Original Article

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Therapeutic effects of three trichothecenes in the silkworm infection assay with *Candida albicans*

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Summary **The silkworm infection assay is a useful method for directly evaluating the *in vivo* therapeutic effects of drug candidates. In the present study, 3 known trichothecenes, trichodermin, epiisororidin E, and verrucarin A, were evaluated as antifungal agents in the silkworm-*Candida albicans* assay. Trichodermin and epiisororidin E yielded effective therapeutic effects, while verrucarin A exhibited no efficacy in this assay system. These results strongly suggest that trichodermin and epiisororidin E are the lead compounds for developing a new antifungal agent.**

Keywords: Antifungal, silkworm infection assay, trichothecenes, *Candida albicans*, cytotoxicity

1. Introduction

Antibiotic candidates against pathogenic microorganisms are generally screened using *in vitro* assay systems such as the paper disk method and microdilution method; however, in most cases, they show no therapeutic effects in *in vivo* assays using mammals because the pharmacokinetics, systemic absorption, and stability of candidates are poor in the mammal body. To overcome these issues, an *in vivo*-mimic infection assay using the silkworm has been introduced to screening at the early stage and is expected to be a relevant strategy for the discovery of therapeutically effective candidates (*1*). In this assay, candidate compounds or test samples are injected into pathogenic microorganism-infected silkworm larvae and the survival rate over a few days is then evaluated. In a silkworm infection model with methicillin-resistant *Staphylococcus aureus* (MRSA), nosokomycins (*2-4*), and lysocins in bacterial culture broths were identified as effective *in vivo* anti-MRSA antibiotics (*1*).

In the course of our screening for antifungal antibiotics using the silkworm infection assay with pathogenic *Candida albicans*, three known trichothecenes, trichodermin (**1**) (*5*), epiisororidin E (**2**) (*6*), and verrucarin A (**3**) (*7*) (Figure 1), were isolated from the culture broths of *Trichoderma* sp. FKI-6009 and *Myrothecium* sp. FKI-5047. Compounds **1** and **2** showed therapeutic effects in *Candida*-infected silkworm, whereas **3** was toxic to the silkworm. In the present study, we described the *in vitro* antifungal activities and cytotoxic and therapeutic activities of **1** to **3** in the silkworm assay. The possibility of trichothecenes as antifungal antibiotics was discussed.

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2. Materials and Methods

2.1. *Materials*

Trichoderma sp. FKI-6009, which was isolated from a soil sample collected in Okinawa main Island, Okinawa, Japan, was used to produce trichodermin (**1**). *Myrothecium* sp. FKI-5047, which was isolated from a soil sample collected in Minato-ku, Tokyo, Japan, was used to produce epiisororidin E (**2**) and verrucarin A (**2**). Miconazol was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. *Silkworm*

Fertilized silkworm eggs, *Bombyx mori* (Hu•Yo × Tukuba•Ne), were purchased from Ehime Sansyu (Ehime, Japan).

2.3. *Assay to determine in vitro antifungal activity*

The following fungal strains were used in the *in vitro* assay; *Candida albicans* ATCC90029, *C. glabrata* ATCC90030, *C. parapsilosis* ATCC90018,

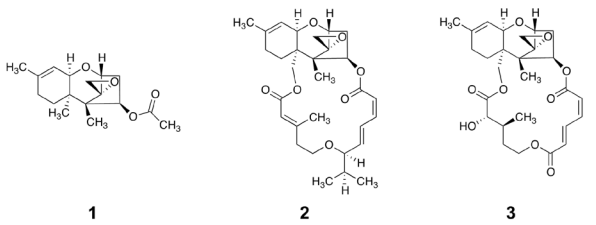

Figure 1. Structures of trichodermin (1), epiisororidin E (2), and verrucarin A (3).

Cryptococcus neoformans ATCC90113, *Aspergillus fumigatus* NBRC33022, *A. flavas* NBRC6343, *A. niger* NBRC105649, *A. terreus* NBRC7078, *Rhizopus oryzae* NBRC4705, *R. microsporus* IFM46417, *R. pusillus* var. *rhizopodiforms* NBRC9744, and *Absidia corymbifera* IFM5335.

Yeast-like fungi including *Candida* spp. and Cryptococcus sp. were subcultured on Potato-dextrose agar (PDA; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) plates at 35°C. Five colonies of ~1 mm in diameter on PDA after a 24-hour incubation at 37°C were suspended in sterile 0.85% saline and the turbidity of the suspension was adjusted at MacFarland 0.5. The suspension was diluted to 1/2,000 in RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA). The minimum inhibitory concentration (MIC) values of **1**, **2**, and **3** against the yeast were determined by the broth dilution assay according to the standard guidelines described in the Clinical and Laboratory Standards Institute (CLSI) documents M27-A3 method (*8*).

Filamentous and Zygomycetous fungi including *Aspergillus* spp., *Rhizopus* spp., and *Absidia* sp. were subcultured on PDA at 35°C until good sporulation was obtained. The sporulating colonies were collected with 1 mL of sterile 0.85% saline, the homogeneous suspension containing spores and conidia was left to stand for 5 min to settle heavy particles, and the upper homogeneous suspension was transferred to a sterile tube. The suspension was adjusted to optical density (OD) at 530 nm, ranging from 0.09 to 0.13 for filamentous fungi and from 0.15 to 0.17 for Zygomycetous fungi. The inocula for the homogeneous suspensions were diluted to 1/50 in RPMI 1640 medium. The MIC values of **1**, **2**, and **3** against filamentous and Zygomycetous fungi were determined by the broth dilution assay according to the standard guidelines described in the CLSI documents M38-A2 method (*9*).

2.4. *Assay for antibacterial activity*

Antibacterial activity against 9 species of bacteria was measured by the paper disk method. Media for microorganisms were as follows: GAM agar (Nissui Seiyaku Co., Tokyo, Japan) for *Bacteroides fragilis*; Waksman agar for *Mycobacterium smegmatis*; Bacto PPLO agar (Becton Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with horse serum 15%, glucose 0.1%, phenol red (5 mg/mL) 0.2%, and agar 1.5% for *Acholeplasma laidlawii*; nutrient agar for *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Xanthomonas oryzae*. A paper disk (i.d. 6 mm, Advantec, Tokyo, Japan) containing 10 µg of a sample was placed on the agar plate. Bacteria, except for *Xanthomonas* oryzae, were incubated at 37°C for 24 hours. *X. oryzae* was incubated at 27°C for 24 hours. Antimicrobial activity was expressed as the diameter (mm) of the inhibitory zone.

2.5. *Silkworm infection assay with C. albicans*

C. albicans TIMM1778 subcultured on a Sabouraud agar plate was inoculated into Sabouraud liquid media and grown for 24 hours at 27°C on a reciprocating shaker at 180 rpm. The culture was diluted in Sabouraud liquid medium at the required concentration (5.0×10^8 cells/mL). The silkworm infection assay with *C. albicans* was carried out by the established method with some modifications (*3*). Hatched silkworm larvae were raised by feeding an artificial diet containing antibiotics (Silk Mate 2S, Nihon Nosan Kogyo, Kanagawa, Japan) in an incubator at 27˚C until the fourth molting stage. On the first day of fifth-instar larvae, silkworms were fed an antibiotic-free artificial diet (Silk Mate, Katakura Industries, Saitama, Japan). On the second day, *C. albicans* (2.5×10^7 cells in 50 µL of Sabouraud liquid medium) was injected into the hemolymph through the dorsal surface of the silkworm using a disposable 1-ml syringe with a 27G needle (TERUMO, Tokyo, Japan). A test compound or sample (in 50 µL of 10% DMSO) was immediately (within one hour of the *C. albicans* injection) injected into the hemolymph, and silkworms were maintained in an incubator at 27˚C. All *C. albicans*-infected silkworm larvae not administered the drug died within 48 hours. When miconazol (50 µg/larva) was injected under these conditions, all silkworms survived and lived after 80 hours.

2.6. *Assay for toxicity to silkworm larva*

A compound (10-100 µg in 50 µL of 10% DMSO) was injected into the hemolymph of silkworm larvae (n = 5), and larvae were maintained in an incubator at 27˚C. The concentration of a compound at which all larvae died within 3 days was defined as a toxic concentration.

2.7. *Assay for cytotoxicity to Chinese Hamster Ovary (CHO)-K1 cells*

Chinese Hamster Ovary (CHO)-K1 cells were cultured in Ham's F-12 (Sigma-Aldrich, St Louis, MO, USA) supplemented with MEM vitamins (Sigma-Aldrich),

geneticin (300 µg/mL, Life Technologies), and 10% heat inactivated FBS (Biowest, Nuaille, France) at 37°C in a humidified atmosphere of 5% CO_2. CHO cells (5.0×10^4 cells/mL) were treated with a compound for 24 to 48 hours. MTT (5.0 µg/mL, Sigma-Aldrich) was added and cells were incubated for approximately 3 hours (*10*). After the cells were lysed with 40% N,N-dimethylformamide, 20% sodium dodecyl sulfate (SDS), 2.0% acetic acid, and 0.030% hydrochloric acid, the absorbance of the solution was read at 550 nm using POWER WAVE X340 (Bio-Tek Instruments, Winooski, VT, USA).

3. Results

3.1. *In vitro antifungal activity.*

The MIC values of **1**, **2**, and **3** against various pathogenic fungi are summarized in Table 1. The MIC value of clinically used miconazol is also shown for comparative purposes. Compounds **1** to **3** exhibited antifungal activities against the yeast-like fungi, and showed moderate activity against some of the filamentous and Zygomycetous fungi. MIC values showed that the antifungal activities of **1** and **3** were similar while **2** exhibited the weakest antifungal activity. However, miconazol exhibited very potent antifungal activity against all the fungi listed in Table 1.

3.2. *Therapeutic effects in the silkworm infection assay with C. albicans*

Compounds **1**, **2**, and **3** were evaluated in the silkworm-*C. albicans* infection assay ($n = 5$). As shown in Figure 2, all infected silkworms not administered a drug (control) died within 48 hours. When **1** and **2** were injected into silkworms under these conditions, they dose-dependently survived. Compound **2** exhibited the most potent efficacy because all silkworms survived 72 hours after the injection at a dose of 50 µg/larva. Compounds **1** and **2** exhibited no toxic effect on silkworm even at 100 µg/larva, whereas **3** was toxic at 100 µg/larva because all silkworms died soon after the injection.

3.3. *Cytotoxicity to CHO-K1 cells*

Cytotoxic effects on the growth of CHO-K1 cells

Table 1. *In vitro* antifungal activity of 1, 2, 3 and miconazol

Items	1	2	3	Miconazol
Yeast-like fungus				
Candida albicans ATCC90029	0.125	1.0	0.125	0.0039
C. glabtata ATCC90030	2.0	16	4.0	0.0078
C. parapsilosis ATCC90018	0.5	4.0	0.125	0.031
Cryptococcus neoformans ATCC90113	2.0	4.0	2.0	0.125
Filamentous fungus				
Aspergillus fumigatas NBRC33022	> 32	> 32	> 32	1.0
A. flavas NBRC6343	> 32	> 32	> 32	2.0
A. niger NBRC105649	4.0	> 32	32	2.0
A. terreus NBRC7078	> 32	> 32	> 32	1.0
Zygomycetous fungus				
Rhizopus oryzae NBRC4705	> 32	> 32	> 32	0.5
R. microsporus IFM46417	> 32	> 32	> 32	2.0
Rhizomucor pusillus NBRC9744	8.0	16	4.0	1.0
Absidia corymbifera IFM5335	8.0	> 32	8.0	4.0

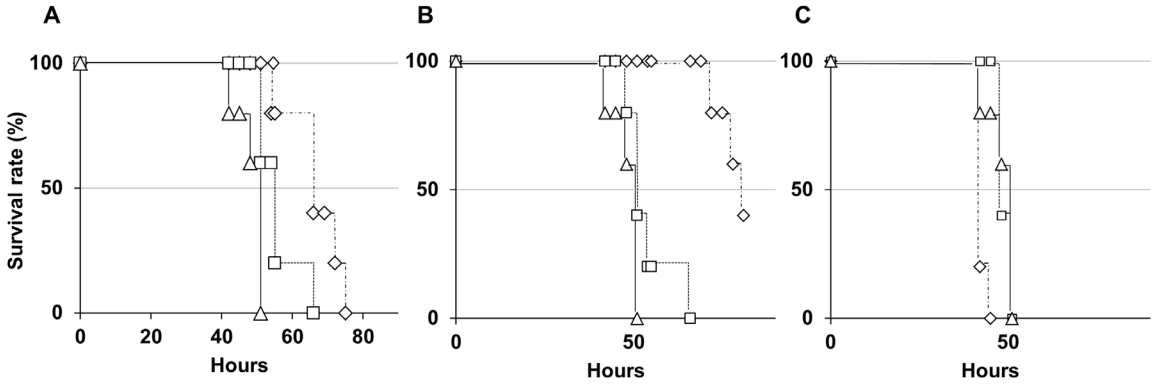

Figure 2. Therapeutic effects of trichodermin (1), epiisororidin E (2), and verrucarin A (3) in the silkworm infection assay with *C. albicans*. (A) 1, (B) 2, (C) 3, ◊: 50, □: 5, Δ: 0 µg/larvae.

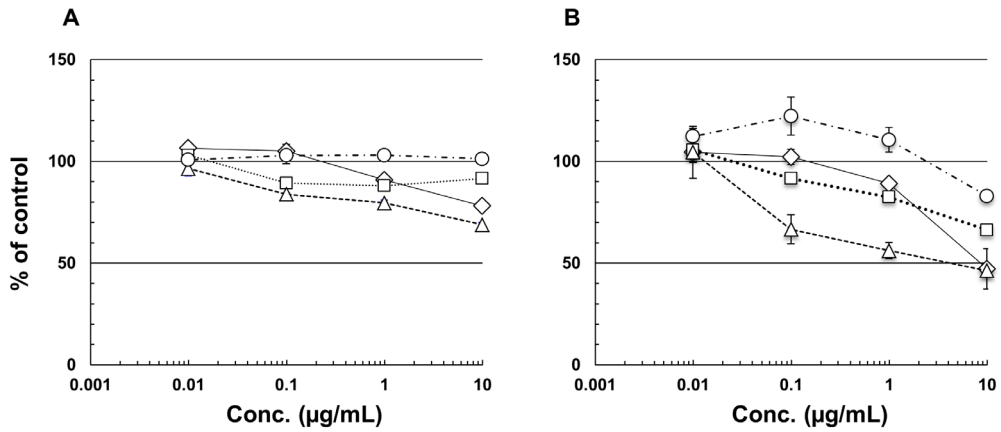

Figure 3. Cytotoxicities of trichodermin (1), epiisororidin E (2), verrucarin A (3), and miconazol (4) in CHO-K1 cells. (A) After a 12-hour incubation. **(B)** After a 24-hour incubation. □◊: **1**, □: **2**, Δ: **3**, ○: **4**.

are shown in Figure 3. After a 12-hour incubation at 10 μg/mL, **1** (22% inhibition), **2** (8%), **3** (31%), and miconazol (0%) exhibited low cytotoxicity. After 24 hours, the cytotoxicities of **1** and **3** were clearly observed with IC_{50} values of 8.8 and 4.2 μg/mL, respectively. However, **2** and miconazol showed low cytotoxicities to CHO-K1 cells (IC_{50}, > 10 μg/mL). These results indicated that **2** showed the lowest cytotoxicity among the three trichothecenes.

3.4. *Antibacterial activity*

The paper disk method revealed that **1** to **3** showed no antibacterial activity at 10 μg/6 mm disk against the 9 bacteria listed in section 2.4.

4. Discussion

In the present study, an *in vivo*-mimic silkworm infection assay with *C. albicans* was performed for the primary screening of antifungal antibiotics, and three trichothecenes, **1** to **3**, were isolated from fungal culture broths. Trichothecenes are generally known as mycotoxins that have toxic effects on eukaryotic cells by inhibiting protein, DNA or RNA synthesis to induce apoptosis (*11,12*). In the present study, the known trichothecenes **1** to **3** exhibited antifungal activities. Although the *in vitro* antifungal activity of **2** was the lowest among the three trichothecenes (Table 1), it exhibited the highest efficacy in the silkworm infection assay with *C. albicans* (Figure 2). These results were similar to those of clinically used fluconazole (FCZ); the *in vitro* antifungal activity of FCZ was previously shown to be less than those of other azoles, but it still exerted beneficial *in vivo* effects because it is metabolically stable and exhibits low plasma protein binding activity (*13*). This may also be the case for the potent therapeutic effects of **2** in silkworm.

The silkworm infection assay may be applied to determining the *in vivo* therapeutic effects and toxicities of candidate compounds. Therefore, trichothecene **2** may be a promising lead for the development of antifungal therapeutics.

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