LY121019 INHIBITS *NEUROSPORA CRASSA* GROWTH AND (1-3)-β-D-GLUCAN SYNTHASE

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Aculeacin A and echinocandin B are cyclopeptide antibiotics with a long-chain fatty acid (palmitic acid and linoleic acid, respectively[1~4]) that are active against some yeasts and a few filamentous fungi[4~9]. Both aculeacin A and echinocandin B have been shown to affect cell-wall synthesis[5,8,10~12]. Under *in vitro* conditions, aculeacin A and echinocandin B inhibit (1-3)-β-glucan synthase activity: Aculeacin A – noncompetitive inhibitor, *Geotrichum lactis*, Ki_{app} 60 μM[13]; *Neurospora crassa*, Ki_{app} 12 μM (C. S. TAFT; unpublished results); echinocandin B – noncompetitive inhibitor, *Candida albicans*, Ki_{app} could not be determined[14]; uncompetitive inhibitor, *N. crassa*, Ki_{app} 12 μM[15]. These results have led to the attractive hypothesis that aculeacin A and echinocandin B exert their antifungal activity *in vivo* by inhibiting (1-3)-β-D-glucan synthase (EC 2.4.1.34; UDP-Glucose: 1,3-β-D-glucan 3-β-glucose transferase), thus resulting in osmotically sensitive cells.

LY121019 is a semisynthetic, novel analog of echinocandin B that has *in vitro* and *in vivo* activity against *C. albicans*[16]. Preliminary results have shown that LY121019 affects cell-wall synthesis (R. S. GORDEE *et al.*; personal communication). Given the structural similarity between LY121019 and echinocandin B, it seemed likely that LY121019 would inhibit (1-3)-β-D-glucan synthase activity *in vitro*. In this paper, we report that, indeed, LY121019 is a noncompetitive inhibitor of *N. crassa* (1-3)-β-D-glucan synthase activity with a Ki_{app} of 16 μM.

Materials and Methods

Chemicals

UDP-[^{14}C]Glucose (250 mCi/mmol) was purchased from ICN; LY121019 was obtained from Dr. C. J. FOUTS-JOHNSON of Eli Lilly and Company, Indianapolis, Indiana, U.S.A. Papulacandin B, aculeacin A and echinocandin B were generous gifts of Ciba-Geigy Limited, Basel, Switzerland, Toyo Jozo Co., Ltd., Tokyo, Japan, and Sandoz, Ltd., Basel, Switzerland, respectively. All other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and distilled-deionized water was used throughout.

Growth of *N. crassa*

Wild-type (OR-748-1a; Fungal Genetics Stock Center No. 988) and protoplasts of *os-1* (NM233t); *nic-1* (S1614), a, (referred to as *os-1*) were grown and maintained as previously described[17]. Growth inhibition experiments were performed as described previously[18]. Briefly, for wild-type, macroconidial suspensions were obtained from 5~7 days (25°C) slant cultures of agar-solidified VOGEL's medium N[19] plus 1.5% sucrose. Suspensions were filtered through sterile cotton, to exclude hyphal fragments and preconidial chains, and added (final concentration, 1×10^4 conidia/ml) to molten (45°C) VOGEL's medium N containing sorbitol 7.5%, sucrose 1.5% (SS medium) and agar 1.2%. Seeded medium was added to sterile petri dishes (25 ml per 150× 10 mm dish) and allowed to cool at room temperature. Sterile filter-paper disks (0.6 cm) containing various concentrations of inhibitors were gently placed on the agar surface and plates incubated right-side up at 25°C. For *os-1*, protoplasts were obtained from exponentially growing cultures (in SS medium containing 10 μg/ml nicotinamide) at 37°C[18]. Cell suspensions were filtered through sterile glass wool (to exclude cell aggregates) and cells added to molten (45°C) SS medium containing 10 μg/ml nicotinamide and 1.2% agar. Disks containing inhibitors were gently placed on the agar surface (after cooling) and resulting plates were incubated right-side up at 25°C to permit protoplast growth and regeneration.

(1-3)-β-D-Glucan Synthase *In Vitro* Assay

(1-3)-β-D-Glucan synthase activity of wild-type protoplast lysates was determined as previously described[17]. Briefly, protoplasts of wild-type were obtained by treating 16-hour germinated macroconidia with Novozym 234 as described by QUIGLEY *et al.*[20]. Protoplasts were stored frozen (−70°C) until used. Frozen cell pellets were lysed in solubilization buffer (GEF (HEPES 25 mM, glycerol 0.1 M, sodium fluoride

10 mM, phenylmethylsulfonylfluoride 1 mM, EDTA 5 mM, pH 7.4) containing phosphate (monobasic) 200 mM, dithiothreitol 1 mM, glycerol 0.5 M and GTP 10 μM, pH 7.4) at a protein concentration of 6~10 mg/ml. (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 of UDP-[^{14}C]glucose (~50,000 cpm) various concentrations of inhibitors (dissolved in 50% DMSO). Incubations were started by the addition of cell protein (90~150 μg) to ice-cold reaction mixtures, and after incubation at 25°C for 0, 1.5 and 3 minutes, reactions were stopped by the addition of 50 μl 5% 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[21].

Other Procedures

Resulting kinetic data were processed[15] using the ROSFIT computer program described by GRECO *et al.*[22]. Photographs were taken using a Zeiss Axiophot microscope with Tri-X pan film. Contact prints of petri dishes were made using Kodabromide F-5 paper. Protein content of cell lysates was determined using the method of BRADFORD[23].

Results and Discussion

LY121019 Inhibits *N. crassa* Growth

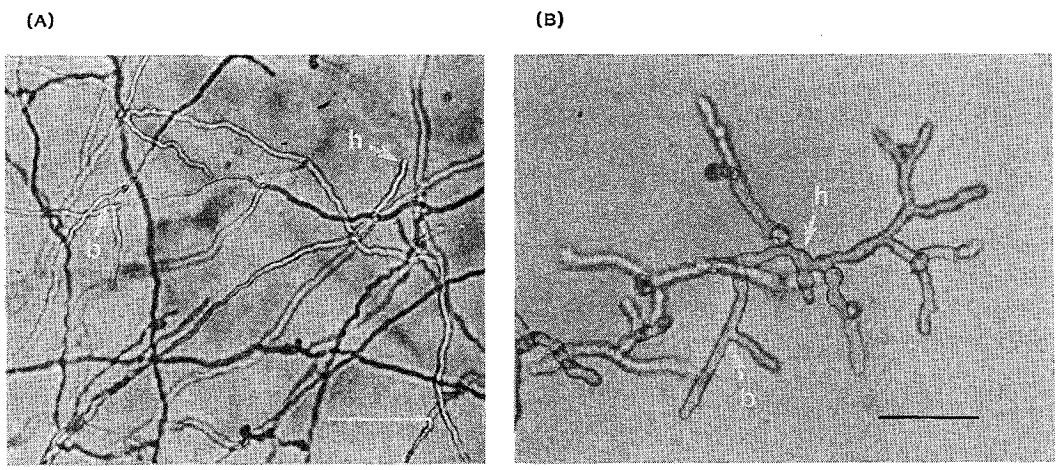
Echinocandin B, aculeacin A, papulacandin B

Table 1. Effect of various antifungal antibiotics on the growth of wild-type *Neurospora crassa* and *os-l*[a].

Compound	Concentration (μg/disk)	Zone diameter (mm)	
		Wild-type	*os-l*
DMSO	10	0	0
	15	0	0
LY121019	20	30	30
	40	32	34
	80	36	40
Echinocandin B	20	23	26
	40	24	24
	80	28	30
Papulacandin B	16	25	22
	32	25	24
	96	27	28
Aculeacin A	16	16	15
	32	20	20
	96	22	26

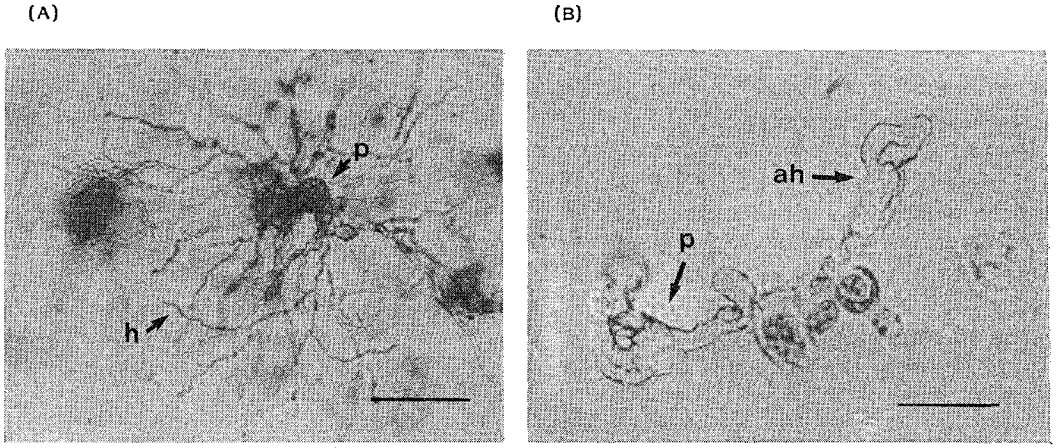
[a] Agar-solidified medium containing wild-type macroconidia and *os-l* protoplasts were prepared as described in Materials and Methods and disks containing the indicated amount of the above compounds were placed on the surface. Zones of inhibition diameters were measured after 24~36 hours incubation at 25°C.

Fig. 1. Effect of LY121019 on *Neurospora crassa* wild-type growth.



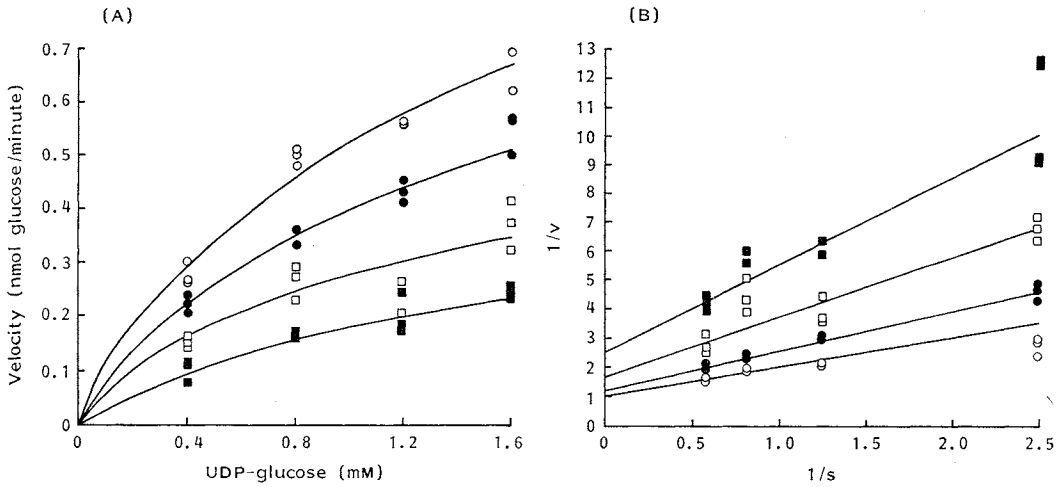
Photomicrographs of untreated wild-type hyphae (A) and within the zone of inhibition by LY121019 (B). Note hyphae (h) and branch point (b). Bar in A represents 150 μm while bar in B represents 100 μm.

Fig. 2. Effect of LY121019 on regeneration *os-1* protoplasts.



Photomicrographs of regenerating *os-1* protoplasts untreated (A) and within the zone of inhibition by LY121019 (B). Note primary protoplast (p), hyphae (h) and abnormal hyphae (ah). Bar in A represents 200 μm while bar in B is 40 μm.

Fig. 3. Effect of LY121019 on (1-3)-β-glucan synthase activity.



Protoplasts were isolated, lysed and (1-3)-β-glucan synthase activity assayed as described in Materials and Methods. Reaction mixtures contained the indicated UDP-[^{14}C]glucose concentrations (50,000 cpm/assay), 90 μg cell protein, and the following concentrations of LY121019; (A): ○ 0 μm, ● 5 μm, □ 15 μm, ■ 30 μM, (B): double reciprocal replot of the data of A.

and LY121019 are reported to inhibit a number of yeasts and a few filamentous fungi[4,5,7~12,16,24]. To determine the effects of these compounds on *N. crassa*, both germinating macroconidia of wild-type and regenerating protoplasts of *os-1* were embedded in agar and disks containing these drugs placed on the surface. These results are summarized in Table 1, and show that all four antifungal compounds inhibit *N. crassa* (wild-type and *os-1*). When cells within zones of inhibition were examined by light microscopy, they were found to have altered hyphal morphologies: Fig. 1A shows untreated wild-type; note the long, sparsely branched hyphae. Fig. 1B shows an LY121019-inhibited wild-type colony; note that the hyphae are irregularly shaped and that branching is greater than in untreated controls. Regenerating protoplasts of *os-1* were also inhibited by LY121019 (Fig. 2). Note the aberrant hyphae that regenerated within

the zone of inhibition. Clearly, LY121019 inhibits *N. crassa* growth resulting in morphologically abnormal hyphae.

LY121019 Inhibits *N. crassa* (1-3)-β-D-Glucan Synthase Activity

The effect of LY121019 on (1-3)-β-D-glucan synthase activity present in crude lysates of wild-type protoplasts is shown in Fig. 3A; the double reciprocal replot is presented in Fig. 3B. A plot of 1/v intercept vs. [I] and 1/slope vs. [I] were linear (not shown) and revealed a Ki_{app} of 16± 1.1 μM. These data indicate that LY121019 is a noncompetitive inhibitor of (1-3)-β-D-glucan synthase activity.

Taken together, our data are consistent with the idea that LY121019 inhibits fungal growth by inhibiting (1-3)-β-D-glucan synthase *in vivo*. Definitive proof of this hypothesis awaits the isolation of LY121019-resistant mutants and the demonstration that (1-3)-β-D-glucan synthase is resistant to inhibition by LY121019 *in vitro*. A search for these mutants is presently underway.

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