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ORIGINAL PAPER

In Vitro Susceptibility of Berberine Combined with Antifungal Agents Against the Yeast Form of *Talaromyces marneffei*

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Abstract *Talaromyces* (*Penicillium*) *marneffei* can cause fatal disseminated infection in immunocompromised hosts. However, therapeutic strategies for the mycosis are limited. Reports of the other fungi suggest that berberine, a component of traditional herb, inhibitors interact with antifungal agents to improve the treatment outcomes. In the study, we evaluated the in vitro efficacy of berberine in combination with conventional antifungal agents against the pathogenic yeast form of *T. marneffei.* We demonstrate the synergistic effect of combination of berberine with fluconazole (52.38%), itraconazole (66.67%), voriconazole (71.43%), amphotericin B (71.43%) or caspofungin (52.38%) of *T. marneffei* strains, respectively. Time–kill curves confirmed the synergistic interaction, and no antagonistic was observed in all of the combinations. In conclusion, berberine could enhance the efficacy of conventional antifungal agents against the yeast form of *T. marneffei* in vitro. The results indicated berberine might have a potential role in combination therapy for talaromycosis.

Keywords *Talaromyces marneffei* · Antifungal susceptibility · Berberine · Combined therapy

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Introduction

Talaromyces marneffei (*T. marneffei*) is a thermal-dependent dimorphic fungus which is endemic in Southeast Asia, such as southern China and Thailand. The pathogen can cause systemic disseminated infections in immunocompromised patients, especially in HIV patients [1]. Up to now, the use of a single drug to treat systemic talaromycosis has not been completely satisfactory, and amphotericin B is still the drug of choice for the primary treatment of this mycosis. However, the strong toxicity associated with amphotericin B limited its use [2]. Thus, it is necessary to develop new therapeutic strategies to increase clinical outcome, reduce the amount of drugs and thus reduce the toxic side effects of drugs [3, 4].

Berberine is an alkaloid extracted from plants such as *Coptis chinensis* and *Phellodendron amurense*. It has a remarkable antibacterial effect and has a long history to treat the bacterial gastroenteritis and dysentery [5, 6]. In recent years, berberine's other pharmacological actions have been discovered gradually. Studies showed that berberine has significant effects against *Candida albicans* and *Cryptococcus* alone or in combination with conventional antifungal agents [7, 8].

However, the activity of berberine alone or in combination with antifungal agents against *T. marneffei* has not yet been reported. In this study, we evaluated the in vitro combined activity of berberine with fluconazole, itraconazole, voriconazole, amphotericin B and caspofungin against the yeast form of 21 isolates of *T. marneffei*.

Materials and Methods

Strains

A total of 21 *T. marneffei* strains stored at the Department of Dermatology and Venereology in the First Affiliated Hospital of Guangxi Medical University were assessed. Twenty isolates were obtained from clinical patients or isolated from bamboo rats; *T. marneffei* type strain FRR 2161 was also evaluated in this study; *Candida parapsilosis* ATCC 22019 was included as a control. Yeast cells of *T. marneffei* were prepared as previously described [3]. In brief, *T. marneffei* isolates were cultured on brain heart infusion agar (BHI) at 37 °C and maintained by continuous weekly passages, and the yeast-like cells were harvested and suspended in sterile water and thoroughly vortexed. The number of yeast cells was estimated by counting with a hemocytometer in lactophenol cotton blue. The suspensions were added to RPMI 1640 medium to obtain a stock of $1–5\times10^6$ CFU/mL and then were diluted 1000-fold, resulting in a working stock of $1–5\times10^3$ CFU/mL.

Agents

Antifungal agents used in this study included fluconazole (FLC), itraconazole (ITC), voriconazole (VOC), amphotericin B (AMB), caspofungin (CAS) and berberine (BBR), all of which were obtained from Sigma-Aldrich, USA, as pure powders. AMB, ITC, VOC and BBR were diluted in 100% dimethyl sulfoxide (DMSO). CAS and FLC were prepared in sterile distilled water. Stock solutions were diluted in RPMI 1640 medium (Gibco, USA) and then further serially diluted twofold, yielding four times the final strength required for the test.

Antifungal Susceptibility Assays

Drug interactions were assessed by a checkerboard microdilution method that also included the determination of the MIC of each drug alone in the same plate using the guidelines presented in the CLSI document M27-A3 with minor modifications [9]. The final drug concentrations were 1–128 μg/mL for FLC, 0.00078–1 μg/mL for ITC and VOC, 0.0625–8 μg/mL for AMB, 0.5–64 μg/mL for CAS and 4–512 μg/mL for BBR, depending on the susceptibility of *T. marneffei* to these drugs. The plates were incubated at 37 °C for 48 h. MICs of agents were read as the lowest drug concentration with no visible growth except FLC, which was defined as the lowest concentration necessary to inhibit 80% of growth compared with the control. Duplicated testing was performed on different days.

The fractional inhibitory concentration index (FICI) was used to classify drug interaction. And the FICI = MIC (A combo)/MIC (A alone) + MIC (B combo)/MIC (B alone). Synergy and antagonism were defined by FICI of ≤ 0.5 and > 4, respectively. An FICI result of > 0.5 but ≤ 4 was considered indifferent.

Table 1 Susceptibilities of tested drugs alone against 21 isolates of yeast form of *T. marneffei* (μg/ml)

Agent[a]	MIC range	GM MIC[b]	MIC_{90}	MIC_{50}
FLC	2–64	3.39	4	2
ITC	0.06–0.25	0.10	0.125	0.125
VOC	0.06–0.125	0.08	0.125	0.06
AMB	1–2	1.75	2	2
CAS	16–32	23.78	32	32
BBR	32–64	41.67	64	32

GM geometric mean

[a]*FLC* fluconazole, *ITC* itraconazole, *VOC* voriconazole, *AMB* amphotericin B; *CAS* caspofungin, *BBR* berberine

[b]Value in micrograms per milliliter

Time–Kill Curves

Talaromyces marneffei type strain FRR 2161 in RPMI 1640 medium was prepared at the starting inoculum of 10^3 CFU/mL [10]. The concentrations were 8 μg/mL for BBR, 0.5 μg/mL for FLC, 0.015 μg/mL for ITC and VOC, 0.25 μg/mL for AMB and 4 μg/mL for CAS, in vivo achievable concentration of the drugs. DMSO comprised < 1% of the total test volume. At predetermined time points (0, 8, 12, 24, 36 and 48 h after incubation with agitated culture at 37 °C), a 100-μl aliquot was taken from each solution and diluted tenfold in sterile water. A 100-μl aliquot from each dilution was streaked on the yeast peptone dextrose (YPD) agar plate. Colonies counted were determined after incubation at 37 °C for 48 h. The experiment was performed in triplicate. Synergism and antagonism were defined as a respective increase or decrease of ≥ 2.0-$\log_{10}$-CFU/mL in antifungal activity produced by the combination compared with that by the more active agent alone after 24 h, while a change of < 2-$\log_{10}$-CFU/mL was considered indifferent [8].

Results

The restraint of each drug alone or in combination against the 21 strains of *T. marneffei* in vitro is given in Tables 1, 2 and 3. When used alone, the MIC range of BBR is 32–64 μg/ml and the MICs of other antifungal agents were similar to previous studies [3, 4, 11], while in the combination, synergy was observed in BBR combined with all of the five antifungal agents. The highest synergy was observed with the combination of BBR/VOC and BBR/AMB (both percentage of strains indicating synergy of drug combinations were 71.43%), followed by BBR/ITC (66.67%), BBR/FLC (52.38%) and BBR/CAS (52.38%), respectively. The time–kill curves for the five combinations all showed a reduction of ≥ 2.0-log10-CFU/ml at 24 h compared to use alone (Fig. 1), which also confirmed the synergistic interactions among the five combinations.

In all of the combinations showing synergy, significantly lower MICs were achieved with FLC (64–4 μg/mL), VOC (0.125–0.015 μg/mL), ITC (0.125–0.015 μg/mL), AMB (2–0.25 μg/mL) and CAS (32–2 g/mL). The geometric mean (GM) MICs decreased, on average threefold for BBR in the combinations. Furthermore, antagonism was not observed for any combination.

Discussion

BBR is an alkaloid component of Chinese traditional herbs. The crude drugs containing BBR have been used as antifungal agents for 1000 years in China and Japan [12]. In recent years, the potent antifungal activity of berberine was observed on *Candida albicans* and *Cryptococcus* in vivo and in vitro [7, 13–15]. In our study, the results are also

Table 2 MICs of drug combination, FICI ranges and ratios of strains for which the drug combinations showed synergy

Antifungal combination	MICs in μg/ml(range)	FICI[a] range	GM FICI	Synergy(%)[b]
BBR–FLC	8–32 (0.5–8)	0.25–0.75	0.53	52.38
BBR–ITC	4–8 (0.015–0.06)	0.245–0.75	0.44	66.67
BBR–VOC	4–16 (0.015–0.03)	0.303–0.75	0.49	71.43
BBR–AMB	8–16 (0.25–1)	0.25–0.75	0.47	71.43
BBR–CAS	8–16 (2–8)	0.25–1.0	0.49	52.38

[a]*FICI* fractional inhibitory concentration index (synergy, FICI ≤ 0.5; indifferent, FICI > 0.5 and< 4.0; and antagonism, FICI ≥ 4.0)

[b]Percentage of strains indicating synergy of drug combinations

Table 3 In vitro interactions of berberine with fluconazole, itraconazole, voriconazole, amphotericin B and caspofungin against the pathogenic yeast form of *T. marneffei*

Isolates	MIC (μg/mL)			FICI	MIC (μg/mL)		FICI	MIC (μg/mL)		FICI
	BBR	FLC	BBR/FLC		ITC	BBR/ITC		VOC	BBR/VOC	
WT	32	2	8/0.5	0.5	0.125	4/0.03	0.365	0.125	8/0.03	0.49
CI-1	32	2	8/1	0.75	0.25	4/0.03	0.245	0.06	8/0.015	0.5
CI-2	64	4	8/0.5	0.25	0.125	8/0.03	0.365	0.06	16/0.015	0.5
CI-3	32	2	8/1	0.75	0.125	4/0.06	0.605	0.125	8/0.03	0.49
CI-4	32	2	8/0.5	0.5	0.06	8/0.015	0.5	0.125	8/0.03	0.49
CI-5	32	2	8/1	0.75	0.125	8/0.03	0.49	0.06	16/0.015	0.75
CI-6	32	2	8/1	0.75	0.125	4/0.06	0.605	0.125	8/0.03	0.49
CI-7	64	4	8/0.5	0.25	0.125	8/0.03	0.365	0.06	8/0.03	0.625
CI-8	32	4	16/1	0.75	0.06	8/0.03	0.75	0.06	4/0.03	0.625
CI-9	32	64	16/8	0.625	0.06	4/0.015	0.375	0.06	8/0.015	0.5
CI-10	64	64	16/4	0.3125	0.125	8/0.06	0.605	0.06	16/0.03	0.75
BrI-1	64	2	8/1	0.625	0.125	4/0.03	0.3025	0.06	8/0.03	0.625
BrI-2	64	2	8/1	0.625	0.06	8/0.015	0.375	0.06	16/0.015	0.5
BrI-3	32	2	8/0.5	0.5	0.125	4/0.03	0.365	0.125	4/0.015	0.245
BrI-4	32	4	8/0.5	0.375	0.125	4/0.03	0.365	0.06	8/0.015	0.5
BrI-5	32	2	8/0.5	0.5	0.125	4/0.03	0.365	0.125	8/0.015	0.37
BrI-6	64	2	32/0.5	0.75	0.06	8/0.03	0.625	0.125	4/0.03	0.3025
BrI-7	64	4	16/1	0.5	0.06	4/0.03	0.5625	0.06	8/0.015	0.375
BrI-8	32	2	8/1	0.75	0.125	4/0.03	0.365	0.125	4/0.03	0.365
BrI-9	32	4	8/0.5	0.375	0.125	8/0.03	0.49	0.06	8/0.015	0.5
BrI-10	64	2	8/1	0.625	0.06	8/0.03	0.625	0.06	8/0.03	0.625

Isolates	MIC(μg/mL)			FICI	MIC(μg/mL)		FICI
	BBR	AMB	BBR/AMB		CAS	BBR/CAS	
WT	32	2	8/0.5	0.5	16	16/8	1
CI-1	32	2	16/0.5	0.75	32	16/4	0.625
CI-2	64	2	8/0.5	0.375	32	8/4	0.25
CI-3	32	2	16/0.25	0.625	16	8/2	0.375
CI-4	32	1	16/0.25	0.75	16	16/2	0.625
CI-5	32	2	8/0.5	0.5	16	8/4	0.5
CI-6	32	2	8/0.5	0.5	32	8/2	0.3125
CI-7	64	2	8/0.25	0.25	32	16/2	0.3125
CI-8	32	1	8/0.25	0.5	32	16/4	0.625
CI-9	32	2	8/0.5	0.5	16	16/2	0.625
CI-10	64	2	8/0.25	0.25	16	16/2	0.375
BrI-1	64	2	8/0.25	0.25	32	16/2	0.3125
BrI-2	64	2	8/0.5	0.375	16	16/4	0.5
BrI-3	32	2	8/1	0.75	16	16/4	0.75
BrI-4	32	2	8/0.25	0.375	16	8/8	0.75
BrI-5	32	2	8/0.5	0.5	16	16/4	0.75
BrI-6	64	2	16/0.5	0.5	16	16/2	0.375
BrI-7	64	2	8/0.5	0.375	16	16/8	0.75
BrI-8	32	1	8/0.5	0.75	16	8/2	0.375

Table 3 continued

Isolates	MIC(μg/mL)			FICI	MIC(μg/mL)		FICI
	BBR	AMB	BBR/AMB		CAS	BBR/CAS	
BrI-9	32	1	16/0.25	0.75	32	16/4	0.625
BrI-10	64	2	8/0.5	0.375	16	16/2	0.375

WT wide type, *CI* clinical isolates, *BrI* bamboo rats isolates

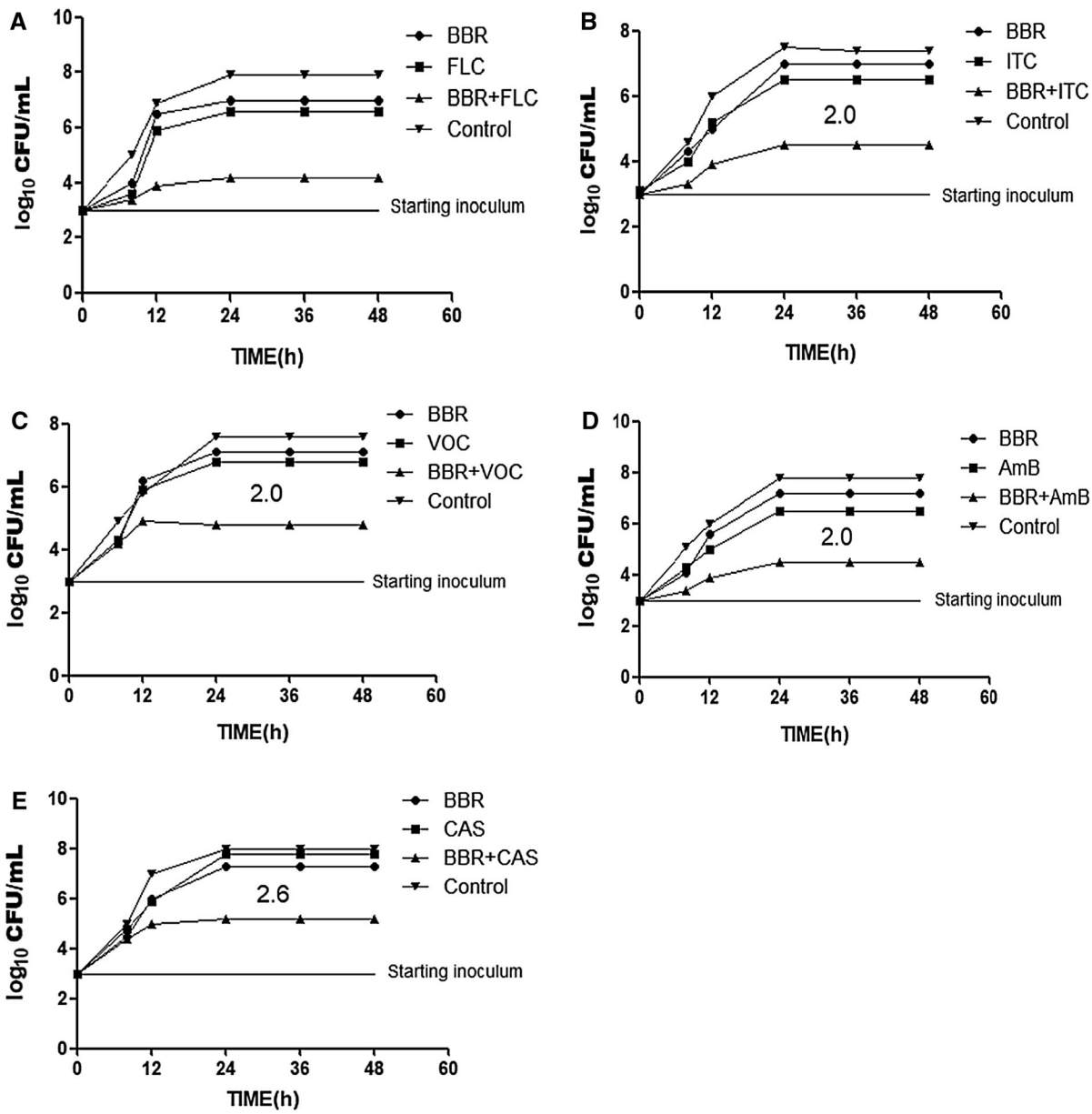

Fig. 1 Time–kill curves of *T. marneffei* type strain FRR 2161 that were obtained by using initial inoculums of 10^3 CFU/ml. BBR (8 μg/ml); FLC (0.5 μg/ml); AMB (0.25 μg/ml); CAS (4 μg/ml); ITC (0.015 μg/ml); VOC (0.015 μg/ml)

encouraging. Synergistic activity for berberine combined with conventional antifungal drugs is observed in more than half of the tested strains of *T. marneffei*, especially in BBR combined with AMB or VOC, which showed the highest synergistic activity. Our in vitro data indicated BBR could enhance the activity of the antifungal agents against pathogenic yeast form of *T. marneffei*, and the results suggest the therapies combined with BBR would reduce the dosage of antifungal agents without the loss of a clinical response.

BBR is absorbed on the intestine and has been used to treat the bacterial gastroenteritis and dysentery [16]. It would be noteworthy that about 12.5% *T. marneffei*-infected patients would develop diarrhea when the digestive tract was invaded [17, 18]. Our study demonstrated BBR had antifungal activity against yeast form of *T. marneffei*, especially in combination with antifungal agents. The results indicated BBR might have a potential role in combination therapy among the patients who had severe gastrointestinal invaded.

In summary, our study shows berberine could enhance the efficacy of antifungal agents against the yeast form of *T. marneffei* in vitro. A therapeutic strategy of combination of BBR together with conventional antifungal drugs might improve treatment regimens for *T. marneffei* infection. However, the clinical relevance of these findings is not yet clear. Further animal models and clinical data are needed for validation.

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Authors' Contributions Gang Liang and Cun-wei Cao designed this study and drafted the article. Hong Luo and Kai-su Pan performed the antifungal susceptibility assays and time–kill curves and data collection and edited the article. Dong-yan Zheng, Yan-qing Zheng and Xiu-ying Li isolate and identify strains. Xiao-lu Luo Alex Andrianopoulos and Le-min Wen performed the data analysis and calculated the statistics. Jing Guo, Chun-yang Huang and Justin Joseph critically revised the article. Rong Hu, Yu-jiao Li and Tian-min Li provided valuable advice and supported the clinical protocol. Study funding was secured by Cunwei Cao and Gang Liang. All of the authors have read and approved the final manuscript.

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