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ORIGINAL ARTICLE

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In vitro antifungal activity of luliconazole (NND-502), a novel imidazole antifungal agent

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Abstract The in vitro activity of luliconazole (NND-502), a novel imidazole antifungal agent, against dermatophytes and several other groups of medically important fungi including the rare causative agents of dermatomycoses, was studied. The luliconazole susceptibility tests were performed with a total of 58 fungal strains of 23 species of fungi grouped into dermatophytes, dematiaceous fungi, hyaline hyphomycetes, yeastlike fungi, and zygomycetes using a broth microdilution method with RPMI 1640 medium. The minimum inhibitory concentration (MIC) values for luliconazole were compared with those of three reference drugs, lanoconazole (LCZ), bifonazole (BFZ), and terbinafine (TBF), all of which have been popular for the topical treatment of dermatophytosis, cutaneous candidiasis, and other superficial fungal infections in Japan. Luliconazole inhibited growth of all filamentous fungi except zygomycetes at low concentrations (MIC, ≦0.004–0.125 μg/ml), with dermatophytes being most susceptible (MIC, ≦0.004–0.008 μg/ml). The susceptibility of these filamentous fungi to luliconazole was almost equal to that to LCZ, and surpassed TBF and BFZ, although to a lesser extent; yeastlike fungi were also susceptible to luliconazole (MIC, 0.125–4 μg/ml). Again the antiyeastlike fungi activity of luliconazole was at the same level as LCZ and was greater than that of BFZ and TBF. In contrast to BFZ and TBF, however, luliconazole and LCZ were virtually inactive against zygomycetes.

Key words Luliconazole (NND-502) · Imidazole · Antifungal spectrum · Dermatomycosis

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Introduction

Luliconazole, with the chemical formula of (*R*)-(−)-(*E*)-[4-(2,4-dichlorophenyl)-1,3-dithiolan-2-ylidene]-1-imidazolylacetonitrile and the structure shown in Fig. 1, is a novel optically active antifungal imidazole compound whose preparations of topical use are under clinical trial for treatment of dermatophytosis and other superficial cutaneous fungal infections in Japan. Although luliconazole is structurally related to its predecessor lanoconazole, which has been clinically used for the topical treatment of dermatophytosis as well as of cutaneous candidiasis and malasseziosis in this country, they are chemically different in that the former is an *R*-enantiomer and the latter a racemic compound consisting of an active *R*-enantiomer and *S*-enantiomers.[1]

To learn the antifungal potency and spectrum of luliconazole, we studied its in vitro activity against dermatophytes and a variety of causative agents of superficial cutaneous and/or subcutaneous fungal infections in comparison with lanoconazole, bifonazole, and terbinafine, all of which are widely used for the topical treatment of superficial dermatomycoses in Japan.

Materials and methods

Antifungal agents and concentration ranges tested

Luliconazole (NND-502; Lot no. 96R01-014-DT), lanoconazol (LCZ; Lot no. 1D1001Y), bifonazol (BFZ; Lot no. 28F0004), and terbinafine hydrochloride (TBF; Lot no. 2) were supplied by the Research Center, Nihon Nohyaku Co. (Osaka, Japan). Each compound was dissolved in dimethyl sulfoxide (DMSO) and stored at −80°C.

The testing drug concentration ranges in susceptibility tests were 0.004–8 μg/ml for luliconazole, 0.004–8 μg/ml for LCZ, 0.004–8 μg/ml for BFZ, and 0.001–2 μg/ml for TBF.

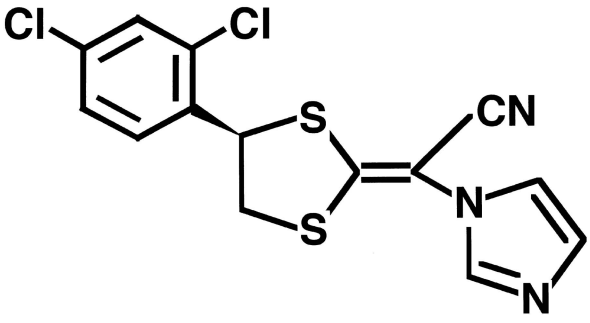

Fig. 1. Chemical structure of luliconazole

Fungal strains

A total of 58 fungal strains used in the present study were stock cultures preserved at the Teikyo University Institute of Medical Mycology. They comprised 29 strains of dermatophytes and 29 strains of other groups of pathogenic or opportunistic fungi.

Determination of antifungal activity

Minimum inhibitory concentration (MIC) determination for yeastlike fungi was measured based on the standard broth microdilution method of antifungal susceptibility testing for yeasts, approved by the National Committee for Clinical Laboratory Standards (NCCLS) M27-A2 method,[2] and filamentous fungi was measured by the Japanese Society for Medical Mycology (JSMM) method.[3]

Culture media

Modified 1/10 Sabouraud dextrose agar (peptone 0.2%, glucose 0.1%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_20$ 0.1%, agar 1.5%, pH unadjusted), Sabouraud dextrose agar, potato dextrose agar, and YM medium were used to grow test strains for preparing inocula. RPMI 1640 medium (+L-glutamine, $-NaHCO_3$, −phenol red; Sigma-Aldrich, MO, USA) buffered with 0.165 M 3-morpholinepropansulfonic acid (MOPS; Sigma-Aldrich) were used as assay media in antifungal susceptibility tests.

Preparation of fungal inocula

Dermatophytes and dematiaceous fungi

Dermatophytes were grown on modified 1/10 Sabouraud dextrose agar or Sabouraud dextrose agar and dematiaceous fungi on Sabouraud dextrose agar at 27°C for 1–4 weeks, and then conidial suspensions were prepared in physiological saline containing 0.05% Tween 80. After gauze filtration, the number of conidia in the suspensions was counted using a Burker-Turk hemocytometer and adjusted to 1.2×10^6/ml with RPMI 1640 medium.

Filamentous fungi other than dermatophytes and dematiaceous fungi

The test strains were grown on potato dextrose agar at 27°C for 1–2 weeks, and conidial suspensions were prepared as in the previous paragraph. The number of conidia in the suspensions was adjusted to 1.2×10^5/ml with RPMI 1640 medium.

Yeastlike fungi

The test strains were grown on YM agar at 35°C for 1–5 days, and suspensions containing 1.2×10^4 yeast cells/ml were prepared with RPMI 1640 medium.

Inoculation and incubation

One-hundred-milligram (100-μg) aliquots of drug solutions at various concentrations were dispensed into wells of microplates (Multi Well Plate, Sumilon MS-8196F; Sumitomo Bakelite, Tokyo, Japan) for antifungal susceptibility testing. For filamentous fungi, 10 μl each of the fungal inoculum and Alamar blue solution were added to each well. A growth control well, containing 100 μl RPMI 1640 medium, 10 μl of each fungal inoculum and 10 μl Alamar blue solution, and a negative control well, containing 110 μl RPMI 1640 medium and 10 μl Alamar blue solution, were included for each fungal strain tested. In the test for yeastlike fungi, Alamar solution was replaced by 10 μl RPMI 1640 medium, and growth control and negative control were similarly prepared and included. The final inocula were 1×10^5 for dermatophytes and dematiaceous fungi, 1×10^4 for filamentous fungi other than dermatophytes and dematiaceous fungi, and 1×10^3 for yeastlike fungi.

After inoculation, the microplates were incubated in a humidified incubator at 30°C for filamentous fungi and 35°C for yeastlike fungi for a maximum of 1 week. During the experimental period, the plates were visually observed or spectrophotometrically measured every 24 h. Endpoints were determined when the growth controls of filamentous fungi changed to a definite red color, or when the growth controls of yeastlike fungi showed turbidity of optical density (OD) 0.15 or above at 620 nm.

Endpoint determination

Filamentous fungi

Endpoints were in principle read visually. If there was difficulty in visual reading, the absorbance at 570 nm was measured using a microplate reader. The endpoint was a well showing a negative control well, or a well showing 50% growth inhibition (IC_{50}) compared to the growth control determined from absorbance measurement. The drug concentration at the endpoint was defined as the minimum inhibitory concentration (MIC).

Table 1. In vitro antifungal activities of luliconazole and three reference drugs against stock strains of dermatophytes and other groups of pathogenic fungi

Species (no of strains)	MIC or MIC range (μg/ml)			
	Luliconazole	Lanoconazole	Bifonazole	Terbinafine
Dermatophytes				
Trichophyton rubrum (10)	≦0.004	≦0.004	0.031–0.25	0.008–0.016
T. mentagrophytes (10)	≦0.004–0.008	≦0.004–0.008	0.25–2	0.016–0.031
T. violaceum (1)	≦0.004	≦0.004	0.125	0.016
T. verrucosum (1)	≦0.004	≦0.004	0.125	0.016
T. tonsurans (1)	≦0.004	≦0.004	0.016	0.004
Microsporum canis (2)	≦0.004	≦0.004	0.125, 1	0.016, 0.031
M. gypseum (2)	≦0.004	≦0.004	0.25, 0.5	0.016, 0.031
Epidermophyton floccosum (2)	≦0.004	≦0.004	0.031, 0.063	0.031, 0.125
Dematiaceous fungi				
Hortaea werneckii (3)	≦0.004–0.008	0.008–0.016	4–8	0.125–0.25
Alternaria alternata (2)	0.063	0.125	4	0.25, >2
Hyaline hyphomycetes				
Aspergillus fumigatus (3)	≦0.004	≦0.004	1	0.125
A . flavus (1)	0.008	0.016	>8	0.031
A. terreus (1)	≦0.004	0.008	2	0.031
Paecilomyces lilacinus (2)	0.031	0.125	4, 8	0.125, 0.25
Fusarium solani (2)	0.125	0.25	>8	1, >2
Scopulariopsis brevicaulis (1)	≦0.004	0.008	2	0.5
Yeastlike fungi				
Candida albicans (5)	0.125–0.5	0.125–0.5	4–8	>2
C. tropicalis (1)	4	2	>8	>2
C. parapsilosis (1)	4	2	>8	1
C. glabrata (1)	1	1	4	>2
Cryptococcus neoformans (2)	0.25	0.25, 1	4, 8	>2
Trichosporon asahii (2)	0.125, 0.25	0.125, 0.25	4, 8	>2
Zygomycetes				
Mucor circinelloides (2)	>8	>8	4	0.5, >2

Yeastlike fungi

The absorbance at 620 nm was measured using a microplate reader. The endpoint was a well showing 80% growth inhibition compared to the growth control (IC_{80}) and defined as the MIC.

Results

Table 1 shows the MIC ranges of luliconazle and the three reference agents against 29 strains of 8 species of dermatophytes, 5 strains of 2 species of dematiaceous fungi, 10 strains of 6 species of hyaline hyphomycetes, 12 strains of 6 species of yeastlike fungi, and 2 strains of 1 species of zygomycetes.

The MIC of luliconazole against dermatophytes was less than the lowest drug concentration tested (≦0.004 μg/ml) for almost all the test strains, showing its extremely potent antidermatophytic activity. Such activity of luliconazole was closely similar to that of LCZ and substantially greater than that of BFZ (MIC range, 0.016–2 μg/ml) and TBF (MIC range, 0.008–0.125 μg/ml).

The activity of luliconazole also showed a potent activity against dematiaceous fungi and hyaline hyphomycetes (MIC range, ≦0.004–0.125 μg/ml), which was at the same level as that of LCZ and surpassed BFZ (MIC range, 1 to >8 μg/ml) and TBF (MIC range, 0.031 to >2 μg/ml). A characteristic finding was that *Fusarium solani* with the lowest susceptibility to BFZ (MIC, >8 μg/ml) and TBF (MIC, 1 to >2 μg/ml) among these two groups of filamentous fungi, showed moderate susceptibility to luliconazole (MIC, 0.125 μg/ml).

The activity of luliconazole against yeastlike fungi (MIC range, 0.125–4 μg/ml) was again comparable to that of LCZ and superior to that of both BFZ (MIC range, 4 to >8 μg/ml) and TBF (MIC range, 1 to >2 μg/ml).

The zygomycetous fungus *M. circinelloides* was not inhibited by luliconazole or LCZ even at the highest concentration tested (8 μg/ml).

Discussion

Dermatophytosis caused mainly by *Trichophyton rubrum* and *T. mentagrophytes* remains a troublesome cutaneous disease because the incidence is still very high and a complete cure is not easily achievable by current antifungal chemotherapy. A new antifungal agent with increased therapeutic efficacy is needed. Next to dermatophytosis, cutaneous candidiasis due to *Candida albicans* and occasionally to non-*albicans Candida* is the second most fre-

quent superficial cutaneous fungal infection and is followed by cutaneous malasseziosis caused by several lipophilic *Malassezia* spp. Moreover, although their occurrence is infrequent or even rare, some different groups of pathogenic or opportunistic fungi, including dematiaceous fungi (e.g., *Hortaea werneckii* and *Alternaria alternate*), hyaline hyphomycetes (e.g., *Aspergillus* spp., *Paecilomyces* spp., *Fusarium* spp., and *Scopulariopsis* spp.), zygomycetes (e.g., *Mucor* spp. and *Rhizopus* spp.), and yeastlike fungi other than the genus *Candida* (e.g., *Cryptococcus* spp. and *Trichosporon* spp.), also cause superficial cutaneous and/or subcutaneous fungal infections.[4] Therefore, if a given antidermatophytic agent is also active against *Candida* spp. as well as against all or most of the infrequent or rare pathogens of cutaneous fungal infections, its clinical usefulness should be greater.

Luliconazole is a novel antifungal imidazole compound whose 1% cream and liquid preparations are under clinical trial in Japan for the topical treatment of dermatophytosis, cutaneous candidiasis, and cutaneous malasseziosis. There are two earlier laboratory studies demonstrating potent in vitro and in vivo activities of luliconazole against the two major dermatophytic pathogens, *T. rubrum* and *T. mentagrophytes*, as well as against *C. albicans* and *Aspergillus fumigatus*.[5–7] However, because only a limited number of fungal species and/or strains were investigated in these studies, it remains to be answered whether and how luliconazole is active against other species or groups of pathogenic fungi.

The present study was undertaken with the aim of learning the in vitro activity of luliconazole against a wide range of pathogenic or opportunistic fungi, mainly those potentially causing superficial dermatomycoses. For this purpose, substantial numbers of strains of such fungal species were subjected to antifungal susceptibility testing using LCZ, BFZ, and TBF, all of which are major topical antifungal drugs in this country, as the reference agents.

We used the NCCLS M27-A2 microbroth dilution method,[2] which is now generally accepted as the standard reference procedure for the antifungal susceptibility testing technique of all yeastlike fungi. By contrast, no reference method has yet been standardized for filamentous fungi, although the NCCLS M38-P macrobroth dilution technique was proposed for the susceptibility testing of filamentous fungi.[8] Subsequently, three new drugs were added and the antifungal susceptibility testing method (NCCLS M38-A)[9] by similar procedures was approved. The laboratory usefulness of the M38-A method appears to be hampered, however, by difficulty in turbidity-based determination of the endpoint. For this reason, the Standardization Committee of the Japanese Society of Medical Mycology proposed a modified micro-broth dilution method in which the endpoint is read colorimetrically using a redox indicator Alamar blue and the antifungal susceptibility of a large variety of filamentous fungi can be determined (referred to as the JSMM method).[3] As it was confirmed that the JSMM method is easier to use to simultaneously test a large number of fungal strains with diverse growth rates than is the NCCLS M38-P method and that both methods gave virtually an identical MIC value for micafungin (FK463) and several existing systemic antifungal drugs,[10,11] we adopted the JSMM method to test all filamentous fungi in the present study.

We demonstrated here that luliconazole was highly active against all fungal strains and species except zygomycetous fungi, inhibiting the growth of dermatophytes, dematiaceous fungi, hyaline hyphomycetes, and yeastlike fungi at concentrations of 0.008, 0.063, 0.25, and 4 μg/ml or below, respectively. Such activity was superior to that of BFZ and TBF, and almost identical in potency to LCZ, which is known as the most potent topically applied antifungal drug currently available. The similarity in both antifungal spectrum and potency between luliconazole and LCZ is probably due to the analogous structures of the two compounds.[1]

The results of the present study on the in vitro activity of luliconazole suggest that this compound is a promising candidate for the clinical development of a novel antifungal agent particularly useful in the treatment of dermatomycoses.

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