

Efficacy of Luliconazole Against Broad-Range Filamentous Fungi Including *Fusarium solani* Species Complex Causing Fungal Keratitis

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Purpose: Fungal keratitis can be difficult to medically treat. Topical antifungals are usually applied empirically as the initial option in treating fungal keratitis. Natamycin (NAT) and/or voriconazole (VRCZ) have been widely used in the treatment of fungal keratitis. However, *Fusarium solani* species complex (FSSC), which are the dominant species of fungal keratitis, are resistant to VRCZ. This study investigated in vitro efficacy of luliconazole (LLCZ), a new imidazole antifungal, against FSSC and other filamentous fungi.

Methods: A total of 18 *Fusarium* isolates and 7 others were grown on potato dextrose agar at 30 and 37°C. For *Fusarium*, species identification and phylogenetic tree analysis were performed based on elongation factor-1 α (EF-1 α) DNA sequencing. The broth microdilution method was used for antifungal susceptibility testing of 11 antifungal drugs including LLCZ.

Results: The 18 identified *Fusarium* isolates belonged to FSSC (n = 13), *Fusarium oxysporum* species complex (FOSC; n = 2), *Fusarium chlamydosporum* species complex (FCSC; n = 1), *Fusarium incarnatum-equiseti* species complex (FIESC; n = 1), and *Fusarium fujikuroi* species complex (FFSC; n = 1). We further divided 13 FSSC isolates into 3 clades, FSSC5 (n = 8), FSSC3 + 4 (n = 4), and FSSC9-a (n = 1), with 8 FSSC strains growing at 37°C. LLCZ showed lowest minimum inhibitory concentrations (MICs) against all tested filamentous fungi, with a MIC₉₀ against the

Fusarium species of 0.06 μ g/mL, whereas MIC₉₀ for NAT and VRCZ were 4 and 8 μ g/mL, respectively.

Conclusions: LLCZ has the strongest in vitro antifungal activity among all drugs used against broad-range filamentous fungi including FSSC. LLCZ may potentially be a new medical treatment option for fungal keratitis.

Key Words: luliconazole, *Fusarium solani*, fungal keratitis (Cornea 2019;38:238–242)

Infectious keratitis is a major worldwide cause of corneal blindness that especially occurs in developing regions of the world.¹ Causative organisms of infectious keratitis include bacteria, virus, fungi, *Acanthamoeba*, and other rare protozoa. Among these organisms, the treatment of fungal keratitis is the most challenging. Fungal keratitis requires surgical treatment including penetrating keratoplasty and evisceration to a greater degree than that observed for other causes of keratitis.² The frequency of fungal keratitis in infectious keratitis varies according to geography, climate, occupation, and healthcare exposure.³ In tropical or agricultural countries, it has been reported that the proportion of fungal keratitis to total infectious keratitis is more than 60%.^{4,5} In many countries, the most predominantly reported fungal species is *Fusarium*, with proportions ranging from 25% to 73.3%.^{2,4–8} Thus, *Fusarium* keratitis is one of the most important types of infectious keratitis.

Recently, topical voriconazole (VRCZ) has been widely used in the treatment of fungal keratitis, and there are many reports on the successful treatment of fungal keratitis caused by *Aspergillus fumigatus*, *Purpureocillium lilacinum*, *Alternaria* species, and *Beauveria bassiana*.^{9–14} However, minimum inhibitory concentrations (MICs) of VRCZ against *Fusarium* species are higher than those of the other fungal species.¹⁵ It has been reported that polyenes such as amphotericin B and natamycin (NAT) are still most susceptible for the *Fusarium* species in vitro.^{16–20} Indeed, randomized clinical trials showed that NAT was more effective than VRCZ for *Fusarium* keratitis.^{21,22} However, disadvantages of using polyenes are that they are strongly toxic to human cells and their corneal penetration as drugs is poor.²³ Therefore, there is a need for

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new antifungal ophthalmic solutions that can be used as a first-line drug for broad-range filamentous fungi.

Luliconazole (LLCZ) is a new imidazole antifungal agent with broad-spectrum antifungal activity that is structurally related to its predecessor, lanconazole. LLCZ, lanconazole and efinaconazole have been clinically used as topical drugs in the treatment of onychomycosis and dermatophytosis. These drugs are also known to be highly active against dermatophytes, with geometric mean MICs of LLCZ, lanconazole, and efinaconazole reported to be 0.0005, 0.002, and 0.007 $\mu\text{g}/\text{mL}$, respectively.²⁴ Posaconazole and ravuconazole are also new azoles that have yet to be topically applied in an ophthalmic setting. In our present study, we investigated the *in vitro* antifungal activity of LLCZ, lanconazole, and other azoles against the *Fusarium* species isolated from fungal keratitis and other species of reference strains.

MATERIALS AND METHODS

Fungal Isolates, Colony Morphologies, and Growth Temperatures

A multicenter study of fungal keratitis in Japan performed by the Japanese Association for Ocular Infection collected 14 *Fusarium* isolates.⁸ Four culture collection strains of *Fusarium* species and other species (*Aspergillus flavus*, *Alternaria alternata*, *B. bassiana*, and *Scedosporium apiospermum*) were obtained from the Biological Resource Center, NITE (NBRC). We also examined *P. lilacinum*, which was isolated from fungal keratitis, and 2 *Candida* reference strains that were obtained from the American Type Culture Collection (ATCC) (Table 1).

The colony morphology on the surface and reverse side was observed on potato dextrose agar plates after a 4-day incubation at 30 and 37°C. Colony diameters on potato dextrose agar plates were measured.

Species Identification and Phylogenetic Tree Analysis

Genomic DNA of *Fusarium* isolates was extracted using a ZR Fungal/Bacterial DNA Kit in accordance with the supplier's instruction. DNA sequencing of the PCR product amplifying the elongation factor-1 α (EF-1 α) gene was performed with primer pairs, using a previously described method.²⁵ Species identification was determined by comparing similar sequences with the *Fusarium* MLST database (<http://www.cbs.knaw.nl/fusarium/>).

The phylogenetic tree based on EF-1 α sequences was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0.26 (<http://megasoftware.net>) by the Neighbor-Joining method.²⁶

Antifungal Agent and Antifungal Susceptibility Testing

Susceptibilities of 11 antifungal drugs (micafungin, caspofungin, amphotericin B, NAT, VRCZ, miconazole, LLCZ, lanconazole, efinaconazole, posaconazole, and

ravuconazole) in 3 major classes (echinocandins, polyenes, and azoles) were tested by the broth microdilution method that is based on the CLSI M38-A2 standard.²⁷ MIC endpoints for newer azoles, which included LLCZ, lanconazole, and efinaconazole, were defined according to those used for VRCZ. Endpoints for echinocandins were defined by the minimal effective concentration. *Candida parapsilosis* ATCC22019 and *Candida krusei* ATCC6258 were used as quality control strains. LLCZ, lanconazole, and efinaconazole were provided by Nihon Nohyaku Co, Ltd (Tokyo, Japan), and ravuconazole was provided by Seren Pharmaceuticals Inc (Tokyo, Japan), with all other agents purchased from Sigma-Aldrich (St. Louis, MO).

RESULTS

Species Identification and Phylogenetic Tree Analysis of *Fusarium* Isolates

The DNA sequence-based species identification revealed that the 18 total *Fusarium* isolates belonged to 5 species complexes; *Fusarium solani* species complex (FSSC; n = 13), *Fusarium oxysporum* species complex (FOSC; n = 2), *Fusarium chlamydosporum* species complex (FCSC; n = 1), *Fusarium incarnatum-equiseti* species complex (FIESC; n = 1), and *Fusarium fujikuroi* species complex (FFSC; n = 1). The 13 FSSC strains were further divided into 3 clades; FSSC5 (n = 8), FSSC3 + 4 (n = 4), and FSSC9-a (n = 1) (Table 1 and Fig. 1).

Growth Temperatures and Colony Morphologies

All tested *Fusarium* isolates grew well at 30°C, but exhibited variable growth at 37°C in accordance with the strain (Table 1). Eight strains of FSSC grew at 37°C. Of these, only one strain GK10 (FSSC3 + 4) grew a colony that was larger than 10 mm in diameter. All other strains exhibited colonies that were smaller than 10 mm. None of the strains belonging to species complexes other than FSSC grew at 37°C. The strain GK14, which belongs to FSSC5, showed a villous colony appearance, whereas all other FSSC strains showed a felt-like cream-colored colony appearance. The GK12 (FFSC) colony strain also showed a villous appearance. Purplish colonies were observed in both FOSC strains.

Antifungal Susceptibility Testing

Table 1 presents results of the antifungal susceptibility test and MICs/MECs for all strains. Table 2 shows the MIC₅₀, MIC₉₀, and MIC ranges for polyenes and azoles against the *Fusarium* species. LLCZ showed the lowest MIC₉₀ (0.06 $\mu\text{g}/\text{mL}$) against the *Fusarium* species, followed by lanconazole (0.12 $\mu\text{g}/\text{mL}$). The MIC₉₀ for NAT and VRCZ was 4 and 8 $\mu\text{g}/\text{mL}$, respectively. VRCZ and efinaconazole exhibited moderate MICs for the *Fusarium* species, whereas they had low MICs against the non-*Fusarium* strains. Posaconazole and ravuconazole exhibited high MICs against the *Fusarium* species, especially for FSSC (Table 1). Cumulative

TABLE 1. Results of Species Identifications, Growth Rates, Colony Morphologies, and Antifungal Susceptibilities of Isolates Included in the Study

Strain No.	Origin/Strain Name	Species	Growth Rates†		MECs (µg/mL)					MICs (µg/mL)					
			30°C	37°C	MCFG	CPFG	AMPH	NAT	VRCZ	MCZ	LLCZ	LCZ	EFCZ	PCZ	RVCZ
1	Keratitis/GK10	FSSC 3 + 4	+++	++	>16	>16	2	4	1	1	0.03	0.03	0.5	1	>16
2	Keratitis/GK6	FSSC 3 + 4	+++	+	>16	>16	2	4	4	>16	0.03	0.06	1	>16	>16
3	Keratitis/GK5	FSSC 3 + 4	+++	+	>16	>16	2	4	4	>16	0.03	0.06	1	>16	>16
4	Keratitis/GK8	FSSC 3 + 4	+++	+	>16	>16	2	4	4	>16	0.06	0.12	2	>16	>16
5	Keratitis/GK11	FSSC 5	+++	-	>16	>16	2	4	8	>16	0.06	0.12	2	>16	>16
6	Keratitis/GK18	FSSC 5	+++	+	>16	>16	2	4	4	>16	0.12	0.25	4	>16	>16
7	NBRC5232	FSSC 5	+++	-	>16	>16	1	4	4	>16	0.03	0.06	1	>16	>16
8	NBRC7153	FSSC 5	+++	-	>16	>16	1	4	8	>16	0.06	0.12	4	>16	>16
9	NBRC5890	FSSC 5	+++	+	>16	>16	2	4	4	>16	0.06	0.12	2	>16	>16
10	Keratitis/GK17	FSSC 5	+++	+	>16	>16	2	4	4	>16	0.06	0.12	2	>16	>16
11	Keratitis/GK14	FSSC 5	+++*	-	>16	>16	2	4	4	>16	0.03	0.03	1	2	>16
12	Keratitis/GK7	FSSC 5	+++	+	>16	>16	2	4	8	>16	0.06	0.12	4	4	>16
13	Keratitis/GK3	FSSC 9-a	+++	-	>16	>16	2	4	8	>16	0.12	0.25	4	>16	>16
14	NBRC9955	FCSC	+++	-	4	8	1	4	0.5	1	0.015	0.03	0.06	0.5	1
15	Keratitis/GK4	FIESC	+++	-	1	16	2	4	4	2	0.06	0.12	2	2	>16
16	Keratitis/GK12	FFSC	+++*	-	8	>16	2	4	4	>16	0.03	0.06	1	2	16
17	Keratitis/GK1	FOSC	+++	-	>16	>16	4	4	4	>16	0.03	0.03	0.5	8	4
18	Keratitis/GK2	FOSC	+++	-	>16	>16	2	4	4	>16	0.03	0.03	0.5	8	8
19	NBRC8558	<i>A. flavus</i>	+++	+++	0.015	0.5	2	>16	0.5	1	0.008	0.015	0.25	0.5	0.5
20	NBRC4026	<i>A. alternata</i>	++	-	0.5	1	1	2	0.5	1	0.015	0.12	0.25	0.25	1
21	Keratitis/GU1	<i>P. lilacinum</i>	++	+	>16	>16	>16	>16	0.25	4	0.03	0.06	0.12	0.5	1
22	NBRC31676	<i>B. bassiana</i>	+	-	1	1	2	2	0.5	1	0.015	0.015	0.12	0.25	1
23	NBRC31146	<i>S. apiospermum</i>	++	++	0.5	2	4	2	0.12	0.25	0.015	0.015	0.03	0.5	1
24	ATCC22019	<i>C. parapsilosis</i>	NT	NT	1	1	1	4	0.03	1	2	2	0.03	0.25	0.06
25	ATCC6258	<i>C. krusei</i>	NT	NT	0.12	0.25	2	4	0.25	2	2	2	0.12	0.5	0.5

*These strains grew colonies with a villous appearance.

†Growth rates were defined based on colony diameter at 4 days as follows: ≥30 mm (+++), ≥10 mm and <30 mm (++) , <10 mm (+) and no growth (-).

AMPH, amphotericin B; EFCZ, efinaconazole; MEC, minimum effective concentration; CPFG, caspofungin; LCZ, lanoconazole; MCZ, miconazole; MIC, minimum inhibitory concentration; MCFG, micafungin; PCZ, posaconazole; RVCZ, ravuconazole; FSSC, *F. solani* species complex; FCSC, *F. chlamydosporum* species complex; FIESC, *F. incarnatum-equiseti* species complex; FFSC, *F. fujikuroi* species complex; FOSC, *F. oxysporum* species complex; *A. flavus*, *Aspergillus flavus*; *A. alternata*, *Alternaria alternata*; *P. lilacinum*, *Purpureocillium lilacinum*; *B. bassiana*, *Beauveria bassiana*; *S. apiospermum*, *Scedosporium apiospermum*; *C. parapsilosis*, *Candida parapsilosis*; *C. krusei*, and *Candida krusei*.

MIC curves for the in vitro testing showed that LLCZ was most effective for the *Fusarium* species among all antifungal drugs examined in this study (Fig. 2). MICs for the *Fusarium* species of amphotericin B and NAT were low regardless of the species. *Fusarium* species were not susceptible to the Echinocandins (Table 1). LLCZ also exhibited the lowest

MICs against other filamentous fungi including *A. flavus*, *A. alternata*, *P. lilacinum*, *B. bassiana*, and *S. apiospermum* (Table 1). However, the antifungal activity of LLCZ for the *Candida* species was lower than that for echinocandins and triazoles, such as efinaconazole, VRCZ, posaconazole, and ravuconazole.

FIGURE 1. Phylogenetic diversity of *Fusarium* species isolates included in this study based on elongation factor-1α (EF-1α) sequence data. The percentages of replicate trees in which associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Evolutionary distances were computed using the p-distance method and are presented in units of base differences per site. The sequence of *Fusarium illudens* NRRL22090 was used as an out-group.

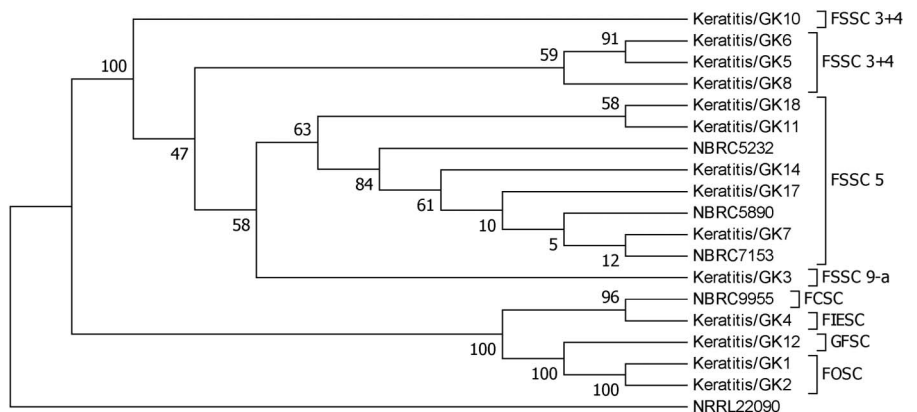


TABLE 2. Comparison of Antifungal Susceptibility of Polyenes and Azoles Against *Fusarium* Species

Antifungal Drugs	MIC ₅₀	MIC ₉₀	Range
AMPH	2	2	1–4
NAT	4	4	4
VRCZ	4	8	0.5–8
MCZ	>16	>16	1–16
LLCZ	0.03	0.06	0.015–0.12
LCZ	0.06	0.12	0.03–0.25
EFCZ	1	4	0.06–4
PCZ	>16	>16	0.5–16
RVCZ	>16	>16	1–16

AMPH, amphotericin B; EFCZ, efinaconazole; LLCZ, luliconazole; LCZ, lanocanazole; MIC, minimum inhibitory concentration; MCZ, miconazole; PCZ, posaconazole; and RVCZ, ravuconazole.

DISCUSSION

This study tested the in vitro activity of LLCZ, a new imidazole antifungal, against various fungi including the keratitis-derived *Fusarium* species. Results showed that LLCZ had lowest MICs for a broad-range of filamentous fungi among all tested antifungal agents. Notably, the MIC₉₀ for the *Fusarium* species was 0.06 µg/mL, which was far lower than that observed for NAT (4 µg/mL) and VRCZ (8 µg/mL) (Table 2). If further studies including corneal penetration, absorption, tissue compatibility studies, and stability of drugs are completed, LLCZ could potentially be a new medical treatment option for fungal keratitis.

Although initial medical treatments for fungal keratitis are usually empirical, management of the disease is difficult, as there has yet to be any single agent found that is universally effective.²⁸ For *Fusarium* keratitis, NAT has

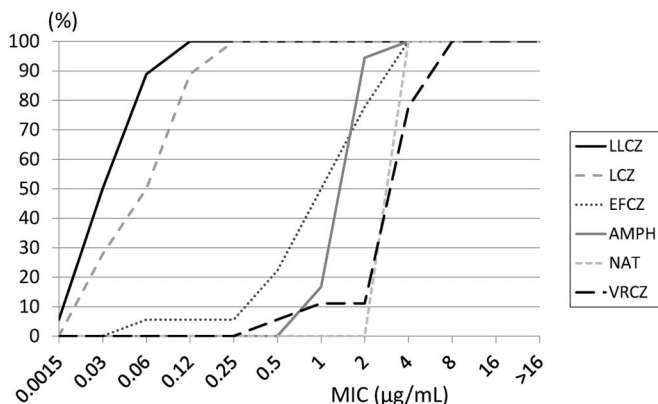


FIGURE 2. Cumulative minimum inhibitory concentration curves of antifungal drugs tested against the *Fusarium* species (n = 18). The 18 total isolates included 14 clinical strains from keratitis and 4 strains from a culture collection. The vertical axis shows the percentage of isolates (%), whereas the horizontal axis shows the minimum inhibitory concentrations (MICs, µg/mL). Luliconazole (LLCZ) exhibited the lowest MIC among all antifungal drugs tested. LCZ, lanocanazole; EFCZ, efinaconazole; AMPH, amphotericin B; NAT, natamycin; and VRCZ, voriconazole.

been shown to be more effective than VRCZ.²¹ However, in *Aspergillus* keratitis, more cases treated with NAT were reported to develop perforation or required therapeutic keratoplasty than VRCZ-treated cases.²² Furthermore, ulcers caused by *Aspergillus* were more refractory to primary treatments with topical 5% NAT monotherapy compared with those caused by *Fusarium*.²⁹ *P. lilacinum* is also one of the important species of fungal keratitis that is known to be resistant to polyenes and sensitive only to VRCZ in most of the strains.^{13,30} The findings of all previous studies suggest that there is a strong need to create a new antifungal drug for potential use as a first-line topical drug.

About frequency and severity of fungal keratitis, the *Fusarium* species remain the most important cause of this disease. The genus *Fusarium* is composed of several species complexes, which indicate there are groups of closely related species. There have been 7 species complexes previously shown to be related to human infections and they include: *Fusarium dimerum* species complex (FDSC), FSSC, FOFC, FFSC, FIESC, FCSC, and the complex including *Fusarium sporotrichoides* (FSAMSC).³¹ Among these, FSSC is the most dominant species complex in keratitis, accounting for 75% to 88% of all *Fusarium* keratitis cases.^{19,31} FSSC has been further divided into several phylogenetic groups by phylogenetic tree analysis. Hassan et al reported that FSSC3 + 4 (also called *Fusarium falciforme*) was predominantly isolated from keratitis,⁶ whereas Muraosa et al³² reported that FSSC3 + 4 was the most frequent isolate collected from patients with superficial fusariosis in humans, followed by FSSC5. In our present study, 10 keratitis-derived FSSC isolates were distributed among the FSSC5 (n = 5), FSSC3 + 4 (n = 4), and FSSC9 (n = 1) complexes (Table 1). Our current results do not contradict any of the aforementioned reports. All FSSC isolates in our study showed high MICs for VRCZ, with the exception for the GK10 strain (FSSC3 + 4) with low azole MICs. Moreover, this strain also showed differences in growth at 37°C from the other 3 strains belonging to FSSC3 + 4 (GK10, GK6, and GK5). To differentiate these strains, additional molecular identification by sequencing the RNA polymerase II largest subunit (RPB1) and RNA polymerase II second largest subunit (RPB2) will need to be undertaken.

LLCZ is a novel imidazole antifungal compound that was originally synthesized in Japan³³ and which is now widely used as a 1% cream, 1% solution, and a 1% ointment in the topical treatment of onychomycosis and dermatophytosis.^{34,35} Furthermore, our present study also showed that LLCZ is active not only against dermatophytes but also against a broad-range of filamentous fungi that includes the *Fusarium* species, *A. flavus*, *P. lilacinum*, *B. bassiana*, and *S. apiospermum*, which cover most of the isolates from fungal keratitis. In addition, the advantage of using LLCZ is its small molecular weight (MW), as the MWs of LLCZ, VRCZ, NAT, amphotericin B, and micafungin are 354, 349, 665, 924, and 1270 Da, respectively. Because of the small MW and lipophilicity, topical VRCZ exhibits good drug penetration into the deep corneal stroma and anterior chamber.³⁶ Thus, topical LLCZ may be a potential candidate as a new antifungal ophthalmic solution with a broad-spectrum, strong antifungal activity, and high corneal penetration. Although

efinaconazole exhibited a slightly lower MIC₉₀ against FSSC (4 µg/mL) versus VRCZ (8 µg/mL), its potential activity was less than that observed for LLCZ. Posaconazole and ravuconazole were not active against FSSC (Table 1). However, the disadvantage of using LLCZ is that MICs against the *Candida* species were higher than those for VRCZ and echinocandins. Thus, it should be noted that LLCZ is a less effective drug when used in the treatment of candidiasis.

The major limitation of this study is that we only tested the efficacy of LLCZ in vitro. Thus, corneal toxicity, corneal permeability, and drug stability when using LLCZ in an ophthalmic solution will need to be examined in the future. Another limitation is that we failed to include several important fungi such as *Curvularia* and *Exerohilum* species in antifungal susceptibility testing. These fungi also should be tested in future studies.

In conclusion, we showed that LLCZ exhibited the strongest antifungal activity among all existing antifungals against a broad-range of filamentous fungi including FSSC, which is the most important cause of fungal keratitis. LLCZ could potentially be a new medical treatment option for fungal keratitis.

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