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FR901469, a Novel Antifungal Antibiotic from an Unidentified Fungus No.11243

II. *In Vitro* and *In Vivo* Activities

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FR901469 is a water-soluble macrocyclic lipopeptidolactone ($C_{71}H_{116}N_{14}O_{23}$) that has inhibitory activity against 1,3-β-glucan synthase and exhibits *in vitro* and *in vivo* antifungal activity against both *Candida albicans* and *Aspergillus fumigatus*. The MICs of FR901469 against *Candida albicans* FP633 and *Aspergillus fumigatus* FP1305 in a micro-broth dilution test were 0.63 and 0.16 μg/ml, respectively. FR901469 showed excellent efficacy by subcutaneous injection against both *Candida albicans* and *Aspergillus fumigatus* in a murine systemic infection mode, with ED_{50}s of 0.32 and 0.2 mg/kg, respectively. This compound also showed potent anti-*Pneumocystis* activity in the nude mice model with experimental *Pneumocystis* pneumonia. The hemolytic activity of FR901469 towards mouse red blood cells is about 30-fold weaker than that of amphotericin B.

FR901469 that is a non-echinocandin type lipopeptide antibiotic was isolated as a novel antifungal antibiotic from an unidentified fungus No.11243.[1] This compound is a water-soluble macrocyclic lipopeptidolactone ($C_{71}H_{116}N_{14}O_{23}$) and has inhibitory activity against 1,3-β-glucan synthase from *Candida albicans*. This activity was greater than for the echinocandin-like lipopeptides[2~6] and for the glycolipid papulacandin B[7], which are known 1,3-β-glucan synthase inhibitors. Although the natural echinocandin-like lipopeptides, except for WF11899A with a sulfonate moiety, lack appreciable water-solubility, FR901469 showed excellent water-solubility, comparable to WF11899A.

The fungal cell wall is an essential structure to fungi and is not present in mammalian cells[8,9]. As such, it is expected to be a suitable target for antifungal agents and to fulfil the criteria for a safe drug. The cell walls of most fungi contain chitin and β-glucans, and in *Candida albicans*, the cell wall macromolecules consist mainly of 1,3-β-glucan. The echinocandin-like lipopeptides[10~14] and papulacandins[15] indeed displayed excellent anti-*Candida* activity. Similarly, studies on the cell wall composition of *Pneumocystis carinii*, known as the cause of *P. carinii* pneumonia and now classified as a fungus, revealed that the 1,3-β-glucan target is an important component of the cell wall in the cyst form, and it is known that inhibitors of glucan synthesis are lethal to *P. carinii*[7,16,17]. The present report describes the *in vitro* and *in vivo* antifungal activities and hemolytic activity of FR901469.

Materials and Methods

Compounds

Aculeacin A was a generous gift from Asahi Chemical Industry 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. Amphotericin B was purchased from Sigma. Fluconazole was synthesized in the Medicinal Chemistry Research Laboratories of Fujisawa Pharmaceutical Co.,

Ltd., Osaka, Japan.

In Vitro Antifungal Activity

Antifungal activity was measured by microbroth dilution method assay using 96-well titer plates. Each inoculum was prepared as followed. The *Candida* cultures were incubated in yeast-maltose (YM) broth medium for 20 hours at 37°C in a standing condition. The culture of *Cryptococcus neoformans* was grown in YM broth medium 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* sp.) were cultured on YM agar slants for 7 days. The spores were harvested in sterile saline, and filtered through gauze. Finally, the fungal cells or spores were resuspended in yeast nitrogen base-glucose (YNBD) medium for inoculation. Test compounds were dissolved in methanol, and diluted serially two-fold with YNBD. The test microorganisms were inoculated to each well to yield 1×10^4 cfu/well in 100 μl. The plates were incubated for 20 hours at 37°C (*Candida* sp. and *Aspergillus* sp.) or 48 hours at 37°C (*Cryptococcus neoformans*). MIC was determined by microscopic observation.

In Vivo Antifungal Activities of FR901469 against *Candida albicans* and *Aspergillus fumigatus* in Murine Infection Models

The *in vivo* anti-*Candida* and anti-*Aspergillus* activities were evaluated in a murine model of systemic infection. *Candida albicans* FP-633 and *Aspergillus fumigatus* FP1305, both of which are clinical isolates in the Fujisawa culture collection, were used in these studies. For anti-*Candida* activity, 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. For anti-*Aspergillus* activity, the inoculum was prepared from a seven-day old culture of YM agar slant. ICR mice (female, four weeks old), administered cyclophosphamide (200 mg/kg) intraperitoneally for 4 days before challenge, were intravenously injected with 1×10^6 spores of the fungi. Five mice were used in each group. Test compounds were dissolved in 20% polyethylene glycol 400/saline, 10% HCO-60/saline or saline in each experiment, and administered subcutaneously one hour after challenge and once a day for three consecutive days. The ED_{50} was determined at day 14.

Effect of FR901469 against *Pneumocystis carinii* in the Nude Mice Model

BALB/c athymic nude mice (female, four weeks old), maintained in vinyl isolators placed in P2 facilities, were inoculated intranasally with 1×10^4 cysts of *Pneumocystis carinii* under anethesia. Four months later, prior to the start of drug treatment, some of the infected mice were randomly chosen and examined for the number of cysts in the lungs. FR901469 was dissolved in saline, and administered subcutaneously once a day for fourteen consecutive days. A 10% lung homogenate in phosphate-buffered saline (pH 7.2) was made by using a glass homogenizer after the lung specimen was weighed, and 25 μl of the homogenate was smeared onto a slide glass. The total number of cysts on the smear was counted by microscopic observation after staining with toluidine blue O (TBO) and were expressed as the numbers of cysts per lung.

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 heparinized conditions, and washed with saline three times. The resultant red blood cells (RBC) were suspended with saline to yield a 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 settle RBC. The hemolytic activity was determined by visual observation. The minimum lytic concentration (MLC) is defined as the lowest concentration at which the compounds lyse red blood cells.

Statistical Analysis

For statistical analysis, the Student's t test was used. P values of <0.05 were considered significant.

Results

In Vitro Antifungal Activity

Table 1 shows the antifungal spectrum of FR901469 and other reference antifungal agents against various yeast-like and filamentous fungi. FR901469 displays a broad spectrum and has potent activity against a variety of fungal species. FR901469 was more active than WF11899A and FLCZ against most *Candida* species, and all *Aspergillus*. However, FR901469 was inactive against *Cryptococcus neoformans*.

Table 1. *In vitro* antifungal activities of FR901469 and other compounds by microbroth dilution method.

Test organism	MIC (μg/ml)[a]			
	FR901469	WF11899A	Echinocandin B	Amphotericin B
Candida albicans FP633	0.10	0.31	2.5	1.25
(serum)	0.10	1.56	3.13	0.39
C. albicans FP629	0.63	0.31	2.5	1.25
C. krusei YC109	0.63	0.63	5.0	5.0
C. utilis YC123	0.63	0.63	2.5	1.25
C. tropicalis IFO-0006	0.31	0.63	2.5	1.25
C. parapsilosis OUT-6016	0.20	0.05	0.78	0.25
Cryptococcus neoformans YC203	>50	>50	>50	2.5
Aspergillus fumigatus FP1305	0.16	0.31	1.25	2.5
(serum)	0.16	0.16	6.25	0.39
Aspergillus fumigatus 8004	0.08	0.16	0.63	0.31
A. fumigatus FP163	0.005	0.08	0.63	0.16
A. niger ATCC9642	0.63	0.005	5.0	0.31

Micro-broth dilution test in YNBD medium at 37°C for 20 hours except for *Cryptococcus neoformans* for 48 hours.

[a]MIC values (μg/ml) were determined using broth (YNBD) microdilution assay.

Table 2. *In vivo* antifungal activities of FR901469 and other compounds against *Candida albicans* in murine infection model.

	Compound	ED_{50} (mg/kg) [a]
Experiment 1[b]	FR901469	0.32
	WF11899A	2.7
	Aculeacin Aγ	6.4
	Fluconazole	4.5
Experiment 2 [c]	FR901469	0.63
	Echinocandin B	>32

[a] ED50 was calculated based on the survival rate at 14 days after infection.

[b] Vehicle: 20% PEG

[c] Vehicle: 10% HCO-60

In vivo Antifungal Activities of FR901469 against *Candida albicans* and *Aspergillus fumigatus* in Murine Infection Models

The protective efficacy of FR901469 administered subcutaneously against murine systemic infection with *C. albicans* was examined. The ED_{50}s of FR901469 in experiment 1 and experiment 2 at day 14 after challenge were 0.32 mg/kg and 0.63 mg/kg, respectively. As shown in Table 2, FR901469 was superior to the echinocandin-like lipopeptides and fluconazole in this model. Survival curves for the *in vivo* candidiasis model (experiment 2) are shown in Fig. 1.

The protective efficacy of FR901469 subcutaneously

Fig. 1. Antifungal activities of FR901469 and echinocandin B against *Candida albicans* in murine infection model.

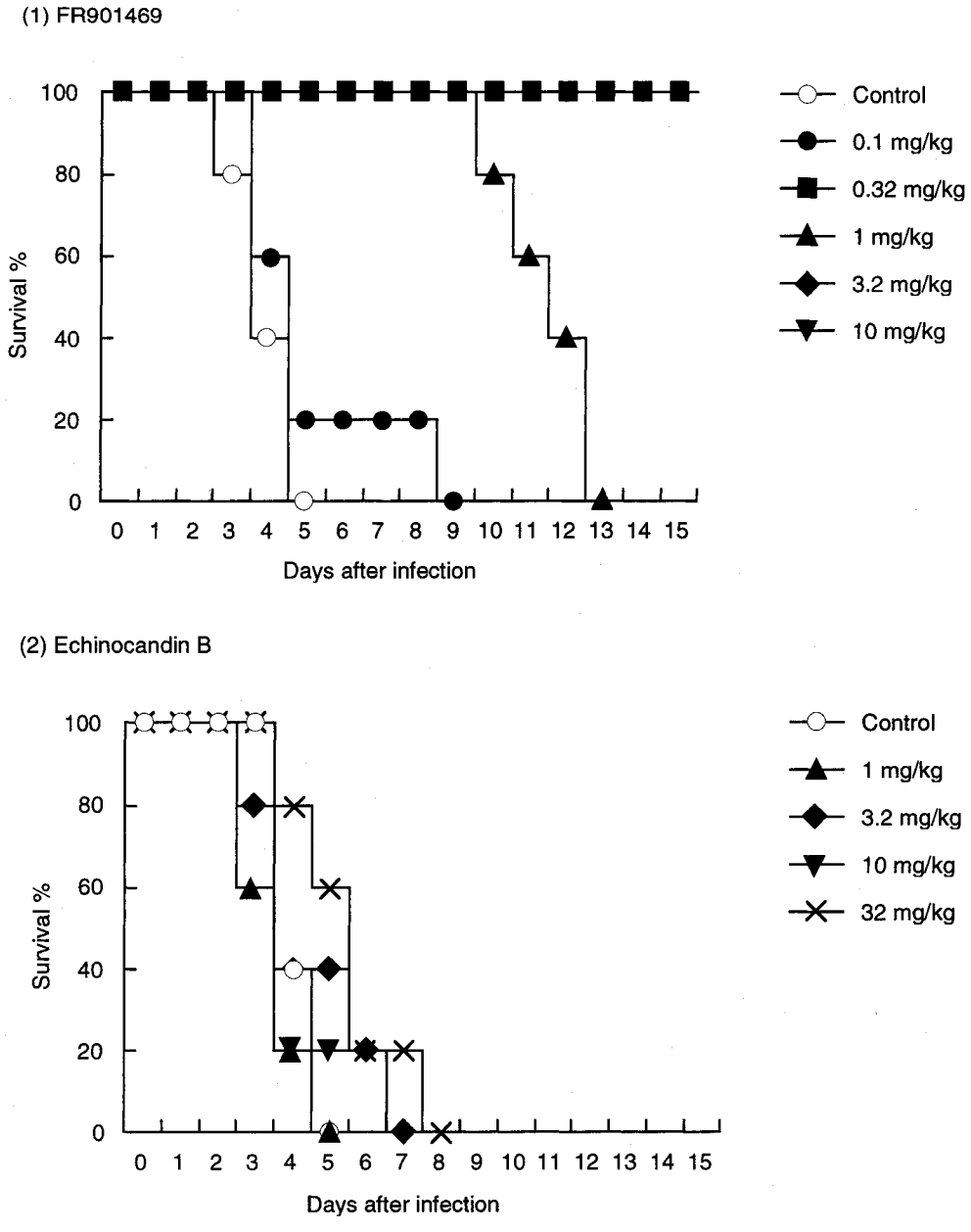


Table 3. *In vivo* antifungal activity of FR901469 and other compounds against *Aspergillus fumigatus* in murine infection model.

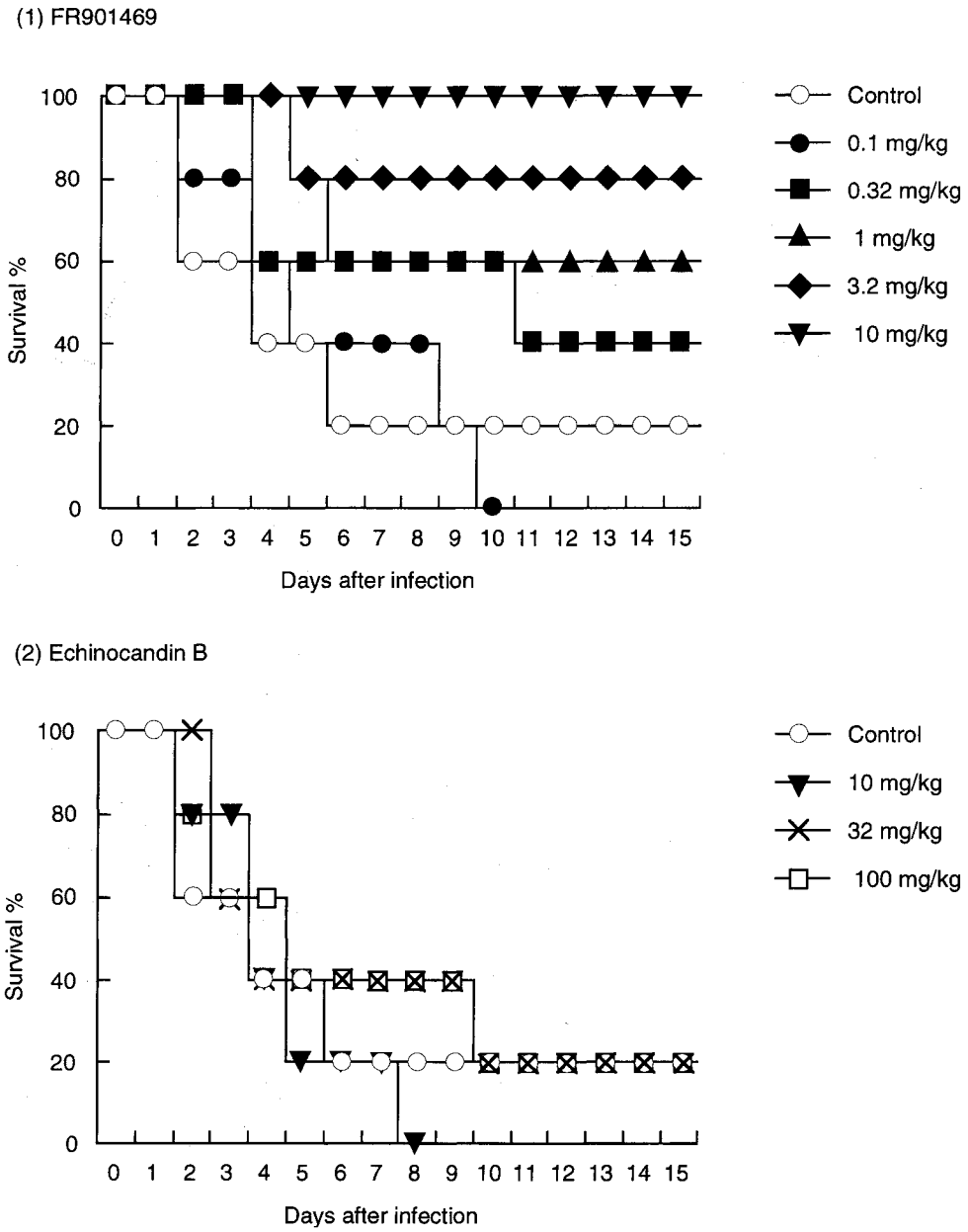
	Compound	ED_{50} (mg/kg) [a]
Experiment 1 [b]	FR901469	0.5
	WF11899A	>32
Experiment 2 [c]	FR901469	0.52
	Echinocandin B	>100

[a] ED50 was calculated based on the survival rate at 14 days after infection.

[b] Vehicle: 20% PEG

[c] Vehicle: 10% HCO-60

Fig. 2. Antifungal activities of FR901469 and echinocandin B against *Aspergillus fumigatus* in murine infection model.



administered against murine systemic infection with *A. fumigatus* was also examined and compared with those of WF11899A and echinocandin B (Table 3). The ED_{50}s of FR901469 in experiment 1 and experiment 2 at day 14 after challenge were 0.5 mg/kg and 0.52 mg/kg, respectively. The efficacy of FR901469 was superior to those of WF11899A and echinocandin B. These two lipopeptide compounds were inactive in this model at doses of 32 mg/kg and 100 mg/kg, respectively. Survival curves for the *in vivo* aspergillosis model (experiment 2) are shown in Fig. 2.

Effect of FR901469 against *Pneumocystis carinii* in the Nude Mice Model

FR901469 was administered to *P. carinii*-infected nude mice to examine its efficacy on the growth of *P. carinii* cysts in the lungs. As shown in Table 4, the number of *P.*

Table 4. Effect of FR901469 on *Pneumocystis carinii*-infected nude mice.

	No. of cysts/lung (log_{10})
Before treatment (4 months after infection)	6.3
14 days after treatment	
control	6.31±0.01
FR901469 10mg/kg	2.46 ± 0.36 [a]

a) Significantly different from the values for the saline-treated control group ($p < 0.01$)

carinii cysts in the lungs of infected mice was very high at the start of drug administration. At 4 weeks after administration, a significant therapeutic effect was observed with a daily dose of 10 mg/kg.

Hemolytic Activity

The hemolytic activities of FR901469 and other antifungal agents were tested using mouse red blood cells. As shown in Table 5, FR901469 was about 30-fold less hemolytic than amphotericin B, but almost the same as echinocandin B.

Table 5. Hemolytic activity of FR901469 and other compounds.

compound	MLC (μg/ml)[a]
FR901469	250
Echinocandin B	125
Amphotericin B	8

[a] Minimum lytic concentrations for mouse red blood cells

Discussion

In this study, the *in vitro* and *in vivo* antifungal activities of FR901469 were evaluated and compared with those of other antifungal antibiotics. The results of *in vitro* study indicated that FR901469 has a favorable spectrum of antifungal activity, especially against the clinically more prevalent fungi such as *Candida* and *Aspergillus* spp. This activity was superior to those of echinocandin-like lipopeptides and amphotericin B. Furthermore, an outstanding feature of FR901469 was the good activity in serum against the strains of *Candida albicans* and *Aspergillus fumigatus*. Both FR901469 and the echinocandin-like compounds were ineffective against *Cryptococcus neoformans*. This result suggests that poor penetration or access of the compound to the target and the metabolic state of the yeast in broth culture may be related to relative resistance[18].

FR901469 showed good efficacy in systemic infection models in mice infected by clinically relevant *C. albicans* and *A. fumigatus*, in correlation with its potent activity against these organisms. In particular, the ED_{50} value for FR901469 was over 10 times lower than fluconazole in the mouse model of systemic candidiasis. This result suggests that FR901469 may have advantages over fluconazole in the therapy of life-threatening fungal infections in immunocompromised patients. In the immunosuppressed aspergillosis model, FR901469 was much more active than WF11899A and echinocandin B. FR901469 is also effective in decreasing the numbers of *P. carinii* organisms in the lungs of infected nude mice. Echinocandin-like lipopeptides have been reported to be active against the cysts of *P. carinii* pneumonia[7,9,11,16,17]. In our experiments, it seems that the efficacy of FR901469 against cysts in *P. carinii* pneumonia is almost as potent as WF11899A (data not shown). These results indicate that FR901469 may be a potent parenterally administered therapeutic agent for systemic candidiasis and aspergillosis, and *P. carinii* pneumonia.

The hemolytic activity of FR901469 towards mouse red blood cells was about 30-fold weaker than that of amphotericin B. Furthermore, the data indicated that the hemolytic activity of FR901469 is almost the same as that of echinocandin B. In the case of echinocandin-like lipopeptides, it has been demonstrated that the antifungal

activity is not linked to the hemolytic activity[19~21]. In studies of derivatives of WF11899A[22~25], chemical modification of the side chain of WF11899A indeed reduced the hemolytic toxicity, maintaining good water-solubility and improving anti-fungal activity. Therefore, if the acyl side chain of FR901469 is modified, there is the possibility that a FR901469-derived agent would be a more effective and safer antifungal agent.

In conclusion, the results of this study suggest that FR901469 is a promising compound for further evaluation as a new antifungal candidate.

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