WF11899A, B AND C, NOVEL ANTIFUNGAL LIPOPEPTIDES

II. BIOLOGICAL PROPERTIES

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WF11899A, B and C, novel water-soluble lipopeptides related to the echinocandins, possess potent anti-*Candida* activities. The IC_{50}s of the compounds against four clinical isolates of *Candida albicans* ranged from 0.004 to 0.03 μg/ml by microbroth dilution assay. These compounds mildly suppressed the growth of *Aspergillus fumigatus* and *A. niger*. WF11899A, B and C showed a potent *in vivo* anti-*Candida* activity. Particularly, WF11899A was superior to cilofungin, and equal to fluconazole. 1,3-β-glucan synthase was inhibited by these compounds at the IC_{50}s of 0.7, 0.7 and 1.8 μg/ml for WF11899A, B and C, respectively. However, they hemolysed mouse red blood cells *in vitro* at the concentration of 62 μg/ml.

WF11899A, B and C are novel echinocandin type of lipopeptide antibiotics produced by *Coleophoma empetri* F-11899[1]. These compounds were structurally characterized by having a sulfate moiety. Although the other related natural lipopeptides lacked water-solubility, WF11899A possessed excellent water-solubility ascribed to the sulfate moiety.

Echinocandin B and aculeacin A, which are representative of natural antifungal lipopeptides, are well known as 1,3-β-glucan synthesis inhibitors[2~4]. The glucan exists in fungal cell wall, not in mammalian cells. It is, therefore, expected to be a suitable target for antifungal agents. These antibiotics indeed showed an excellent anti-*Candida* activity by disturbing fungal cell wall biosynthesis, but they had hemolytic potential[5]. Semi-synthetic cilofungin[6], an echinocandin B derivative having a 4-octyloxybenzoyl side chain, showed the possibility that the hemolytic toxicity can be reduced.

In this paper, WF11899A, B and C were compared with the related lipopeptides for *in vitro* and *in vivo* antifungal activity, inhibitory activity on 1,3-β-glucan synthesis and hemolytic activity.

Materials and Methods

Compounds

Aculeacin A was a generous gift of Toyo Jozo Co., Ltd., Tokyo, Japan. Echinocandin B was isolated from the cultural broth of *Aspergillus nidulans* var. *roseus* A42355 NRRL-11440. Cilofungin was generously provided by Eli Lilly and Company, Indianapolis, Indiana, U.S.A., and further, the compound was semi-synthesized in our laboratory from echinocandin B according to the method described by Debono *et al.*[7] Amphotericin B was obtained from Sigma. Fluconazole was synthesized in the New Drug Research Laboratories of Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan.

In Vitro Antifungal Activity

Microbroth dilution assay: Each inoculum was prepared as followed. The *Candida* cultures were incubated in yeast-maltose (YM) broth for 20 hours at 37°C at the standing condition. The culture of *Cryptococcus neoformans* was grown in YM broth for 20 hours at 30°C with shaking. The cell suspension

was prepared by washing the cultured cells with sterile saline. The filamentous fungi (*Aspergillus* spp.) were cultured on the YM agar slants for seven days. The spores were harvested in sterile saline, and filtered through gauze. Finally, the fungal cells or spores were resuspended in yeast nitrogen base (YNB) containing 0.5% glucose for inoculation.

Antifungal activity was measured in YNB containing 0.5% glucose by microbroth dilution assay using 96-well microtiter plates. The compounds were dissolved in methanol, and diluted serially two-fold with YNB in microplates. The test microorganisms were inoculated to each well to yield 1×10^4 cfu/well in 100 μl. The plates were incubated for 22 hours at 37°C (*Candida* spp. and *Aspergillus* spp.) or 48 hours at 30°C (*C. neoformans*). IC_{50} was determined by measuring turbidity at 600 nm on microplate reader.

Agar dilution assay: Compounds were diluted two-fold in YNB agar containing 0.5% glucose, and their concentrations ranged from 10 to 0.08 μg/ml. The fungal cells or spores suspensions ($1 \times 10^5 \sim 1 \times 10^6$ cfu/ml) in sterile saline were inoculated on the surface of the prepared plate by stamp method. The plate was incubated for 36 hours at 37°C, and then the MICs were determined.

In Vivo Efficacy

The *in vivo* anti-*Candida* activity were evaluated in a mouse model of systemic infection. *C. albicans* FP-633, a clinical isolate in Fujisawa culture collection, was used in this study. The inoculum was prepared from a three day old culture of YM agar slant. ICR mice (female, four weeks old) were intravenously injected with 2×10^6 cells of the yeast. All compounds were solublized in 20% polyethylene glycol 400 saline, and administered subcutaneously one hour after challenge and once a day for three consecutive days.

Glucan Synthase Assay

Enzyme (membrane fraction) preparations and assay procedures were conducted according to the method described by SAWISTOWSKA-SCHRÖDER *et al.*[4] with some modifications. *C. albicans* 6406 kindly supplied from Dr. D. KERRIDGE of University of Cambridge was used in this study. Briefly, the yeast cells were grown to logarithmic phase (absorbance at 660 nm; 0.42) in YNB medium supplemented with 2% glucose at 30°C under shaking. The cells were harvested by centrifugation, washed and suspended in ice-cold buffer A (50 mM Tris-HCl buffer (pH 7.5), 1 mM EDTA, 1 mM β-mercaptoethanol, 1 M sucrose and 25 μM GTP). The cells were broken by mixing with 0.4 mm glass beads on a vortex mixer and the glass beads were washed with ice-cold buffer B (buffer A minus 1 M sucrose), and the cell debris was removed by centrifugation. The supernatant fluids were centrifuged at $100,000 \times g$ for 45 minutes at 4°C. The pellet was washed by ultracentrifugation in buffer B, resuspended in buffer C (buffer B - glycerol, 2:1) at 10 mg protein/ml and stored at −80°C as a source of enzyme.

Measurement of inhibitory activity on glucan synthase was carried out as follows. Two and a half μl of test compound solution or vehicle was incubated with 25 μl of the reaction mixtures (50 mM Tris-HCl buffer (pH 8.0), 0.8% BSA, 0.1 mM GTP, 0.1% CHAPS, 0.05% Tween 80 and the particulate enzyme (40 μg protein)) for 15 minutes at room temperature. After the incubation, 25 μl of UDP-[U-^{14}C]glucose (0.35 μCi/ml, 1 mM) was added to the reaction vessel to react for 60 minutes at room temperature. The reaction was terminated by the addition of 100 μl of ice-cold 5% trichloroacetic acid (TCA) and allowed to stand on the ice. The resultant precipitate was collected on a GF/C glass filter and counted for radiolabel activity in toluene scintillator.

Hemolytic Activity

Fresh red blood cells prepared from ICR mouse (female, four weeks old) were used in the hemolytic assay. The whole blood cells were collected under the heparinized condition, and washed with saline three times. The resultant red blood cells (RBC) were suspended with saline to yield 2% (v/v) suspension. Fifty μl of RBC suspension was added to 50 μl of the compound solution which were serially two-fold diluted with saline in U-bottom microtiter plates. The plate was incubated for two hours at room temperature with gentle shaking, and then allowed to stand for a while to sediment RBC. The hemolytic activity was determined by visual observation. The minimum lytic concentration (MLC) is defined as the lowest concentration of a compound which lyse red blood cells[8].

Results and Discussion

In Vitro Antifungal Activity

The *in vitro* antifungal activities of WF11899A, B and C were compared with that of aculeacin A using microbroth dilution method (Table 1). These compounds showed a similar anti-yeast spectrum to aculeacin A, that is, highly active against *Candida* spp., but not active against *Cryptococcus neoformans*. WF11899A, B and C exhibited the almost same activity against *Candida albicans*, the IC_{50}s of them against four clinical isolates of *C. albicans* ranged from 0.004 to 0.03 μg/ml. The IC_{50}s of aculeacin A was from 0.008 to 0.06 μg/ml. WF11899A, B and C appeared to be more active against the other three *Candida* spp. than aculeacin A, and were effective also against *Aspergillus* spp. Judging from microscopic observations, these compounds were fungicidal against *Candida* spp., but they were mildly fungistatic against filamentous fungi even at the highest concentration (5 μg/ml), as well as aculeacin A. Echinocandin/aculeacin type of lipopeptides were reported to be antibiotics with narrow spectrum by agar dilution method. Therefore, we compared the MICs by agar dilution method with the IC_{50}s by microbroth dilution method. Table 2 shows the MICs and the IC_{50}s of WF11899A, echinocandin B, cilofungin and amphotericin B. The antifungal spectra of the structurally related lipopeptides, including WF11899A by agar dilution method,

Table 1. *In vitro* antifungal activity of WF11899A, B, C and aculeacin A by microbroth dilution method.

Test organism	IC_{50} (μg/ml)			
	WF11899A	WF11899B	WF11899C	Aculeacin A
Candida albicans FP578	0.008	0.008	0.008	0.008
C. albicans FP582	0.025	0.015	0.03	0.06
C. albicans FP629	0.008	0.004	0.008	0.015
C. albicans FP633	0.025	0.025	0.03	0.06
C. tropicalis YC118	0.025	0.05	0.015	0.31
C. krusei YC109	0.16	0.16	0.16	0.62
C. utilis YC123	0.03	0.003	0.003	0.06
Cryptococcus neoformans YC203	>2.5	>2.5	>2.5	>2.5
Aspergillus fumigatus FD050	1.9	1.6	0.62	2.5
A. niger ATCC9642	0.03	0.03	0.03	2.5

Table 2. Comparisons of the IC_{50}s by microbroth dilution method with the MICs by agar dilution method for WF11899A, echinocandin B, cilofungin and amphotericin B against *Candida* spp. and *Aspergillus* spp.

Test organism	IC_{50} (μg/ml)/MIC (μg/ml)			
	WF11899A	Echinocandin B	Cilofungin	Amphotericin B
Candida albicans FP578	0.025/0.16	0.24/0.31	0.36/0.31	0.39/0.16
C. albicans FP582	0.004/0.16	0.16/0.31	0.24/0.62	0.55/0.31
C. albicans FP629	0.018/0.16	0.24/0.31	0.47/0.31	0.39/0.31
C. albicans FP633	0.018/0.16	0.12/0.31	0.39/0.31	0.39/0.16
C. tropicalis YC118	0.09/<0.08	0.47/0.31	0.94/0.62	0.62/0.16
C. krusei YC109	0.14/0.62	1.1/2.5	3.2/5.0	1.6/1.25
C. utilis YC123	0.03/0.16	0.55/0.31	0.94/1.25	0.47/0.31
Aspergillus fumigatus FD050	1.3/>10	5.0/>10	<0.003/>10	0.62/0.62
A. niger ATCC9642	0.004/>10	5.0/>10	0.08/>10	1.9/0.62

These determinations were measured in YNB containing 0.5% glucose.

Fig. 1. Protective effect of WF11899A, B and C against systemic infection of *C. albicans* FP633.

—— WF11899A, — — WF11899B, --— WF11899C and ------ Vehicle.

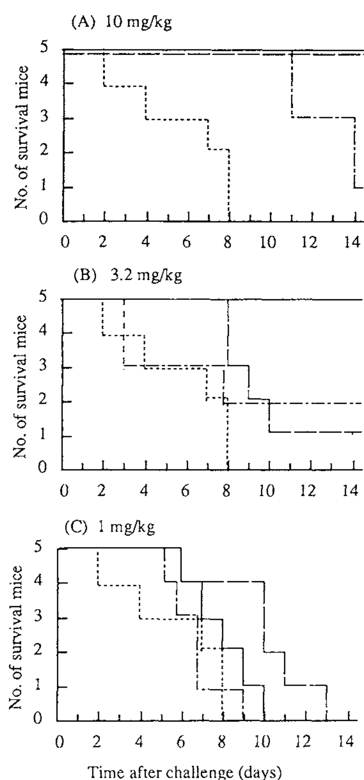

Table 3. *In vivo* anti-*Candida* activity of WF11899A, echinocandin B, cilofungin and fluconazole in a murine systemic model.

Compound	ED_{50} (mg/kg)	
	Day 10[a]	Day 14
WF11899A	1.1	2.9
Echinocandin B	>10.0	>10.0
Cilofungin	5.0	>10.0
Fluconazole	<1.0	2.4

Tested compound was subcutaneously administered to ICR mice (n=8) infected with *C. albicans* FP633.
[a] The day at which all non-treated infected mice died.

Table 4. Inhibitory activity of WF11899A, B, C and the related other lipopeptides on 1,3-β-glucan synthase prepared from *C. albicans* 6406.

Compound	IC_{50} (μg/ml)
WF11899A	0.7
WF11899B	0.7
WF11899C	1.8
Aculeacin A	1.3
Echinocandin B	2.6
Cilofungin	2.9

were consistent with the results previously reported, which showed that the antifungal activity of the lipopeptides limited to *Candida* spp. The MICs of these lipopeptides against *Aspergillus fumigatus* and *A. niger* were >10 μg/ml. However, IC_{50}s (microbroth dilution method) of them were diversified and the range was from <0.003 to 5 μg/ml. By contrast, the IC_{50}s of amphotericin B was nearly identical with its MICs.

In Vivo Activity

The protective efficacy of WF11899A, B and C against murine systemic infection with *C. albicans* was examined (Fig. 1). These compounds significantly prolonged the survival of infected mice. In particular, all mice treated with WF11899A at the dose of 3 mg/kg survived during the observations. The ED_{50}s of WF11899A, B and C at day 14 after challenge were 2.7, 4.6 and >10 mg/kg, respectively. In an additional experiment, the efficacy of WF11899A was compared with those of the other lipopeptides and an azole compound. As shown in Table 3, the result demonstrate that WF11899A is superior to echinocandin B and cilofungin, and as active as fluconazole.

Inhibitory Activity on Glucan Synthase

The mode of action of echinocandin analogs was reported to be inhibition of 1,3-β-glucan synthase. Inhibitory activity of WF11899A, B, C and the related lipopeptides on incorporation of UDP-[^{14}C]glucose into TCA insoluble fractions were examined. The IC_{50}s of them were listed in Table 4. WF11899A, B and C inhibited the glucan synthesis which was catalyzed by the particulate enzyme prepared from *C. albicans*

6406. The potency of WF11899A and B seemed to be slightly higher than those of echinocandin B and cilofungin.

Hemolytic Activity

The hemolytic activities of the lipopeptides and amphotericin B were tested using mouse red blood cells (Table 5). WF11899A, B and C were 8-fold less hemolytic than amphotericin B, but almost the same as aculeacin A and echinocandin B. Cilofungin, the derivative overcoming hemolysis of echinocandin B, was not hemolytic as previously reported by Lilly's researchers[5].

Table 5. Hemolysis by WF11899A, B, C, the related other lipopeptides and amphotericin B.

Compound	MLC[a] (μg/ml)
WF11899A	62
WF11899B	62
WF11899C	62
Aculeacin A	31
Echinocandin B	125
Cilofungin	>500
Amphotericin B	8

[a] Minimum lytic concentrations for mouse red blood cells.

Discussion

The *in vitro* and *in vivo* antifungal activities of WF11899A, B and C were evaluated and compared with those of other antifungal antibiotics. The results indicated that these compounds had a potent anti-*Candida* activity. In particular, WF11899A showed the most efficacy on a murine model of systemic candidiasis. The spectrum of echinocandin analogs was considered to be limited in *Candida* spp., but the growth of *Aspergillus fumigatus* and *A. niger* was mildly suppressed by WF11899A, B and C as well as the other lipopeptide antibiotics at the much lower concentration than their MICs obtained by the conventional agar dilution method. The evaluation of cilofungin indicated that it had anti-*A. fumigatus* activity on a systemic aspergillosis mouse model despite its high MIC[9], and its efficacy was correlated with its inhibitory activity against 1,3-β-glucan synthase prepared from *A. fumigatus*[10]. In our preliminary experiment, WF11899A also showed *in vivo* anti-*A. fumigatus* activity. These observations suggested that it was difficult to evaluate the effect of echinocandin analogs against filamentous fungi by conventional agar dilution method. The microbroth dilution method, by which we can observe mild growth inhibition, seems to be the alternative for the evaluation of echinocandin analogs.

WF11899A, B and C, as well as aculeacin A and echinocandin B, lysed mouse red blood cells *in vitro*. To decrease the hemolytic toxicity, echinocandin B was modified to cilofungin by enzymatic deacylation[11] and chemical reacylation[7]. On the other hand, natural echinocandin analogs, pneumocandins, were reported to be nor or little hemolytic[8,12]. These compounds demonstrated that the antifungal activity was not linked to the hemolytic toxicity, and substitution of the side chain let this class of compounds proceed to further evaluations. The chemical modifications of WF11899A indeed reduced the hemolytic toxicity, keeping its good water-solubility and anti-*Candida* activity. These results will be published elsewhere.

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