L-671,329, A NEW ANTIFUNGAL AGENT

III. *IN VITRO* ACTIVITY, TOXICITY AND EFFICACY IN COMPARISON TO ACULEACIN

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L-671,329 is a novel, echinocandin-like natural product that possesses potent anti-*Candida* activity, including activity against *Candida parapsilosis*. The *in vitro* MICs of L-671,329 were comparable to aculeacin against 18 yeasts and three filamentous fungi in an agar dilution assay. L-671,329 lysed mouse red blood cells (RBCs) at a concentration of 400 μg/ml, but not at 50 or 12.5 μg/ml. Aculeacin lysed RBCs at 400 and 50 μg/ml.

L-671,329 significantly prolonged survival of mice infected with *Candida albicans* (ED_{50} 3.38 mg/kg) following twice-daily intraperitoneal dosing for five consecutive days. The prolongation observed was greater than that seen with aculeacin therapy (ED_{50} 6.44 mg/kg). No acute or chronic toxicities of L-671,329 or aculeacin (as measured by mortality) were detected at a concentration of 100 mg/kg following intraperitoneal administration (TD_{50} >100 mg/kg). Both L-671,329 and aculeacin eradicated cells of *C. albicans* from the kidneys of infected mice. L-671,329 eradicated the yeast at therapeutic concentrations of 12.5 to 100 mg/kg. Aculeacin eradicated yeast cells at therapy concentrations of 25 to 100 mg/kg. L-671,329 has potential as an anti-*Candida* compound.

L-671,329, a novel, echinocandin-like natural product produced by fungal isolate ATCC 20868, was isolated and purified by scientists at the Merck Sharp & Dohme Research Laboratories, Rahway, N.J., and Madrid, Spain. The fermentation and isolation[1] and structure[2] were reported in the preceding papers.

In the present study, L-671,329 was compared to other compounds for spectrum and potency of *in vitro* antifungal activity, red blood cell lysis, toxicity and efficacy in experimental murine candidiasis.

Materials and Methods

Agar Dilution Assay

L-671,329 and aculeacin were solubilized in 10% aqueous DMSO and diluted 2-fold in sterile distilled water. Nystatin (Mycostatin, Calbiochem, San Diego, CA) was solubilized in 10% aqueous dimethylformamide and diluted 2-fold with sterile distilled water. Each diluted drug was added to cooled, molten yeast nitrogen base plus glucose agar (1.0 ml of drug plus 9.0 ml agar). Appropriate solvent and media controls (drug free) also were prepared. Prepared plates were stored in the dark at room temperature overnight prior to use. Drug concentrations tested ranged from 128~0.06 μg/ml.

All fungi used in the assay were from the Merck Culture Collection. The yeast cultures, maintained in yeast-maltose (YM) broth, were transferred to fresh YM medium and incubated overnight at 35°C with shaking (250 rpm). After incubation, each culture was diluted in sterile saline to yield final concentrations of 3×10^5 to 3×10^6 cfu/ml. The filamentous fungi (*Aspergillus* and *Penicillium*) were maintained on potato-glucose agar slants and spore suspensions prepared by washing spores from the agar surface with distilled water. The spore preparations were used as the inocula for the three filamentous fungi tested.

Each prepared plate was inoculated with the test organisms (18 yeasts and three filamentous fungi) using a Denley Multipoint Inoculator (Denley, Sussex, England), which delivers approximately 0.001 ml to the agar surface resulting in an inoculum of from 3×10^2 to 3×10^3 cfu. The plates were incubated at 30°C for 48 hours. The MICs were recorded as the lowest concentration of drug showing no growth or less than 3 cfu/spot.

Red Blood Cell Lysis

A titration/hemolysis assay using fresh blood drawn from CD-1 female mice was used. L-671,329, aculeacin (Toyo Jozo Co., Ltd., Tokyo), amphotericin B (Fungizone, E.R. Squibb & Sons, Inc.) and 5-fluorocytosine (F. Hoffmann-La Roche Inc.) were assayed at concentrations ranging from 0.39~400 μg/ml. The assay was incubated at 25°C for 2 hours after which the tubes were examined visually for complete or partial hemolysis as compared to a drug free control. The minimum lytic concentration (MLC) was defined as the lowest drug concentration that lysed the red blood cells.

Drug Preparation (Toxicity and Efficacy)

L-671,329 and aculeacin were solubilized in 10% DMSO and diluted 2-fold in sterile distilled water to yield concentrations ranging from 4.0 mg/ml to 0.125 mg/ml; these concentrations yield doses ranging from 100 mg/kg to 3.12 mg/kg when 0.5 ml is administered to a 20-g mouse.

Fungus Used for *In Vivo* Study

Candida albicans MY1055 of the Merck Culture Collection was used. The yeast was grown on Sabouraud dextrose agar (Difco; Detroit, Michigan) plates at 30°C for 24 hours. The yeast was removed from the plates using a sterile loop and suspended in sterile physiological saline. The concentration was adjusted to yield an inoculum (5×10^6 cells/mouse) that resulted in 100% mortality within 14 days after intravenous infection.

Determination of Toxicity

CD-1 female mice (Charles River Breeding Laboratories, Wilmington, MA) weighing 20 g were injected ip twice a day (b.i.d.) for five consecutive days with L-671,329, aculeacin or diluent (10% aqueous DMSO). Non-fatal side effects and mortality were assessed at 24 hours (acute toxicity) and for 14 days after initial injection (chronic toxicity). Mean survival was calculated at the end of the test[3]. Surviving mice were sacrificed using CO_2 gas and the internal organs were examined for changes in gross pathology. Livers were removed, fixed in neutral buffered formalin, mounted in paraffin blocks, sectioned, stained with hematoxylin and eosin, and examined microscopically for evidence of tissue changes.

Determination of *In Vivo* Efficacy

L-671,329 and aculeacin were tested against an experimental systemic infection of *C. albicans* MY1055 (5 mice/group). Within 15 minutes of intravenous infection, the first dose of drug was administered ip. The treatment schedule was identical to that described for determination of toxicity. Control groups included sham-infected, sham-treated, and sham-infected/treated. Mortality was assessed for 14 days after infection. Significance of prolongation of survival was determined using a rank sum analysis of the time-to-death data[3].

Quantitation of *C. albicans* in Kidneys

The surviving mice in each group were sacrificed and paired kidneys were cultured for the presence of *C. albicans*. For each animal assayed, the kidneys were removed aseptically, placed in a sterile Whirl-Pak polyethylene bag to which 5.0 ml sterile saline was added. The organs were homogenized in the Whirl-Pak bag and aliquots diluted[4]. The titrated samples were spread on Sabouraud dextrose agar plates, incubated at 30°C for 48~72 hours, and the cfu determined. The number of cfu per g of paired kidneys was calculated.

Results and Discussion

In Vitro Activity: Agar Dilution Assay

The *in vitro* MICs of L-671,329, aculeacin and nystatin obtained against a panel of 18 yeasts and

Table 1. Antifungal activity of L-671,329 determined by an *in vitro* agar dilution assay using yeast nitrogen base glucose agar.

Fungus	MIC (μg/ml)		
	L-671,329	Aculeacin	Nystatin
Cryptococcus neoformans MY1051	>128.0	>128.0	1.0
C. neoformans MY1146	>128.0	>128.0	1.0
Candida albicans MY1058	0.25	0.125	2.0
C. albicans MY1055	0.50	0.25	2.0
C. albicans MY0992	0.50	0.25	2.0
C. albicans MY1013	≤0.06	0.125	2.0
C. albicans MY1029	>128.0[a]	>128.0[a]	2.0
C. parapsilosis MY1009	8.0	4.0	4.0
C. parapsilosis MY1010	8.0	8.0	2.0
C. tropicalis MY1011	0.25	0.125	2.0
C. tropicalis MY1012	128.0	8.0	>128.0
C. pseudotropicalis MY1040	1.0	2.0	1.0
C. krusei MY1020	2.0	0.5	4.0
C. rugosa MY1022	32.0	32.0	4.0
C. guilliermondii MY1019	>128.0	>128.0	4.0
C. stellatoidea MY1017	0.25	0.25	2.0
Torulopsis glabrata MY1059	1.0	0.25	1.0
Saccharomyces cerevisiae MY1027	8.0	2.0	1.0
Aspergillus fumigatus MF4839	>128.0	>128.0	2.0
A. flavus MF0383	>128.0	>128.0	2.0
Penicillium italicum MF2819	>128.0	>128.0	1.0

[a] MIC of >128 μg/ml may be the result of 'Eagle effect'. See text for explanation.

three filamentous fungi are listed in Table 1. The *in vitro* spectrum of both L-671,329 and aculeacin was limited to *C. albicans* and *Candida* species. The MICs against four isolates of *C. albicans* ranged from ≤0.06~0.50 and 0.125~0.25 μg/ml for L-671,329 and aculeacin, respectively (Table 1). The MIC for one isolate of *C. albicans* (MY1029) was >128 μg/ml for both L-671,329 and aculeacin; this high MIC represents a phenomenon known as the 'Eagle effect'[5]; inhibition of the organism at lower drug concentrations, but only reduced growth at higher drug concentrations. L-671,329 also has good activity against isolates of *Candida parapsilosis*, demonstrating a broader spectrum of activity than a structurally related compound under development by Eli Lilly and Company[6]. The MICs against two isolates of *Cryptococcus neoformans* and three filamentous fungi of medical importance all were >128 μg/ml.

Red Blood Cell Lysis

The MLCs of the four drugs tested are listed in Table 2. L-671,329 was 8-fold less lytic than aculeacin, and 32-fold less lytic than amphotericin B. The data suggest that L-671,329 has lower hemolytic potential than aculeacin.

Toxicity

No acute or chronic toxicities, as measured by mortality, were obtained with L-671,329 or aculeacin at a maximum concentration of 100 mg/kg. No side effects following injections were noted. At necropsy, livers were swollen from mice treated with L-671,329 at concentrations of 50 and 100 mg/kg. Livers were swollen from mice treated with aculeacin at concentrations of 25, 50 and 100 mg/kg. However, no significant changes in the histology of the affected livers were observed.

Table 2. Minimum lytic concentrations (MLCs) of L-671,329, aculeacin, amphotericin B and 5-fluorocytosine.

Drug	MLC (μg/ml)[a]
L-671,329	400
Aculeacin	50
Amphotericin B	12.5
5-Fluorocytosine	>400

[a] MLC was defined as the lowest concentration of drug that lysed red blood cells.

In Vivo Efficacy

At doses of 12.5~100 mg/kg, L-671,329 significantly prolonged the survival of mice infected with *C. albicans* MY1055 (Table 3). The compound also was effective in reducing the number of yeast cells recovered from the kidneys of infected mice (Table 4). All infected mice that were treated with L-671,329 at concentrations ranging from 6.25~100 mg/kg survived. The effective dose 50% (ED_{50}) of L-671,329 was 3.38 mg/kg (Table 3).

Aculeacin also prolonged survival and was effective in reducing the number of yeast cells recovered from the kidneys of infected mice (Table 4). All infected mice that were treated with aculeacin at concentrations ranging from 12.5~100 mg/kg survived. The ED_{50} of aculeacin was 6.44 mg/kg (Table 3).

Table 3. Mean survival of mice receiving either L-671,329 or aculeacin (b.i.d. for 5 days, ip) following infection with *Candida albicans* MY1055 (efficacy).

Drug concentration (mg/kg)	Mean survival in days[a]	
	L-671,329	Aculeacin
100	>14	>14
50	>14	>14
25	>14	>14
12.5	>14	>14
6.25	>14	10.0
3.12	10.8	7.6
ED_{50} (mg/kg)	3.38	6.44
TD_{50} (mg/kg)	>100	>100

[a] Mean survival value of >14 means no mortality at 14 days after infection.
TD_{50}: Toxic dose 50%.

Table 4. Clearance of *Candida albicans* from kidney homogenates of mice treated with L-671,329 or aculeacin (b.i.d. for 5 days, ip).

Drug concentration (mg/kg)	No. mice clear of *Candida*[a]/ total mice infected	
	L-671,329	Aculeacin
100	4/5	5/5
50	4/5	4/5
25	5/5	4/5
12.5	3/5	0/5
6.25	1/5	0/5
3.12	0/5	0/5

[a] Cells of *C. albicans* were not isolated from paired kidneys. The limit of detection of the assay is 50 colonies per paired kidneys.

L-671,329 was superior to aculeacin in reducing the number of yeast cells recovered from sampled kidneys. Aculeacin failed to clear the kidneys of yeast cells at drug concentrations below 25 mg/kg; however, L-671,329 cleared kidneys in 3/5 mice at 12.5 mg/kg and in 1/5 mice at 6.25 mg/kg (Table 4).

Thus, L-671,329 significantly prolonged survival of mice infected with an isolate of *C. albicans* (ED_{50} 3.38 mg/kg b.i.d. for 5 days), and eliminated yeast cells from the kidneys of infected/treated mice. Although aculeacin also prolonged life and eliminated yeast cells from the kidneys of infected/treated mice, it was generally less active than L-671,329.

L-671,329 also was 8-fold less hemolytic than aculeacin and 32-fold less hemolytic than amphotericin B, and no significant toxicity as measured by mortality was observed. In addition, good correlation between *in vitro* susceptibility of the *C. albicans* MY1055 and *in vivo* efficacy of L-671,329 was observed, *i.e.*, an MIC 0.50 μg/ml *in vitro* using YNBDA (Table 1) and an ED_{50} 3.38 mg/kg (Table 3). L-671,329 has potential as an anti-*Candida* compound.

References

1) SCHWARTZ, R. E.; R. A. GIACOBBE, J. A. BLAND & R. L. MONAGHAN: L-671,329, a new antifungal agent. I. Fermentation and isolation. J. Antibiotics 42: 163~167, 1989
2) WICHMANN, C. F.; J. M. LIESCH & R. E. SCHWARTZ: L-671,329, a new antifungal agent. II. Structure determination. J. Antibiotics 42: 168~173, 1989
3) KNUDSEN, L. F. & J. M. CURTIS: The use of the angular transformation in biological assays. J. Am. Stat. Assoc. 42: 282~296, 1947
4) WALSH, T. J.; C. MCENTEE & D. M. DIXON: Tissue homogenization with sterile reinforced polyethylene bags for quantitative culture of *Candida albicans*. J. Clin. Microbiol. 25: 931~932, 1987
5) EAGLE, H. & A. D. MUSSELMAN: The rate of bacterial action of penicillin *in vitro* as a function of its concentration, and its paradoxically reduced activity at high concentrations against certain organisms. J. Exp. Med. 88: 99~131, 1948
6) GORDEE, R. S.; D. J. ZECKNER, L. F. ELLIS, A. L. THAKKAR & L. C. HOWARD: *In vitro* and *in vivo* anti-*Candida* activity and toxicology of LY121019. J. Antibiotics 37: 1054~1065, 1984