A9145, A NEW ADENINE-CONTAINING ANTIFUNGAL ANTIBIOTIC

II. BIOLOGICAL ACTIVITY

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A9145 is a new adenine-containing antifungal antibiotic. A9145 showed weak *in vitro* activity against *Candida albicans* in SABOURAUD's medium, but was strongly inhibitory in a chemically defined medium. Viability counts of *C. albicans* following extended exposure to A9145 indicated a fungistatic action. Following a 100 mg/kg dose, a peak of 64 μg/ml of A9145 activity was detected in mouse blood. Administration of 10 mg/kg for 3 days extended the survival time of mice infected with *C. albicans* 128 %; equivalent doses of amphotericin B extended survival time 112 %. A total dose of 31.25 mg/kg administered over a 5-day period reduced by 400-fold the number of *Candida* recovered from kidney homogenates of infected mice. A combination of amphotericin B and A9145 caused a possible synergistic effect against *C. albicans in vitro* and an additive effect *in vivo*.

Polyene antibiotics have been useful chemotherapeutic agents for deep-seated mycoses. Because of toxicity, polyene antibiotics, such as amphotericin B, are not ideal antifungal agents[1]. Non-polyenic compounds such as a polypeptide antibiotic, saramycetin, and 5-fluorocytosine are promising antifungal agents. Saramycetin is active against *Histoplasma capsulatum* and 5-fluorocytosine is active against *Candida albicans* and *Cryptococcus neoformans*[2,3]. A new non-polyenic compound, clotrimidazole (bis-phenyl-(2-chlorophenyl)-1-imidazolyl-methane), has been reported to be effective against *C. albicans* by oral administration[4]. The continuing discovery of compounds of diverse chemical types with useful antifungal activity is encouraging.

A new adenine-containing nucleoside antibiotic, A9145, has been discovered and isolated[5]. The *in vitro* and *in vivo* activity of A9145 against pathogenic fungi is described in this report.

Materials and Methods

In Vitro Studies. All cultures were maintained on SABOURAUD's agar as modified by EMMONS[6]. The minimal inhibitory concentration (MIC) of A9145 was determined using the broth-dilution technique described previously[7]. Cultures of *Histoplasma capsulatum* 26 and *Blastomyces dermatitidis* B6059 were incubated at 37°C while all other cultures were maintained at 30°C. Susceptibility studies on *Candida* species were also performed on filter-sterilized Yeast nitrogen base (Difco) supplemented with dextrose (10 g/liter) and asparagine (1.5 g/liter). The fungistatic effect of A9145 against *C. albicans* was determined by inoculating SABOURAUD's or Yeast nitrogen base broth containing A9145 with inocula differing in concentration by one log. Viable cell counts were performed by plating techniques described previously[8]. The antifungal activity of A9145 in blood was determined by a disc diffusion assay[9]. *Saccharomyces pastorianus*, the assay organism, permitted the detection of 1 μg of activity per ml.

In Vivo Studies. *In vivo* evaluation of A9145 in mice infected intravenously with 1.5×10^6 *C. albicans* A-26, 2.5×10^6 *Cryptococcus neoformans* WS-34, 5×10^5 *B. dermatitidis* B-6059, and 1.5×10^6 *H. capsulatum* 26 was performed as described previously[10,11].

Mice were X-irradiated with 400 r 24 hours prior to infection which was administered in the lateral tail vein with 0.1 ml cell suspension. Antifungal agents were suspended in 0.125 % methyl-

cellulose (Dow Chemical Company) and administered in 0.25 ml volumes to 6 infected mice and 2 uninfected toxicity control animals. All treatments were administered subcutaneously, unless ortherwise noted, at 0 and 2-hours post-infection on the first day of treatment; subsequent treatments on successive days were at 2 hour intervals. The average survival time of treated and untreated animals was compared after 7 days. All animals surviving at 7 days post-infection were arbitrarily given an average survival time of 8 days. The significance of A9145 treatments was determined by the *t* test and P values. An indication of the virulence of *C. albicans* A-26 in each experimental infection was obtained by determining the number of LD_{50}'s[12]. All doses of A9145 administered in this study did not cause death of uninfected toxicity control animals.

The effect of A9145 on the recovery of *Candida* cells from kidney homogenates was performed in unirradiated mice intravenously infected with 10^5 viable cells[18]. After 5 days treatment with A9145, kidney homogenates were plated on SABOURAUD's agar, incubated at 30°C for 48 hours, and the number of cells recovered per gram of kidney determined[7].

Results

In SABOURAUD's broth, A9145 did not show potent antifungal activity against a number of pathogenic fungi (Table 1). In a defined medium, 1.56~3.12 μg of A9145 per ml were inhibitory to *C. albicans*; other *Candida* species were less susceptible (Table 2). The growth of all *Candida* isolates obtained in a defined medium was comparable to that obtained in SABOURAUD's medium. Attempts to identify, by reversal studies, the factor in the complex medium responsible for reduction of A9145 activity have not been successful. Exposure to 1 mg/ml of A9145 in SABOURAUD's broth produced little change in the viable cell count of *C. albicans* inocula compared to growth of controls in antibiotic-free medium (Fig. 1). A similar fungistatic effect was achieved with 10 or 25 μg/ml of A9145 in Yeast nitrogen base broth (Fig. 2). Following 3 and 4 days exposure to A9145 in the defined medium, 25 μg/ml showed a greater reduction in viable counts than 10 μg of the antibiotic.

The MIC of amphotericin B or A9145 caused a greater than 1 log reduction in the viable cell count of *C. albicans* (Table 3). A combination of 0.312 μg amphotericin B per ml with 0.312 μg or 0.078 μg A9145 per ml resulted in a greater than 2 log reduction of viable cells compared to that obtained at the MIC for each antibiotic alone.

A peak of 64 μg of blood activity was observed 15 minutes after a single subcutaneous 100 mg/kg dose of A9145 (Fig. 3). At 90 minutes, approximately 1 μg of A9145 activity was found. A9145

Table 1. *In vitro* antifungal activity of A9145 in SABOURAUD's medium

Organism	Broth dilution MIC (μg/ml)
Candida albicans A-26	>200
C. pseudotropicalis CDCJB	>200
C. tropicalis SBH-65	>200
C. parapsilosis CDCJB	>200
C. quilliermondii CDCJB	>200
Trichophyton rubrum 67-643	>200
T. mentagrophytes #6	>200
Microsporium gypseum 64-350	>200
Sporotrichum schenckii 66-850	>200
Cryptococcus neoformans WS-34	50
Blastomyces dermatitidis B-6059	25
Histoplasma capsulatum #26	100

Table 2. *In vitro* anti-*Candida* activity of A9145 in defined medium*

Organism	Broth dilution MIC (μg/ml)
Candida albicans A-26	1.56
C. albicans A-25	3.12
C. albicans A-23	3.12
C. albicans A-50	3.12
C. albicans A-75	3.12
C. pseudotropicalis CDCJB	12.5
C. tropicalis SBH-65	>200
C. parapsilosis CDCJB	>200
C. quillliermondii CDCJB	>200

* Yeast nitrogen base adjusted to pH 6.6

Fig. 1. Effect of prolonged exposure of varying inocula of *C. albicans* A-26 to 1 mg of A9145 per milliliter in SABOURAUD'S medium

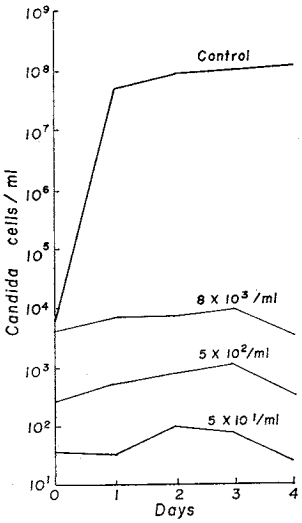

Fig. 2. Effect of prolonged exposure of varying inocula of *C. albicans* A-26 to 10 or 25 μg of A9145 per milliliter in Yeast nitrogen base medium

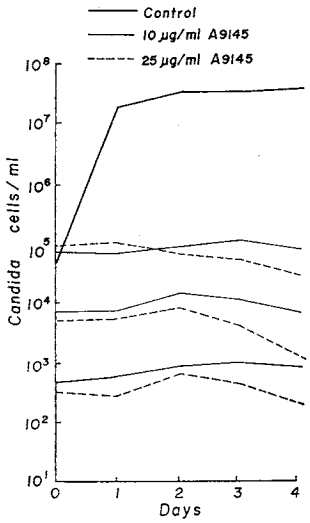

Fig. 3. Antifungal activity of A9145 in mouse blood

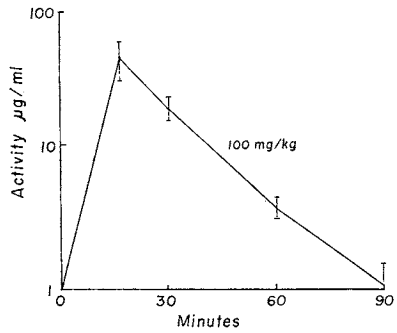

Table 3. Combination of A9145 and amphotericin B against *C. albicans**

Antibiotic combination		Viable cell count (cells/ml)
A9145 (μg/ml)	Amphotericin B (μg/ml)	
0	0	3.6×10^7
2.5**	0	7.0×10^5
0	0.312**	9.0×10^5
0.019***	0.312	1.7×10^6
0.078***	0.312	2.0×10^3
0.312***	0.312	1.4×10^3

* Inoculum=10^3/ml; incubation=30°C for 48 hours
** MIC; lowest antibiotic concentration resulting in no visible growth
*** Exposure of these subinhibitory concentrations did not reduce the viable cell count below controls.

activity was detected in mouse urine and followed the same trend as that seen in blood.

A9145 administered twice daily for 3 days at 40, 20, and 10 mg/kg per day significantly increased the survival time of mice infected with *C. albicans* A-26 (Table 4). Since activity increased with a reduction in dose leves, it would appear that the 40 and 20 mg/kg doses are close to a threshold of toxicity. The 10 mg/kg dose extended survival 124 % beyond controls which was comparable to a 200 mg/kg dose of amphotericin B. Two daily doses of 10, 5, and 2.5 mg/kg per day for 3 days significantly increased survival time; decreasing activity was observed with a decreasing dose of antibiotic (Table 5). The 128 % extension of survival obtained with daily doses of A9145 was comparable to that achieved with amphotericin B. The effectiveness of A9145 by intraperitoneal administration compared with that obtained subcutaneously, while oral doses, though significant, were less effective. A total dose of 40~50 mg/kg of A9145 was ineffective against *C. neoformans*, *B. dermatitidis*, and *H. capsulatum* infected mice.

The recovery of *Candida* cells from kidney homogenates of infected mice was reduced by A9145 (Table 6). Following a total dose of 31.25 mg/kg for 5 days, the number of *Candida* cells recovered was reduced nearly 400-fold from untreated controls. In comparison, amphotericin B at the same level lowered the number of *Candida* cells 100-fold.

The effectiveness of A9145 and amphotericin B administered singly and in combination was

Table 4. *In vivo* activity of A9145 against *C. albicans*

Dose mg/kg twice daily		Average survival time (days ± SE)	Percent increase of average survival time beyond untreated controls
A9145	20 × 3 days	4.0 ± 0.4	38*
	10 × 3 days	5.2 ± 1.0	79*
	5 × 3 days	6.5 ± 0.6	124*
Amphotericin B	100 × 1 day	6.5 ± 1.0	124*
Untreated controls		2.9 ± 0.2	

* $P \leq 0.05$ by "t" test

Table 5. *In vivo* activity of A9145 against *C. albicans*

Dose mg/kg twice daily ×3 days		Total dose mg/kg	Average survival time (days ± SE)	Percent increase of average survival time beyond untreated controls
A9145	5	30	7.3 ± 0.5	128*
	2.5	15	5.3 ± 0.8	66*
	1.25	7.5	4.8 ± 0.8	50*
Amphotericin B	5	30	6.8 ± 1.0	112*
Untreated controls			3.2 ± 0.1	

* $P \leq 0.05$ by "t" test

Table 6. The effect of A9145 on recovery of *Candida* from mouse kidney homogenates

Dose mg/kg twice daily ×5 days		Total dose mg/kg	Viable *Candida* cells recovered per g of kidney
Control	(Saline treated)	0	$3.0\times10^5\pm1.5\times10^5$
A9145	3.12	31.25	$7.9\times10^2\pm1.9\times10^2$
	1.56	15.63	$6.0\times10^4\pm0.9\times10^4$
Amphotericin B	3.12	31.25	3.2×10^8*
	1.56	15.63	$1.1\times10^4\pm0.7\times10^4$

* Determined from a single count

Table 7. Combination of A9145 and amphotericin B against experimental *C. albicans* infections

Doses mg/kg ×no. treatments*		Average survival time (days ± SE)	Percent increase of average survival time beyond untreated controls
A9145	5 × 6	5.0 ± 0.9	61 (P 0.05)
Amphotericin B	12.5 × 1	4.8 ± 1.0	55 (P 0.10)
A9145 plus Amphotericin B	5 × 6 / 12.5 × 1	7.2 ± 0.5	132 (P 0.00005)
Untreated controls		3.1 ± 0.3	

* A9145 was administered twice daily for 3 days; a single dose of amphotericin B was administered.

investigated (Table 7). Multiple doses of A9145 were administered to maintain a high level of activity in blood, while a single dose of the longer lasting amphotericin B was administered. A9145 alone extended survival 61 % beyond controls, while amphotericin B alone extended survival 55 %; the combination of both antibiotics resulted in a 132 % extension of survival time.

Discussion

A9145 showed much greater *in vitro* activity against *C. albicans* in a defined medium than in SABOURAUD's medium. Further study of the structure of A9145 may provide clues for the identity of a factor or factors responsible for diminished antibiotic activity in a complex medium. A fungistatic action was achieved following four days exposure to A9145 with a 40 to 100-fold lower concentration in the defined medium compared to SABOURAUD's medium.

Subinhibitory concentrations of A9145 combined with an inhibitory concentration of amphotericin B resulted in a significant reduction in viable cell count as compared with either antibiotic alone. The greater than 2 log reduction of viable cells suggests a synergistic action between the two antifungal antibiotics.

Low multiple doses of A9145 were most effective against *C. albicans* infected mice. The advantage of this treatment regimen was explained when blood and urine activities were found to peak very soon after injection followed by rapid elimination. Survival time of infected mice treated with A9145 compared favorably with amphotericin B treated controls. Efficacy of this antibiotic was demonstrated in the kidney, the target organ of *Candida* infections. Combination studies with A9145 and amphotericin B doubled the survival of infected mice and indicated the effectiveness of combined therapy for two antifungal antibiotics. Although the spectrum of A9145 antifungal activity appears limited to *C. albicans*, a number of isolates of other pathogenic fungi should be studied for susceptibility to this antibiotic.

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