Note

CILOFUNGIN INHIBITION OF (1-3)-β-GLUCAN SYNTHASE: THE LIPOPHILIC SIDE CHAIN IS ESSENTIAL FOR INHIBITION OF ENZYME ACTIVITY

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Echinocandin B, aculeacin A and cilofungin (*n*-*p*-octyloxybenzoyl echinocandin B nucleus) are similar cyclopeptide antifungal antibiotics which differ in their long-chain fatty acids[1~7]. Each has been reported to interfere with cell-wall biosynthesis and to inhibit (1-3)-β-glucan synthase activity of some yeasts and a few filamentous fungi[5,8~21]. In this paper, we examine the nucleus and fatty acid components of cilofungin through *in vitro* susceptibility tests and *in vitro* inhibition of glucan synthase of *Neurospora crassa* and *Candida albicans*. We report that the components of cilofungin alone do not exert antifungal or anti-glucan synthase activity. In addition, we show that echinocandin B, aculeacin A, and cilofungin differ in their abilities to inhibit glucan synthase activity *in vitro*.

UDP-[^{14}C]Glucose (250 mCi/mmol) was purchased from ICN; cilofungin, echinocandin B nucleus and *p*-octyloxybenzoic acid were obtained from Dr. C. J. FOUTS-JOHNSON of Eli Lilly and Company, Indianapolis, Indiana, U.S.A. Aculeacin A and echinocandin B were generous gifts of Toyo Jozo Co., Ltd., Tokyo, Japan, and Sandoz, Ltd., Basel, Switzerland, respectively. All enzymes and other chemicals were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Distilled-deionized water was used throughout.

To determine the IC_{50} of various antifungal compounds, suspensions of *N. crassa* wild-type (74-OR8-1a) macroconidia were prepared in VOGEL's medium N[22] plus sucrose 1.5% (w/v) from 5~7 days (25°C) slant cultures of agar-solidified VOGEL's medium N plus sucrose 1.5% (w/v). Suspensions were filtered through sterile cotton to exclude hyphal fragments and preconidial chains and inoculated (1×10^4 cells/ml) into VOGEL's medium N plus sucrose 1.5% (w/v). Suspensions (100 μl) were aliquoted into sterile, round bottom 96-well microtiter plates (Corning). In addition, slant cultures of *C. albicans* (B366, ATCC 56884), grown on Bacto-peptone (Difco) 1%, sucrose 4% (w/v) and agar 1.5% (w/v) for 5~7 days at 37°C, were flooded with 5 ml of Bacto-peptone 1% (w/v), sucrose 4% (w/v) and cells resuspended by mixing. The cell concentration of suspensions was adjusted to 1×10^4 cells/ml. Suspensions (100 μl) were aliquoted into sterile, round bottom 96-well microtiter plates (Corning). Cilofungin, echinocandin B nucleus (nucleus), and *p*-octyloxybenzoic acid (fatty acid) were dissolved in 50% DMSO to a final concentration of 80, 500 and 75 μg/ml, respectively. One hundred μl of each solution was added to the first well of each horizontal row, then serially diluted 1 : 2 to the eleventh well. The twelfth well served as a positive control[10]. Microtiter plates were read by a Bio-Rad Microplate Manager (Bio-Rad Data Analysis Software with mode 2550 Microplate Reader, Richmond, CA, U.S.A.) at A_{600}, after 6 days incubation (37°C) for *N. crassa*, and after 2 days incubation (37°C) for *C. albicans*.

(1-3)-β-D-Glucan synthase (EC 2.4.1.34; UDP-Glucose: 1,3-β-D-glucan 3-β-glucose transferase) activity of *N. crassa* wild-type protoplast lysates was determined as previously described[16,23,24]. Briefly, protoplasts of wild-type were obtained by treating 16-hour germinated macroconidia with Novozym 234 for 30 minutes at 25°C. Resulting protoplasts were stored frozen (−70°C) until used. Frozen cell pellets were lysed in GEF buffer (HEPES 25 mM, glycerol 0.1 M, sodium fluoride 10 mM, phenylmethylsulfonylfluoride 1 mM, and EDTA 5 mM, pH 7.4) containing GTP 10 μM. (1-3)-β-D-Glucan synthase activity was assayed in reaction mixtures (26 μl) containing α-amylase (Sigma Type II A) 50 μg, GTP 10 μM, various concentrations (0.4~1.2 mM) of UDP-[^{14}C]glucose (30,000~40,000 cpm/assay), and various concentrations of inhibitors (dissolved in 50% DMSO). Incubations were started by the addition of crude cell lysate to ice-cold reaction mixtures and after incubation at 25°C, reactions were terminated by the addition of 50 μl 5% (w/v) TCA. The incorporation of radioactive glucose into (1-3)-β-D-glucan was determined using

the Millipore filter method described by GOODAY and DE ROUSSET-HALL[25].

(1-3)-β-D-Glucan synthase activity of *C. albicans* protoplast lysates was determined as previously described[19,26,27]. Briefly, cultures, stored at −20°C on corn meal agar (Difco Laboratories, Detroit, Michigan), were thawed by the addition of 5 ml pre-warmed (37°C) PYG (Bacto-peptone (Difco) 1% (w/v), yeast extract 0.3% (w/v), glucose 2% (w/v)). Pre-warmed PYG was inoculated with 1×10^5 cells per ml (final concentration) and incubated for 16~18 hours at 37°C with orbital shaking (140 rpm). Resulting cells were used to inoculate pre-warmed PYG medium at an initial A_{600} of 0.1. Cultures were incubated for 5 hours at 37°C with shaking (140 rpm). Resulting cells were harvested by centrifugation (10,000 × *g*, 5 minutes, 4°C) and cell pellets were suspended in the pretreatment medium of MIRAGAL *et al.*[27] (dithiothreitol 50 mM, EDTA 5 mM, pH 9, 0.5 mg/ml Pronase), and incubated at 25°C for 30 minutes with shaking (140 rpm). Cells were harvested by centrifugation (10,000 × *g*, 5 minutes, 4°C) and washed once with ice-cold 0.6 M KCl by centrifugation. Protoplasts from pretreated cells were released by resuspending cell pellets in 0.6 M KCl containing 10 mg/ml Novozym 234 and incubating at 25°C for 30 minutes with shaking (140 rpm). Protoplasts were harvested by centrifugation (1,500 × *g*, 5 minutes, 4°C), washed twice by centrifugation with ice-cold 1.2 M sorbitol, and cell pellets frozen in dry ice. Cell pellets were stored at −70°C until used. Frozen cell pellets were suspended in buffer A (Tris 50 mM (pH 7.5), EDTA 1 mM, dithiothreitol 1 mM, NaF 10 mM, GTP 100 μM) and lysed by mixing with 0.5 mm glass beads. Resulting crude lysates were mixed with an equal volume of buffer A containing sucrose 2 M. Glucan synthase activity was assayed in reaction mixtures (26 μl) containing α-amylase (Sigma Type IIA) 50 μg, UDP-[^{14}C]glucose (80,000 cpm/assay) 2 mM, crude cell lysate and either cilofungin 30 μM, nucleus 300 μM, or fatty acid 150 μM (dissolved in 50% DMSO). Reactions were started by the addition of cell protein to ice-cold reaction mixtures, incubated at 25°C, and were terminated by the addition of 50 μl 5% (w/v) TCA. The incorporation of radioactive glucose into (1-3)-β-D-glucan was determined by using the Millipore filter method of GOODAY and DE ROUSSET-HALL[25].

The effects of cilofungin and its components on *N. crassa* and *C. albicans* growth were determined and the results presented in Table 1 show that the

Table 1. The effects of cilofungin and its components on *Neurospora crassa* and *Candida albicans* growth[a].

	IC_{50} (μg/ml)	
	N. crassa	*C. albicans*
Cilofungin	0.31	0.078
Nucleus	>31.3	>31.3
Fatty acid	> 4.7[b]	> 4.7
Both nucleus and fatty acid	>15.6 > 4.7	>15.6 > 4.7

[a] The *C. albicans* and *N. crassa* IC_{50} determinations were as described in the text. IC_{50} for *C. albicans* and *N. crassa* is highest dilution resulting in 50% inhibition of growth.

[b] Highest concentration of the fatty acid tested.

IC_{50} for cilofungin was 0.31 μg/ml (*N. crassa*) and 0.078 μg/ml (*C. albicans*), in agreement with previous results[10]. In sharp contrast, neither the nucleus nor the fatty acid alone or in combination showed significant antifungal activity. These results clearly indicate that the intact cilofungin molecule is necessary for inhibition of fungal growth.

We also tested the effect of cilofungin and its components on (1-3)-β-glucan synthase activity of *N. crassa* and *C. albicans*. The results presented in Table 2 show that only intact cilofungin showed significant inhibition of glucan synthase activity. As was found for inhibition of fungal growth, neither the nucleus, nor the fatty acid, nor the two together had significant effect on enzyme activity. Thus, for inhibition of fungal growth and for inhibition of glucan synthase activity, the echinocandin B nucleus and the long chain fatty acid must be covalently bonded.

The effects of aculeacin A, echinocandin B, cilofungin, and its components on glucan synthase activity were tested more rigorously. The results in Table 3 show that the Ki_{app} for cilofungin was 7.5 ± 0.5 μM while that for the components were at least 100 times greater. Interestingly, the Ki_{app} for cilofungin was lower than that of echinocandin B and aculeacin A. In addition, plots of 1/velocity vs. 1/substrate for each inhibitor best fit noncompetitive inhibition (nomenclature of CLELAND[28]), (data not shown). These drugs share the same echinocandin B nucleus, but have different fatty acid side chains (see Fig. 1 and refs 2, 3, 6~8, 10 and 29).

Our results show that for antifungal and anti-glucan synthase activity, the echinocandin B nucleus must be covalently bonded to a fatty acid side chain. Neither the nucleus nor the fatty acid

Table 2. The effects of cilofungin and its components on (1-3)-β-D-glucan synthase activity[a].

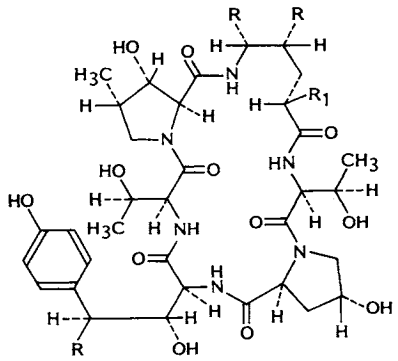
Addition	*Candida albicans*		*Neurospora crassa*	
	Velocity	Inhibition (%)	Velocity	Inhibition (%)
None	0.100±0.013	0	0.527±0.021	0
Cilofungin	0.005±0.002	95	0.133±0.004	75
Nucleus	0.095±0.016	4	0.438±0.024	17
Fatty acid	0.078±0.006	21	0.503±0.033	5
Both (none)[b]	0.073±0.013	0	0.468±0.037	0
Both[b]	0.057±0.006	22	0.378±0.025	19

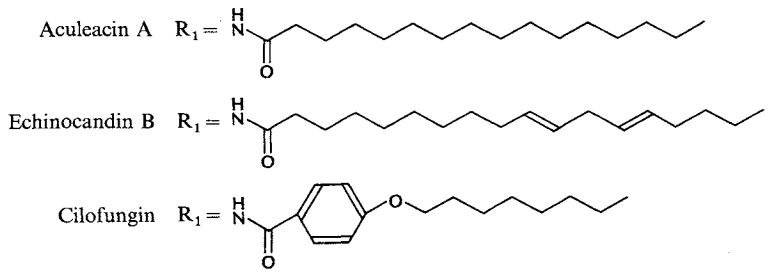
[a] Protoplasts of *C. albicans* and *N. crassa* were isolated, lysed and (1-3)-β-glucan synthase activity assayed as described in the text. Reaction mixtures contained UDP-[^{14}C]glucose (*N. crassa*: 1.2 mM, 30,000 cpm/assay; *C. albicans*: 2 mM, 60,000 cpm/assay), 150 μg cell protein, and the following concentrations of inhibitors: Cilofungin 30 μM, nucleus 300 μM, fatty acid 150 μM, both nucleus 150 μM and fatty acid 150 μM. Reaction mixtures were incubated at 25°C for 0, 5, and 10 minutes for *C. albicans* and 0, 3, and 6 minutes for *N. crassa* and were terminated by the addition of 50 μl 5% (w/v) TCA. The incorporation of radioactive glucose into (1-3)-β-D-glucan and velocities (nmol glucose per minute) were determined as described. ±: standard deviation, N: 4 data points.

[b] Reaction mixtures contained 4 μl 50% DMSO (control: "none") or 2 μl each of nucleus and fatty acid.

Fig. 1. Structures of aculeacin A, echinocandin B, and cilofungin.

R=OH.





These compounds contain the same echinocandin B nucleus but differ in their long chain fatty acids, R_1 (aculeacin A, palmitic acid; echinocandin B, linoleic acid; cilofungin, *p*-octyloxybenzoic acid).

chain component has antifungal or anti-glucan synthase activity. In addition, our results clearly show that the structure of the fatty acid chain is an important determinant in the potency of anti-glucan synthase activity. We suggest that analogues of cilofungin, altered in the fatty acid portion of the molecule, may be even more potent inhibitors of glucan synthase and, hence, fungal growth.

Table 3. Inhibition of *Neurospora crassa* (1-3)-β-glucan synthase activity by cilofungin, echinocandin B nucleus, *p*-octyloxybenzoic acid, echinocandin B and aculeacin A[a].

	Ki_{app} (μM)
Aculeacin A	48 ± 5
Echinocandin B	13 ± 2
Cilofungin	7.5 ± 0.5
Nucleus	1,405 ±350
Fatty acid	1,859 ±818
Both	671 ± 87

[a] Protoplasts of *N. crassa* were isolated, lysed and (1-3)-β-glucan synthase activity was assayed as described in the text. Reaction mixtures contained 0.4, 0.8 or 1.2 mM UDP-[^{14}C]glucose (30,000 cpm/assay), 150 μg cell protein, and 2 μl of 50% DMSO containing the following concentrations of inhibitors: Cilofungin, 0, 15 and 30 μM; nucleus, 0, 150 and 300 μM; fatty acid, 0, 30 and 150 μM; echinocandin B, 0, 7.5 and 15 μM; aculeacin A, 0, 15 and 30 μM. Reaction mixtures were incubated at 25°C for 0, 3, and 6 minutes, and the amounts of glucan formed determined as described. Data were processed and Ki_{app} calculated using the EZ-FIT computer program[30]. ±: standard deviation, N: 20 data points.

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