	S	1.00	0.02	I	32.00	8.00	S
	>64.00	0.50		>64.00	16.00		>64.00	64.00		>64.00	0.50	
64	2.00	0.25	S	4.00	1.00	S	0.50	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	8.00		>64.00	0.50		>64.00	0.50	
65	4.00	0.25	S	8.00	1.00	I	0.50	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	32.00		>64.00	0.50		>64.00	0.50	
66	2.00	0.50	S	8.00	1.00	I	1.00	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	64.00		>64.00	0.50		>64.00	0.50	
67	1.00	0.25	S	4.00	1.00	S	1.00	2.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	16.00		>64.00	0.50		>64.00	0.50	

MICs, minimal inhibitory concentrations as the lowest concentration that showed 100% inhibition of fungal growth ($\mu g\,mL^{-1}$); AMB, amphotericin B; FCZ, fluconazole; ITZ, itraconazole; CAS, caspofungin; FK506, tacrolimus; S, synergism; I, indifference.

Table 3 – Minimal inhibitory concentrations (MICs) and combinations results of antifungal agents and tacrolimus against *Trichosporon asahii* after resistance induction.

Isolates	MICs ($\mu g\,mL^{-1}$) and combinations results after resistance induction											
	AMB/FK506			FCZ/FK506			ITZ/FK506			CAS/FK506		
	On its own	In the combination		On its own	In the combination		On its own	In the combination		On its own	In the combination	
06	1.00	0.50	I	>128.00	32.00	S	>16.00	2.00	S	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
11	0.25	0.25	I	128.00	32.00	S	4.00	1.00	S	8.00	4.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
13	0.50	0.50	I	>128.00	64.00	I	>16.00	4.00	S	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
19	0.50	0.25	I	128.00	64.00	I	4.00	2.00	I	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
21	0.25	0.25	I	128.00	32.00	S	2.00	1.00	I	32.00	8.00	S
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
29	1.00	0.25	S	128.00	128.00	I	>16.00	8.00	I	16.00	32.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
31	0.50	0.13	S	128.00	128.00	I	4.00	0.50	S	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
32	0.25	0.50	I	128.00	128.00	I	>16.00	4.00	S	16.00	32.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
36	0.50	0.25	I	128.00	64.00	I	2.00	2.00	I	16.00	4.00	S
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
43	0.25	0.25	I	128.00	32.00	S	2.00	1.00	I	16.00	4.00	S
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
44	0.25	0.25	I	128.00	32.00	S	>16.00	2.00	S	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
46	1.00	1.00	I	128.00	128.00	I	>16.00	2.00	S	16.00	16.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
47	4.00	0.25	S	64.00	64.00	I	2.00	2.00	I	16.00	16.00	I
	>64.00	5.00		>64.00	0.50		>64.00	0.50		>64.00	0.50	
48	0.50	0.25	I	>128.00	32.00	S	4.00	1.00	S	16.00	4.00	S
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
49	0.50	0.25	I	128.00	64.00	I	8.00	2.00	S	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
50	0.50	0.25	I	128.00	32.00	S	>16.00	1.00	S	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
51	0.25	0.25	I	128.00	32.00	S	2.00	2.00	I	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
53	0.25	0.50	I	128.00	128.00	I	2.00	16.00	A	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
54	0.25	0.13	I	128.00	64.00	I	>16.00	1.00	I	16.00	16.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
56	0.13	0.50	A	>128.00	64.00	I	>16.00	8.00	I	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
58	0.50	2.00	A	128.00	128.00	I	8.00	32.00	A	16.00	1.00	S
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
59	0.50	0.25	I	>128.00	64.00	I	4.00	2.00	I	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
60	0.25	0.50	I	128.00	128.00	I	>16.00	32.00	I	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
61	0.25	0.50	I	>128.00	64.00	I	>16.00	4.00	S	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
62	1.00	0.50	I	>128.00	128.00	I	4.00	2.00	I	16.00	16.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
63	0.25	2.00	A	>128.00	8.00	S	16.00	8.00	I	16.00	2.00	S
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
64	0.25	0.13	I	128.00	64.00	I	2.00	2.00	I	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
65	0.25	1.00	A	>128.00	16.00	S	1.00	8.00	A	16.00	1.00	S
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
66	0.25	0.50	I	128.00	64.00	I	1.00	2.00	I	16.00	2.00	S
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	
67	0.50	0.50	I	128.00	32.00	S	8.00	2.00	S	16.00	8.00	I
	>64.00	0.50		>64.00	0.50		>64.00	0.50		>64.00	0.50	

MICs, minimal inhibitory concentrations as the lowest concentration that showed 100% inhibition of fungal growth ($\mu g\,mL^{-1}$); AMB, amphotericin B; FCZ, fluconazole; ITZ, itraconazole; CAS, caspofungin; FK506, tacrolimus; S, synergism; I, indifference; A, antagonism.

This compound exerts its effects by blocking the immune system through inhibition of calcineurin.[37] Moreover, this protein can also affect essential functions of the fungal cell and it is intrinsically involved in the growth and pathogenesis of three major fungal species: *Cryptococcus neoformans*, *C. albicans*, and *Aspergillus fumigatus*.[36,38] In this study, FK506 showed low antifungal activity when tested alone against FS and FR *T. asahii* isolates (MICs > 64.0 $\mu g\,mL^{-1}$).

However, against the FS group, the results of our study (Table 2) demonstrate strong *in vitro* synergism of FK506 combined with drugs that were ineffective in inhibiting this group of *T. asahii* clinical isolates (Table 1) such as caspofungin (73.33%) and AMB (96.67%). High percentage synergistic interactions for caspofungin plus FK506 have been previously reported against *C. neoformans*, *Fusarium* spp., and *Aspergillus* spp.[11,14,39]

The potential enhanced antifungal activity of caspofungin in combination with other antifungal agents and anti-calcineurin drugs against clinical isolates of *Fusarium* spp. was demonstrated by Shalit et al.[11] The association of this echinocandin with FK506 appeared synergistic against all the isolates tested.[11] The antifungal effect exhibited by immunosuppressants cyclosporin A and FK506 is probably related to calmodulin activated protein phosphatase involved in fungal stress response, virulence, and antifungal resistance.[36] Steinbach et al.[14] also demonstrated a synergistic interaction of cyclosporin and FK506 with caspofungin against *A. fumigatus*. In this study, the calcineurin inhibitors were capable of causing a delay in filamentation of *A. fumigatus*, which suggested that inhibition of this pathway may potentiate the action of conventional antifungal agents in combination therapy against invasive aspergillosis.[14]

The pharmacokinetics of caspofungin is unaltered by coadministration of tacrolimus, but caspofungin may reduce tacrolimus concentrations by up to 20%.[40] Therefore, monitoring standard tacrolimus blood concentrations and appropriate tacrolimus dose adjustments are recommended for patients receiving both therapies.

On the other hand, the synergism observed with the association of FK506 and AMB can benefit the antifungal therapy regimen through the reductions in time to treatment response, dose with associated toxicity, costs, and decreased potential of microorganism-acquired resistance. Although AMB has been reported to have an insignificant effect on tacrolimus metabolism,[41] this polyene is well known to be nephrotoxic. Therefore, monitoring of serum creatinine is probably warranted for patients receiving both drugs.[42]

Studies have also shown that calcineurin inhibitors may exert a synergistic interaction when combined with antifungal azoles against *Candida* species.[15,16] However, our results demonstrated that combinations of FCZ or ITZ with FK506 produced interactions mainly indifferent emphasizing a lack of effect against FS and FR *T. asahii* isolates (Tables 2 and 3). The pharmacokinetics interactions between FK506 and azoles are well known and show that azoles inhibit the metabolism of FK506, requiring monitoring of the plasmatic concentration of FK506.[42]

In conclusion, our findings demonstrated that the combination of FK506 with AMB or CAS leads to high rates of synergism *in vitro*. These combinations against *T. asahii* isolates that presented resistance to both AMB and CAS deserve attention as candidates for *in vivo* studies focusing *T. asahii* experimental infections.

Conflicts of interest

The authors declare no conflicts of interest.

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