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TP-434 is Synergistic *In Vitro* with Amphotericin B, Fluconazole and Caspofungin Against *Candida* spp.

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Revised Abstract

Background. *Candida* species are predominant fungal pathogens associated with nosocomial infections in seriously ill and immunocompromised patients. Amphotericin B (AMB), fluconazole (FLC) and caspofungin (CSP) represent first-line therapies for treatment of *Candida* infections. Use of these drugs is severely limited by intolerable adverse reactions [1] and/or resistance [2, 3]. TP-434 is a novel fluorocycline antibiotic with broad-spectrum antibacterial activity for serious hospital pathogens. To evaluate potential interactions with antifungal drugs, TP-434 was tested in combination against panels of *Candida albicans*, *Candida glabrata* and *Candida krusei* isolates of diverse infection site origin.

Methods. *C. albicans* (n=16), *C. glabrata* (n=13) and *C. krusei* (n=13) isolates were obtained from Tufts Medical Center. Eight *C. albicans* isolates, one *C. glabrata* isolate and all *C. krusei* isolates were FLC-resistant. Microtiter MIC and checkerboard plate assays were performed following CLSI guidances. Results were visually scored at both 24 and 48 hrs. A ≥ 4 -fold change in MIC of antifungal drug, in combination with TP-434, was considered significant.

Results. TP-434 showed no inhibitory activity (MIC >128 μ g/ml) against *C. albicans* and showed moderate to weak (8 – 128 μ g/ml) or no inhibitory activity against *C. glabrata* and *C. krusei*. The MIC ranges for the antifungals alone were 0.5 - 2 μ g/ml, 0.13 - >128 μ g/ml and 0.031 - 2 μ g/ml for AMB, FLC and CSP, respectively. Combinations with 1 to 8 μ g/ml of TP-434 were found to be synergistic with AMB and combinations with 2 – 16 μ g/ml of TP-434 were found to be synergistic with CSP against all three *Candida* species. Combinations with 1 to 8 μ g/ml of TP-434 were synergistic with FLC for *C. albicans* and *C. glabrata*, but not with *C. krusei*.

Conclusion. Despite the absence of, or relatively weak, antifungal activity, TP-434 was found to significantly potentiate the *in vitro* activities of three mechanistically distinct antifungal drugs. Further studies to determine if there is a benefit to adding TP-434 to treatment regimens for single pathogen and mixed infections involving *Candida* spp. are warranted.

Introduction

Candida spp. are the most common fungal pathogens encountered in medical settings. Typically they are associated with nosocomial infections in seriously ill and immunocompromised patients. Amphotericin B (AMB), fluconazole (FLC) and caspofungin (CSP) are three mechanistically distinct antifungal agents that represent first-line therapies for the treatment of *Candida* infections, however the incidence of adverse reactions and resistance limits their clinical utility. Notably, treatment with amphotericin B has been associated with severe infusion-related effects, nephrotoxicity, and elevated risk for hepatotoxicity [1]. While resistance to polyene drugs such as amphotericin B remains rare, resistance of *Candida* spp. to azole drugs such as fluconazole can be >30% in isolates from HIV-infected patients [3].

TP-434 is a novel synthetic tetracycline antibiotic with broad-spectrum activity against pathogenic bacteria that cause serious infections. The primary mechanism of action of tetracycline antibiotics is inhibition of translation through binding to the bacterial 30S ribosomal unit. While tetracyclines have relatively weak affinity for eukaryotic 80S ribosomes, inhibition of mitochondrial protein synthesis in eukaryotic cells, including various fungi and protozoa, has been demonstrated [4]. Oliver et al. have shown that *C. albicans* grown in the presence of high concentrations (200 μ g/ml) of tetracycline for 48 hours had a modest (10-20%) reduction in cellular ergosterol, correlating with a loss of mitochondrial function and increased (32-fold) susceptibility to amphotericin B [5]. This suggested a possible link between mitochondrial inhibition and fungal cell wall composition affecting the antifungal activity of amphotericin B.

For the treatment of mixed infections, TP-434 may be used in combination with other anti-infective drugs. To evaluate potential interactions with antifungal drugs, TP-434 was tested in combination with antifungals against panels of *C. albicans*, *C. glabrata* and *C. krusei* using standard minimal inhibitor concentration (MIC) assays in a checkerboard synergy fashion. Where synergy was observed, the combination with the lowest concentration of TP-434 is reported.

Methods

Strains. *Candida* isolates in this study (n=42) were obtained from Tufts New England Medical Center, Boston, MA. All strains were clinical isolates, many of which were fluconazole-resistant, and included *C. albicans* (n=16), *C. glabrata* (n=13) and *C. krusei* (n=13). *C. albicans* ATCC 90028 and *Candida parapsilosis* ATCC 22019 were purchased from the American Type Culture Collection, Rockville, MD and were used as control organisms in minimum inhibitory concentration (MIC) determinations.

Testing. Microtiter MIC and checkerboard 96-well plate assays were performed using CLSI guidelines [6]. RPMI 1640 medium with glutamine (Lonza, Walkersville, MD) was used for all assays. Media was buffered with 1M MOPS (Sigma) and pH adjusted to 7.0 (± 0.1) using 1N NaOH. A microtiter broth-dilution assay was performed on each isolate prior to evaluation in checkerboard assays to determine appropriate drug concentration ranges for all compounds. For checkerboard assays, the experimental compound was diluted lengthwise at a volume of 25 μ l in a 96-well assay plate starting in column 2 and continuing through column 12, allowing for a known antibiotic control and a check on the sterility of the medium in column 1. Following dilution, 25 μ l pre-diluted TP-434 was added to each row on the plate containing antifungal compound; Twenty-five microliters of RPMI/MOPS was added to wells without TP-434. Inocula were prepared using freshly streaked isolated colonies from Sabouraud dextrose (Sab/dex) agar or YPD agar. Colonies were suspended into 2 ml of sterile 0.85% saline adjusted to a 2 McFarland turbidity standard and vortexed vigorously. Saline suspensions were further diluted 1:2000 in RPMI/MOPS, pH 7.0. Fifty microliters of these dilutions were added to the 96-well assay plates containing the appropriately diluted antibiotic concentrations. Plates were incubated at 35 $^{\circ}$ C and visually scored at both 24 and 48 hours. Plate counts were determined by standard 10-fold serial dilutions in 0.85% saline. Ten microliters of each serial dilution was plated onto Sab/dex agar or YPD agar and incubated for 48 hours at 35 $^{\circ}$ C and colony-forming units per microliter were subsequently enumerated.

Synergy scoring. For each combination, the sum fractional inhibitory concentration (Σ FIC) was calculated according to Reference 7, as follows:

$$\text{FIC of drug} = \text{MIC of drug in combination} / \text{MIC of drug alone}$$

$$\Sigma \text{FIC} = \text{FIC of TP-434} + \text{FIC of antifungal drug}$$

When both drugs showed antifungal activity, synergy was defined as Σ FIC ≤ 0.5 . If the TP-434 MIC was >16 μ g/ml, then synergy was scored as a ≥ 4 -fold potentiation of the antifungal-alone MIC. Where synergy was observed, the most potent combination is reported in green in Tables 1 – 3. A schematic of the checkerboard assay set up is depicted in Figure 1, where yellow indicates fungal growth, orange indicates the lowest inhibitory concentrations in the matrix, and green indicates points of synergy.

Figure 1. Combination Assay Plate Schematic

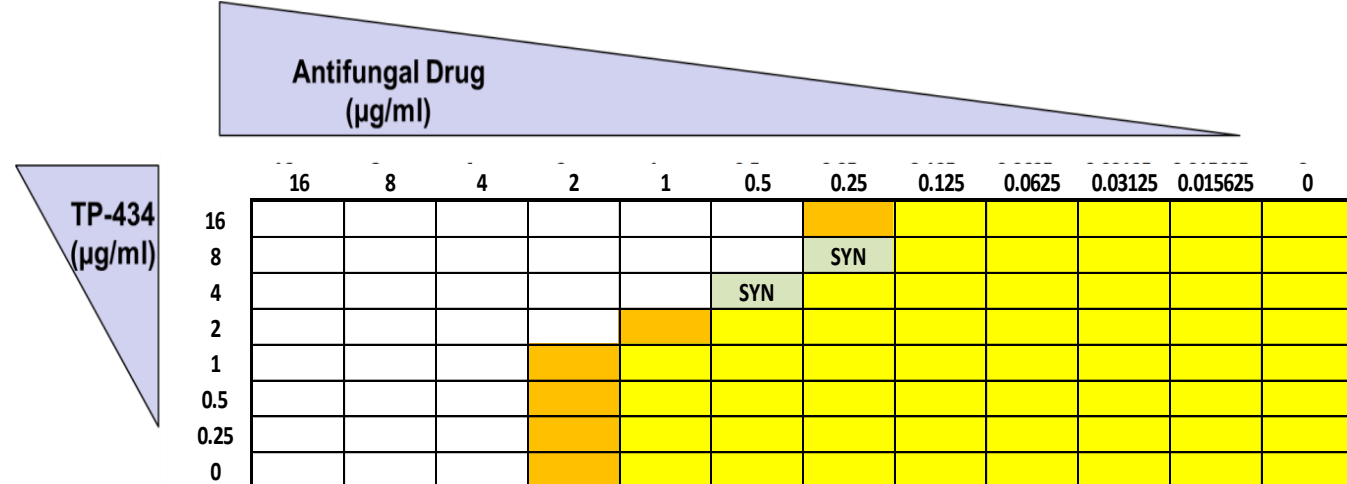


Table 1. Activity of Antifungal/TP-434 Combinations vs. *C. albicans*

A. TP-434 & Amphotericin				green indicates synergistic combinations					
Strain	24 hours		48 hours		Strain	24 hours		48 hours	
	Individual Drug MIC (μ g/ml)	Best Combination (μ g/ml)	Individual Drug MIC (μ g/ml)	Best Combination (μ g/ml)		Individual Drug MIC (μ g/ml)	Best Combination (μ g/ml)	Individual Drug MIC (μ g/ml)	Best Combination (μ g/ml)
CA1158	>16*	0.5	4	0.13	CA1158	>16*	1	4	0.13
CA1159	>16*	1	2	0.25	CA1159	>16*	2	4	0.5
CA1160	>16*	1	2	0.25	CA1160	>16*	2	1	0.5
CA1161	>16*	1	2	0.25	CA1161	>16*	1	8	0.13
CA1162	>16*	1	2	0.25	CA1162	>16*	1	8	0.063
CA1163	>16*	0.5	4	0.13	CA1163	>16*	1	8	0.063
CA1164	>16*	0.5	4	0.13	CA1164	>16*	1	4	0.25
CA1165	>16*	0.5	4	0.13	CA1165	>16*	2	4	0.5
CA1166	>16*	1	1	0.25	CA1166	>16*	1	8	0.063
CA1167	>16*	1	1	0.25	CA1167	>16*	1	8	0.063
CA1168	>16*	1	2	0.25	CA1168	>16*	2	4	0.5
CA1169	>16*	0.5	4	0.13	CA1169	>16*	1	8	0.063
CA1170	>16*	1	2	0.25	CA1170	>16*	2	2	0.5
CA1171	>16*	0.5	4	0.13	CA1171	>16*	1	8	0.063
CA1172	>16*	0.5	2	0.13	CA1172	>16*	1	4	0.25
CA1207	>16*	0.5	2	0.25	CA1207	>16*	1	8	0.13

* value was >128 μ g/ml when tested alone in an independent assay

B. TP-434 & Fluconazole

24 hours				48 hours					
Strain	Individual Drug MIC (μ g/ml)		Best Combination (μ g/ml)		Strain	Individual Drug MIC (μ g/ml)		Best Combination (μ g/ml)	
	TP-434	FLC	TP-434	FLC		TP-434	FLC	TP-434	FLC
CA1158	>16*	2	8	0.25	CA1158	>16*	>16*	4	4
CA1159	>16*	>16*	1	0.5	CA1159	>16*	>16*	2	1
CA1160	>16*	2	2	0.5	CA1160	>16*	>16*	2	1
CA1161	>16*	4	1	1	CA1161	>16*	>16*	2	1
CA1162	>16*	1	2	0.25	CA1162	>16*	>16*	2	1
CA1163	>16*	8	1	1	CA1163	>16*	>16*	2	1
CA1164	>16*	2	2	0.5	CA1164	>16*	>16*	2	1
CA1165	>16*	0.5	no synergy	no synergy	CA1165	>16*	0.5	no synergy	no synergy
CA1166	>16*	0.13	no synergy	no synergy	CA1166	>16*	0.25	no synergy	no synergy
CA1167	>16*	0.25	no synergy	no synergy	CA1167	>16*	0.5	4	0.13
CA1168	>16*	0.25	no synergy	no synergy	CA1168	>16*	0.5	no synergy	no synergy
CA1169	>16*	0.25	no synergy	no synergy	CA1169	>16*	1	4	0.25
CA1170	>16*	0.5	no synergy	no synergy	CA1170	>16*	>16*	4	0.5
CA1171	>16*	0.13	no synergy	no synergy	CA1171	>16*	2	2	0.5
CA1172	>16*	0.5	no synergy	no synergy	CA1172	>16*	2	2	0.5
CA1207	>16*	0.5	no synergy	no synergy	CA1207	>16*	2	no synergy	no synergy

* value was >128 μ g/ml when tested alone in an independent assay

C. TP-434 & Caspofungin

24 hours				48 hours					
Strain	Individual Drug MIC (μ g/ml)		Best Combination (μ g/ml)		Strain	Individual Drug MIC (μ g/ml)		Best Combination (μ g/ml)	
	TP-434	CSP	TP-434	CSP		TP-434	CSP	TP-434	CSP
CA1158	>16*	0.5	4	0.13	CA1158	>16*	0.5	8	0.063
CA1159	>16*	0.25	4	0.063	CA1159	>16*	0.5	8	0.063
CA1160	>16*	0.25	8	0.063	CA1160	>16*	0.5	8	0.063
CA1161	>16*	0.063	16	≤ 0.016	CA1161	>16*	0.063	16	≤ 0.016
CA1162	>16*	0.063	16	≤ 0.016	CA1162	>16*	0.063	no synergy	no synergy
CA1163	>16*	0.063	16	≤ 0.016	CA1163	>16*	0.13	8	0.031
CA1164	>16*	0.5	2	0.13	CA1164	>16*	0.5	4	0.13
CA1165	>16*	0.5	16	0.13	CA1165	>16*	1	16	0.13
CA1166	>16*	0.13	8	≤ 0.031	CA1166	>16*	0.13	16	≤ 0.016
CA1167	>16*	0.13	8	0.031	CA1167	>16*	0.13	16	≤ 0.016
CA1168	>16*	0.13	2	0.031	CA1168	>16*	0.13	16	0.031
CA1169	>16*	0.13	4	0.031	CA1169	>16*	0.13	8	0.031
CA1170	>16*	0.13	4	0.031	CA1170	>16*	0.13	16	≤ 0.016
CA1171	>16*	0.063	16	≤ 0.016	CA1171	>16*	0.063	16	≤ 0.016
CA1172	>16*	0.13	8	0.031	CA1172	>16*	0.13	16	≤ 0.016
CA1207	>16*	0.031	4	0.031	CA1207	>16*	0.5	4	0.063

* value was >128 μ g/ml when tested alone in an independent assay

Results

Table 2. Activity of Antifungal/TP-434 Combinations vs. *C. glabrata*

A. TP-434 & Amphotericin				green indicates synergistic combinations					
Strain	24 hours		48 hours		Strain	24 hours		48 hours	
	Individual Drug MIC (μ g/ml)	Best Combination (μ g/ml)	Individual Drug MIC (μ g/ml)	Best Combination (μ g/ml)		Individual Drug MIC (μ g/ml)	Best Combination (μ g/ml)	Individual Drug MIC (μ g/ml)	Best Combination (μ g/ml)
CG1174	>16	2	4	0.5	CG1174	>16	2	8	0.25
CG1175	>16	1	4	0.25	CG1175	>16	2	4	0.5
CG1176	>16	2	4	0.5	CG1176	>16	2	8	0.25
CG1177	>16	2	2	0.5	CG1177	>16	2	8	0.25
CG1179	16	2	2	0.5**	CG1179	>16	2	8	0.25
CG1180	>16	1	4	0.13	CG1180	>16	2	2	0.5
CG1181	>16	0.5	2	0.13	CG1181	>16	1	4	0.13
CG1182	>16	2	4	0.5	CG1182	>16	2	8	0.25
CG1183	>16	2	2	0.5	CG1183	>16	2	8	0.13
CG1184	16	1	4	0.25***	CG1184	>16	2	8	0.25
CG1185	8	1	no synergy	no synergy	CG1185	>16	2	no synergy	no synergy
CG1186	>16	1	4	0.25	CG1186	>16	2	8	0.13
CG1206	16**	1	4	0.13	CG1206	>16	2	8	0.25

* individual endpoint TP-434 MICs ranging from 8 - 128 μ g/ml in an independent assay

** FIC=0.38

*** FIC=0.5

B. TP-434 & Fluconazole

24 hours				48 hours					
Strain	Individual Drug MIC (μ g/ml)		Best Combination (μ g/ml)		Strain	Individual Drug MIC (μ g/ml)		Best Combination (μ g/ml)	
	TP-434	FLC	TP-434	FLC		TP-434	FLC	TP-434	FLC
CG1174	>16	4	8	1	CG1174	>16	8	no synergy	no synergy
CG1175	>16	4	no synergy	no synergy	CG1175	>16	8	no synergy	no synergy
CG1176	>16	8	8	2	CG1176	>16	16	no synergy	no synergy
CG1177	>16	>16**	no synergy	no synergy	CG1177	>16	>16**	no synergy	no synergy
CG1179	16	2	4	0.5	CG1179	>16	4	4	1
CG1180	>16	2	no synergy	no synergy	CG1180	>16	8	no synergy	no synergy
CG1181	>16	8	no synergy	no synergy	CG1181	>16	8	no synergy	no synergy
CG1182	>16	4	no synergy	no synergy	CG1182	>16	8	4	2
CG1183	>16	4	no synergy	no synergy	CG1183	>16			