In Vitro Antifungal Activity of ZJ-522, a New Triazole Restructured from Fluconazole and Butenafine, against Clinically Important Fungi in Comparison with Fluconazole and Butenafine

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The antifungal activity of ZJ-522, a new triazole antifungal agent restructured from fluconazole and butenafine, was compared to that of fluconazole and butenafine against 43 strains of fungi representing 13 fungal species. MICs were determined by using the National Committee for Clinical Laboratory Standards (NCCLS)-recommended broth microdilution method for yeasts, which was modified for filamentous fungi. ZJ-522 was about 50-fold and 2 to 16-fold more potent than fluconazole against yeasts and filamentous fungi respectively, but it was less active than butenafine against filamentous fungi, although butenafine was inactive against most yeasts. Thus, the fashion of ZJ-522 antifungal activity more similar to that of fluconazole than that of butenafine indicates that ZJ-522 should be an inhibitor of lanosterol 14α-demethylase but not of squalene epoxidase, and should be a candidate for clinical development.

Key words ZJ-522; fluconazole; butenafine; antifungal activity; MIC

In the last 20 years, the frequency of fungal infections has dramatically increased because of factors such as intensive care practices, the use of indwelling catheters, human immunodeficiency virus infection, organ transplantation, therapies using anticancer agents, and other immunosuspressive conditions.[1—4] *Candida albicans* is the organism most often associated with both mucosal and hematogenously disseminated infections,[5—7] and recently, other *Candida* spp. such as *Candida parapsilosis*, *Candida glabrata* and *Candida tropicalis* have emerged as clinically important pathogens.[6,8,9] *Cryptococcus neoformans* and *Aspergillus* spp. are also important pathogens which cause serious infections.[10,11]

Fluconazole has been developed so far to be used widely in antifungal therapies over the last decade because of its high efficiency, low toxicity, and being well delivered and absorbed.[12] Whereas, its activity is limited, especially because of its failure against filamentous fungi[13] and the increasing number of fungi strains resistant to fluconazole.[14] Therefore, great efforts have been made to modify the chemical structure of fluconazole in order to broaden its antifungal spectrum of activity and to increase its potency.[15,16]

As we know, fluconazole has antifungal activity against yeasts because it can inhibit the fungal enzyme lanosterol 14α-demethylase, thereby blocking the biosynthesis of ergosterol, an essential component of fungal cell membranes.[17] Butenafine hydrochloride, a synthetic benzylamine derivative with a mode of action similar to that of the allylamine class of antifungal drugs, also can block the biosynthesis of ergosterol by inhibitting the fungal enzyme squalene epoxidase.[18] Unlike fluconazole, the antifungal spectrum of butenafine includes many filamentous fungi. While fluconazole is generally fungistatic to yeasts, butenafine is fungicidal to many strains of filamentous fungi.[19] Squalene, blocked by butenafine, is believed to have an effect of killing cells of fungi directly when it reaches a high accumulation.

So, a new antifungal agent, ZJ-522 (Fig. 1), restructured from fluconazole and butenafine, was designed and synthesized.[20] It was thought to be a inhibitor of both fungal enzyme lanosterol 14α-demethylase and squalene epoxidase, and have antifungal activity like fluconazole and butenafine.

This study reports the *in vitro* antifungal activity of ZJ-522 against clinical isolates from 13 species of clinically important fungi compared with fluconazole and butenafine.

MATERIALS AND METHODS

Drugs Powder fluconazole and butenafine were obtained from their respective manufacturers and stored at −70 °C until use. ZJ-522 was synthesized in the Department of Medical Chemistry, School of Pharmacy, the Second Military Medical University (Shanghai, China), as its yellow powder. The three powders, whether they were water soluble or not, were prepared in undiluted dimethyl sulfoxide (DMSO) at a concentration of 3.2 mg/ml and stored in 0.1-ml aliquots at −70 °C until it was needed.

Yeast A total of 45 strains from 13 fungal species were used in this study. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 22492 were obtained from the Depart-

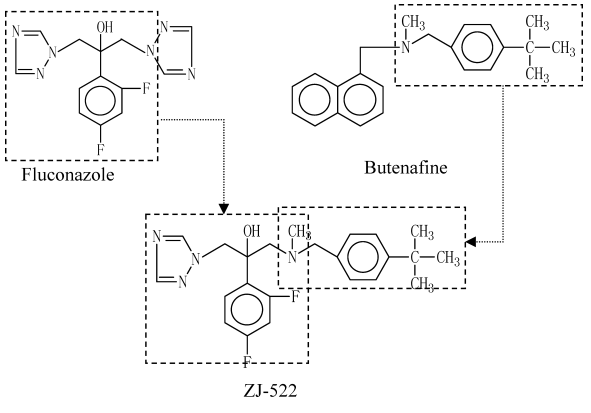

Fig. 1. Structures of Fluconazole, Butenafine and ZJ-522

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ment of Dermatology, the Frist Hospital of Beijing University (Beijing, China). The other 43 clinical isolates, including 12 *Candida albicans*, 7 *Cryptococcus neoformans*, 3 *Aspergillus fumigatus*, and 2 respective *Candida parapsilosis*, *Candida glabrata*, *Candida tropicalis*, *Fonsecaea pedrosoi*, *Fonsecaea compacta*, *Sporothrix schenckii*, *Microsporum canis*, *Microsporum gypseum*, *Trichophyto mentagrophytes* and *Trichophyton rubrum*, were provided by the Department of Dermatology, Changhai Hospital (Shanghai, China). The isolates were stored in yeast nitrogen base (YNB, Difco Laboratories, Detroit, Mich.) with 2% (wt/vol) glucose and 10% (vol/vol) glycerol at −70 °C when they were not in active use, and repeatedly subcultured on Sabouraud dextrose agar plates. They were incubated overnight at 35 °C before test.

Antifungal Susceptibility Testing The MICs for yeast were determined by a broth microdilution method based on the NCCLS method outlined in documents M27-T B[21] and M27-A,[22] with slight modifications.[16,23] RPMI 1640 medium buffered to pH 7.0 with 3-(*N*-morpholino)-propanesulfonic acid (MOPS) at a final concentration of 0.165 M was used as the culture medium, and the inoculum size was 1×10^3 to 5×10^3 cells/ml. The final concentrations of the antifungal agents ranged from 0.0625 to 32 μg/ml for fluconazole and butenafine, 0.001 to 32 μg/ml for ZJ-522. The microdilution plates inoculated with the fungi were incubated at 35 °C. MIC endpoints for *Candida* spp. were determined after incubation for 24 h, and for *C. neoformans*, MIC endpoints were determined after incubation for 72 h. These endpoints were defined as the lowest concentration that produced an 80% reduction of growth compared with that of the drug-free growth control. The MICs for filamentous fungi were determined by the broth microdilution method.[24] RPMI 1640 medium buffered to pH 7.0 with MOPS was used as the culture medium. The inoculum was prepared by suspending conidia of filamentous fungi in phosphate-buffered saline containing 0.05% Tween 80. The conidia were counted using a hemocytometer and then diluted into the growth medium to a concentration of 1×10^4 conidia/ml. A drug free positive control was included for each isolate. The microdilution plates were incubated in moist chambers at 30 °C for 5 d. The MICs were read visually and were defined using a no-growth end point. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as quality control organisms.

RESULTS AND DISCUSSION

Antifungal activities against fungal pathogens of ZJ-522 were investigated by measuring their MIC using the NCCLS microbroth dilution method. Fluconazole and butenafine, from which ZJ-522 was restructured, were used as standard agent for the evaluation of its activities (Table 1). Table 2 summarizes the MICs of ZJ-522, fluconazole, and butenafine for the 45 clinical isolates from 13 fungal species. Overall, ZJ-522 was highly active against all isolates of *Candida* spp. MICs required to inhibit 50 and 90% of the strains (MIC_{50} and MIC_{90}, respectively) of ZJ-522 against *Candida* spp. were 0.031 and 0.125 μg/ml, respectively. *C. albicans* were the most susceptible species (MIC_{50} and MIC_{90} were 0.031 and 0.062 μg/ml, respectively). Fluconazole was less active than ZJ-522 against all isolates of *Candida* spp. (MIC_{50} and MIC_{90} were 8 and 32 μg/ml). All isolates of *Candida* spp. were resistant to butenafine (MIC rang, 16—>64 μg/ml). While all *Cryptococcus neoformans* strains were resistant to Fluconazole (MIC rang, 8—>64 μg/ml), butenafine was more active against them (MIC rang, 1—8 μg/ml), but ZJ-522 was still the most active agent (MIC rang, 0.062—2 μg/ml). Although ZJ-522 was also more active (MIC_{50} and MIC_{90} were 1 and 4 μg/ml, respectively) than fluconazole (MIC_{50} and MIC_{90} were 4 and 16 μg/ml, respectively) against all species of filamentous fungi except *Trichophyton rubrum* and *Aspergillus fumigatus*, especially being 8 to 32 fold superior to fluconazole against *Fonsecaea* spp., it was still far less active than butenafine against them (MIC_{50} and MIC_{90} were ≤0.125 and 1 μg/ml, respectively).

The most exciting observation was the remarkably good activity ZJ-522 demonstrated (MIC rang, 0.031 to 0.125 μg/ml) against 4 strains of *Candida* spp. (3 *C. albicans*, 1 *C. glabrata*) for which fluconazole MICs were high (MICs, ≥16 μg/ml), demonstrating that ZJ-522 essentially had the same activity for both fluconazole-susceptible and fluconazole-resistant strains of *Candida* spp. Moreover, ZJ-522 also demonstrated 32—500 fold superior to fluconazole against 10 isolates of clinical *Cryptococcus neoformans*.

In the present study, ZJ-522 demonstrated great potential as a novel antifungal compound with potent *in vitro* antifungal activity against *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*, the four most commonly isolated species causing disseminated and mucocutaneous candidiasis, and against *Cryptococcus neoformans*, the species causing a severe clinical problem recently. Therefore, ZJ-522 should be particularly useful for the management of these clinically fungal infections.

While inhibitors of lanosterol 14α-demethylase such as azoles always show more active against yeast than against filamentous, inhibitors of squalene epoxidas such as benzylamine and allylamine show more active against filamentous than against yeast. The result that ZJ-522 was more active against yeast than against filamentous fungi demonstrates that the fashion of ZJ-522 antifungal activity is more similar to that of fluconazole than that of butenafine. This indicates that ZJ-522, restructured from both fluconazole and butenafine, shows more likely to be a inhibitor of lanosterol 14α-demethylase as fluconazole than to be a inhibitor of squalene epoxidase as butenafine.

In summary, ZJ-522, a new triazole antifungal agent, restructured from fluconazole and butenafine, was shown to have broad-spectrum and potent activities against clinically important yeast compared with fluconazole and butenafine, and to be a candidate for clinical development. In view of the potent *in vitro* activity demonstrated here, ZJ-522 warrants further investigation for its antifungal activities and the mechanisms.

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Table 1. MICs of ZJ-522, Butenafine, and Fluconazole for 45 Clinical Fungi Isolates of ($\mu g \cdot ml^{-1}$)

Organsim	Strain No.	ZJ-522	Fluconazole	Butenafine
Candida albicans	9306120	0.031	8	>64
	9508212	0.016	0.25	16
	9803104	0.125	32	>64
	9803148	0.031	1	32
	9803152	0.016	1	>64
	9803171	0.031	2	>64
	9803172	0.062	32	>64
	9803173	0.031	16	32
	9803198	0.016	0.25	32
	9803207	0.031	8	>64
	9803230	0.062	32	64
	9805250	0.016	2	>64
Candida parapsilosis	9704040	0.25	8	32
	9809130	0.062	8	>64
Candida glabrata	9605230	0.062	8	64
	9704041	0.125	32	>64
Candida tropicalis	9307240	0.031	4	>64
	9603220	0.016	4	>64
Cryptococcus neoformans	8501200	2	>64	8
	8903250	0.5	16	1
	9406204	0.5	16	1
	9510095	0.5	16	2
	9610310	0.062	32	4
	9611150	0.25	32	2
	9705121	0.125	32	4
	9711050	0.25	16	2
	9802150	0.125	8	1
	9807021	0.5	16	4
Sporothrix schenckii	9802047	2	8	≤0.125
	9805112	0.25	0.25	≤0.125
Fonsecaea pedrosoi	9805253	1	8	0.25
	9809180	4	64	1
Fonsecaea compacta	9307180	1	16	0.25
	9506130	1	32	≤0.125
Microsporum canis	9310140	0.25	0.25	≤0.125
	9503060	0.5	2	≤0.125
Microsporum gypseum	9711205	4	4	≤0.125
	9808050	4	16	0.25
Trichophyto mentagrophytes	9305300	2	4	≤0.125
	9711030	0.5	2	≤0.125
Trichophyton rubrum	9801197	0.5	0.5	≤0.125
	9704021	0.5	0.25	≤0.125
Aspergillus fumigatus	9501130	4	2	1
	9508214	32	8	2
	9807153	16	16	4

Table 2. *In Vitro* Susceptibilities of 45 Clinical Fungi Isolates to ZJ-522, Butenafine, and Fluconazole

Organsim (No. of isolates)	Antifungal agent	MIC (μg/ml)		
		Rang	50%	90%
Candida albicans (12)	ZJ-522	0.016—0.125	0.031	0.062
	Fluconazole	0.25—32	2	32
	Butenafine	16—>64	>64	>64
Candida spp (18)	ZJ-522	0.016—0.25	0.031	0.125
	Fluconazole	0.25—32	8	32
	Butenafine	16—>64	>64	>64
Cryptococcus neoformans (10)	ZJ-522	0.062—2	0.25	0.5
	Fluconazole	8—>64	16	32
	Butenafine	1—8	2	4
Filamentous fungi (17)	ZJ-522	0.25—32	1	4
	Fluconazole	0.25—>64	4	16
	Butenafine	≤0.125—4	≤0.125	1

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