A32390A, A NEW BIOLOGICALLY ACTIVE METABOLITE

III. *IN VITRO* AND *IN VIVO* ANTIFUNGAL ACTIVITY*

J. R. TURNER, T. F. BUTLER, R. S. GORDEE and A. L. THAKKAR
The Lilly Research Laboratories
Eli Lilly and Company
Indianapolis, Indiana 46206, U.S.A.

(Received for publication October 14, 1977)

A32390A, an isonitrile-containing derivative of mannitol, represents a new class of antifungal antibiotics. *In vitro* antifungal activity of A32390A was found against *Candida albicans, Cryptococcus neoformans* and *Histoplasma capsulatum*. *In vivo* antifungal activity of A32390A was demonstrated in mice infected with *C. albicans*. Accumulative doses of 37.5~600 mg/kg, administered subcutaneously over a 24-hour period, showed significant activity without demonstrating toxicity. A32390A was effective, but not as effective as amphotericin B, in reducing the number of *Candida* cells isolated from the kidney of infected mice. Urinary excretion of A32390A accounted for only 10 percent of the administered dose. Improved bioavailability of A32390A was accomplished when the antibiotic was combined with polyvinylpyrrolidone (PVP) in a solid dispersion. Administration of A32390A as a 10 percent dispersion in PVP resulted in increased urinary excretion of the drug and reduced the amount of drug required for *in vivo* activity.

Chemotherapy of systemic fungal infections and the need for effective, nontoxic treatment of these diseases has been extensively reviewed[1~3]. The overriding conclusion voiced in all of these articles is that few antibiotics are available for use against systemic fungal diseases and those currently marketed produce toxic side effects or are limited because of resistance development or metabolic inactivation.

A number of new compounds have been reported to have significant antifungal activity (A25822B, A9145, saramycetin, miconazole, haloprogin) with by far the largest class of compounds being polyenes. Unfortunately, the polyenes, as a class, cause severe nephrotoxicity when administered parenterally[4]. As yet, no compounds have been described which possess both the broad spectrum observed with the polyene antibiotics and the low toxicity of 5-fluorocytosine.

This report describes the *in vitro* and *in vivo* evaluation of a new compound, A32390A [1,6-di-O-(2-isocyano-3-methylcrotonyl)-D-mannitol], a fermentation product of the genus *Pyrenochaeta*, which suggests the possibility of a new class of antifungal compounds. The isolation, characterization, and structure of A32390A are described in an accompanying report[5]. Other biological activities of A32390A include inhibition of dopamine-β-hydroxylase and reduction of heart and adrenal catecholamine levels[6].

Materials and Methods

In vitro Analysis

Susceptibility of *Candida albicans* and *Trichophyton mentagrophytes* to A32390A was determined

* This report was presented in part at the 15th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, D.C., September, 1975.

by disc diffusion tests on agar plates incubated at 30°C for 24 hours. The agar dilution method was employed for *Cryptococcus neoformans, Blastomyces dermatitidis*, and *Histoplasma capsulatum*, in their yeast phases with incubation at 37°C for 48 hours. Yeast nitrogen base (YNB, Difco) was used to maintain *C. albicans* and SABOURAUD's dextrose agar was used for growth of all other organisms. The effect of A32390A on growth kinetics of *C. albicans* was determined in YNB broth incubated at 30°C on a rotary shaker. Samples of the medium containing A32390A and cells were removed at specified intervals, diluted below an inhibitory antibiotic level, and the number of viable cells determined by plate count.

In Vivo Analysis

The *in vivo* evaluation of A32390A was performed in mice as previously described[7]. Infection of the mice was accomplished by injection of *C. albicans*, strain A26, (2.5×10^6 cells), *C. neoformans*, strain WS-34, (2.5×10^6 cells), or *B. dermatitidis*, strain B-6059, (5×10^5 cells) into the lateral tail vein. A32390A was administered to mice as a suspension in 0.125% methyl cellulose (Dow Chemical Co.). Volumes of 0.25 ml were given either intraperitoneally or subcutaneously. Six infected mice and two uninfected (toxicity controls) mice were treated with each dose level at 0, 4, and 24 hours postinfection. The untreated-infected control mice died on the average of 3.5 days postinfection. The significance of antibiotic treatment was determined by STUDENTS *t* test.

A second *in vivo* system was used to evaluate A32390A using unirradiated mice infected with *C. albicans* at 10^6 cells/mouse[8]. Antibiotic treatments were given twice/day for 4 days. The mice were then sacrificed; the kidneys were removed, homogenized, and aliquots of the homogenates were plated for *C. albicans*. The number of yeast cells/g kidney was calculated after a 48-hour incubation.

Preparation and Assay of Antibiotics

A solid dispersion of A32390A in polyvinylpyrrolidone (PVP) was prepared by mixing an acetone solution of A32390A with a chloroform solution of PVP and removing the solvents by evaporation. This solid dispersion was suspended in water for administration to animals.

Assay of antibiotic activity in urine from mice receiving A32390A required extraction of the samples with acetone followed by concentration. Antibiotic activity in the extracted samples was quantified by a disc diffusion technique with *Sarcina lutea* as the assay organism.

Results

A32390A demonstrated the greatest *in vitro* activity against *C. albicans* with a minimal inhibitory concentration (MIC) of 2.5 μg/disc (Table 1). *Cryptococcus neoformans* and *H. capsulatum* were susceptible at 10 μg/ml. *Blastomyces dermatitidis* and the dermatophyte, *T. mentagrophytes*, were not susceptible to A32390A. The MIC of A32390A against 64 clinical isolates of *C. albicans*, determined by the agar dilution method, was 20 μg/ml (Table 2). However, considerable reduction in the growth of these isolates was observed at much lower antibiotic concentrations. Approximately one-third of this group of organisms was partially inhibited at antibiotic concentrations as low as

Table 1. *In vitro* antifungal activity of A32390A

Organism	Minimal inhibitory concentration
Candida albicans, A–26	2.5 μg/disc
Trichophyton mentagrophytes, #6	>40 μg/disc
Cryptococcus neoformans, WS-34	10 μg/ml
Blastomyces dermatitidis, B-6059	>20 μg/ml
Histoplasma capsulatum, #26	10 μg/ml

Table 2. *In vitro* antifungal activity of A32390A

Number of *Candida albicans* isolates tested	MIC	Concentration of A32390A demonstrating activity*
24	20	0.625 μg/ml
18	20	1.25
22	20	5.0

* Concentration which resulted in reduced growth but not complete inhibition.

0.625 μg/ml. An equal number of these isolates showed the same response at 1.25 and 5 μg/ml. Therefore, although the concentration required to completely inhibit growth is relatively high, a significant reduction in growth rates apparently occurs over a wide concentration range below the MIC. This observation was examined further by following the growth of *C. albicans* in broth with and without A32390A (Fig. 1). Medium containing 100 μg/ml of the antibiotic was inoculated with 10^4 cells/ml and viable cell counts were compared to growth in antibiotic-free medium from samples taken at 24-hour intervals. In the absence of antibiotic, *C. albicans* growth reached 10^8 cells/ml in 48 hours. In the presence of A32390A at a concentration of five times the MIC, a population of 10^6/ml was achieved which was 100-fold above the inoculum level, but 100-fold below control growth levels. Since there was no decrease in the viable cell counts in the presence of the antibiotic, it would appear that A32390A has a fungistatic rather than fungicidal action.

The *in vivo* activity of A32390A was assessed by measuring its ability to treat a systemic *C. albicans* infection in mice. Administration of the antibiotic by subcutaneous or intraperitoneal routes, in three doses, extended the survival time of infected mice when compared to untreated controls (Table 3). Significant activity was observed by both routes of administration at doses down to 12.5 mg/kg × 3. Uninfected treated mice were used as toxicity controls at each dose level, and no acute toxicity was observed at these dose rates. No activity was noted after oral administration of the antibiotic. A32390A did not show activity against *C. neoformans* or *H. capsulatum* using the same *in vivo* test system.

A second evaluation system employed for A32390A involved a systemic *Candida* infection in mice. Since the kidney has been implicated as the target organ in *Candida* infections, reduction in numbers of infecting organisms in this organ should be indicative of antibiotic effectiveness. The number of viable yeast cells recovered from kidney homogenates of treated, infected animals is shown in Table 4. A32390A reduced the number of *Candida* cells recovered by 10-fold when compared to untreated infected controls. However, amphotericin B was more effective, reducing cell counts over 100-fold. It has been our experience

Fig. 1. Effect of A32390A on growth of *Candida* in broth.

YNB broth (200 ml) was inoculated with 10^4 *Candida* cells/ml and incubated on a rotary shaker at 30°C. Antibiotic was added at 0 time. Samples were taken at 24-hour intervals for plate counts to determine viable cells.

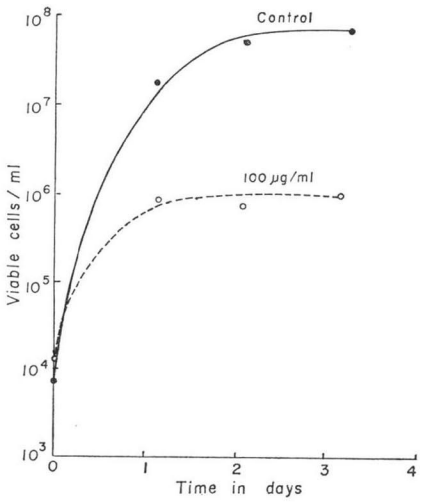

Table 3. *In vivo* antifungal activity of A32390A

Dose administered (mg/kg times three)	Percent extention of survivors beyond controls
SC 100	83%
50	57
25	73
12.5	67
IP 100	50
50	83
25	90
12.5	77

Virulence titration yielded 5.6 LD_{50}'s.
Amphotericin B at 50 mg/kg, IP=107%, SC=140%.

Fig. 2. A32390A recovered from urine of mice treated with A32390A and PVP in various ratios

Mice were given, subcutaneously, 100 mg/kg of A32390A alone or in a dispersion with PVP in a 1:4 or 1:9 ratio. Animals were held individually in glass jars. At indicated times, the mice were sacrificed. Excreted urine was combined with urinary bladder contents. Concentrated acetone extracts of the urine samples were assayed for the antibiotic. Each point represents the determination from one animal.

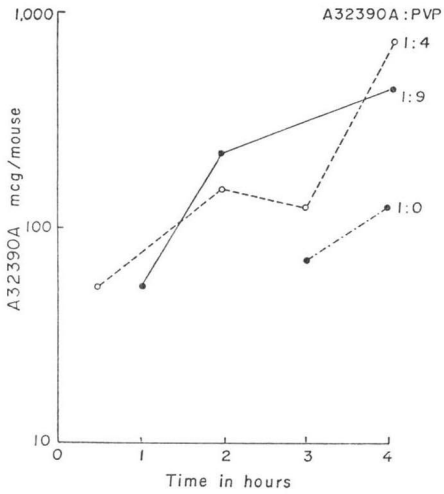

Table 4. Effect of A32390A on *Candida* recovered from mice kidneys

Antibiotic	Dose SC (mg/kg) twice daily for 4 days	*Candida* recovered per gram of kidney
None	—	1.4×10^5
A32390A	25 ×2×4	3.6×10^4
	12.5×2×4	3.2×10^4
Amphotericin B	25 ×2×4	4.1×10^2
	12.5×2×4	5.2×10^2

Table 5. *In vivo* anti-*Candida* activity of A32390-polyvinylpyrrolidone (PVP)

Ratio A32390:PVP	Range of A32390A doses mg/kg×3	Lowest level demonstrating activity mg/kg×3
1:0 (Antibiotic only)	200~25	25
1:1	100~12.5	25
1:2	66~8	66
1:3	50~6.25	12.5
1:4	40~5	15
1:9	20~2.5	7.5
1:24	8~1	No activity
1:49	4~0.5	No activity
0:1 (PVP only)		No activity

that static antibiotics like A32390A are not as effective in this test system as are fungicidal agents such as amphotericin B.

Initial attempts to demonstrate absorption of A32390A in mice by direct assay of blood and urine were unsuccessful. Antibiotic activity was, however, detected in concentrated acetone extracts of urine obtained from mice which were given 100 mg/kg of A32390A subcutaneously. The levels found were low suggesting poor absorption of the drug since only about 6% of the administered dose was recovered in urine. For example, after administration of 1.8 mg A32390A, only 100 μg was recoverable in a 4-hour period (Fig. 2). It was found that the bioavailability of A32390A was improved when the antibiotic was combined with PVP in a solid dispersion. Fig. 2 compares antibiotic recovery in urine after subcutaneous administration of 1.8 mg A32390A alone and mixed with PVP at ratios of 1:4 and 1:9. Administration with PVP enhanced the bio-availability of the drug as judged by higher urine levels and more rapid appearance of the drug in the urine. The amount of drug excreted after administration of either a 1:4 or 1:9 mixture was significantly higher than that found with the drug alone, although the difference between the mixtures was small. No antibiotic activity could be demonstrated in the blood or bile under any of these conditions, again suggesting limited absorption of the drug.

The effect of increased bioavailability of the A32390A: PVP preparation on *in vivo* anti-*Candida* activity was also examined (Table 5). As the concentration of PVP was increased, the amount of antibiotic required to demonstrate activity, based on extension of survival, was reduced. These

results are consistent with the absorption data. A 1: 9 mixture of A32390A with PVP giving a dose rate of 7.5 mg/kg had comparable activity to a dose of antibiotic alone at 50 mg/kg. However, PVP did not improve the therapeutic response observed with this drug, since increasing the amount of antibiotic given in a 1: 9 mixture in excess of 7.5 mg/kg did not show better *in vivo* activity. No change in the acute toxicity was noted with increased absorption of the antibiotic in combination with PVP. At the 1: 24 and 1: 49 ratios, the antibiotic concentrations were too low to be effective in treatment. PVP without antibiotic had no observable effect on the test system.

Discussion

The *in vitro* spectrum of A32390A is limited but includes the most clinically important fungal pathogen, *C. albicans*. A wide range of concentrations of A32390A partially inhibited the growth of *C. albicans* without demonstrating a lethal effect. The levels which cause partial inhibition were found to be more useful in predicting the *in vivo* response than the much higher MIC levels. This became especially significant when pharmacokinetic data indicated A32390A was absorbed poorly but still effectively treated infected mice. Improved bioavailability of A32390A with PVP was observed in two pharmacokinetic properties: detectable urine levels were reached earlier and the levels found were higher than after administration of the antibiotic alone. This change in the pharmacokinetic properties of the antibiotic had an unusual effect on the *in vivo* therapy. Greater absorption of A32390A observed in the presence of PVP did not increase antifungal activity but reduced the amount of drug necessary to demonstrate efficacy normally seen with the antibiotic alone. That is, even with the improved bioavailability conferred by PVP, no improvement in survival was observed. This suggests that bioavailability of the drug remains a limiting factor and that further improvement of the bioavailability by other means may show the intrinsic *in vivo* activity to be considerably higher than observed thus far. No toxic effects were observed even with the increased antibiotic absorption. This study introduces a new agent with potential for treatment of human systemic *Candida* infections and demonstrates a procedure for improving bioavailability of poorly absorbed drugs.

References

1) SEELIG, M. S.: Mechanisms by which antibiotics increase the incidence and severity of candidiasis and alter the immunological defenses. Bact. Reviews 30: 442~459, 1966
2) GARROD, L. P.; H. P. LAMBERT & F. O'GRADY: Antibiotic and Chemotherapy. Edinburg, Churchill Livingstone, London, 1973
3) EMMONS, C. W.; C. H. BINFORD & J. P. UTZ: Medical Mycology. Lea and Febiger, Philadelphia, 1970
4) HAMILTON-MILLER, J. M. T.: Fungal sterols and the mode of action of polyene antibiotics. Advances Appl. Microbiol. 17: 109~134, 1974
5) MARCONI, G. G.; B. B. MOLLOY, R. NAGARAJAN, J. W. MARTIN, J. B. DEETER & J. L. OCCOLOWITZ: A32390A, a new biologically active metabolite. II. Isolation and structure. J. Antibiotics 31: 27~32, 1978
6) HO, P. P. K.; C. MATSUMOTO & R. W. FULLER: Inhibition of dopamine β-hydroxylase and lowering of blood pressure by A32390A. 15th Intersci. Conf. Antimicr. Agents & Chemoth. Abstract 96, 1975
7) GORDEE, R. S. & T. F. BUTLER: New azasteroidal antifungal antibiotics from *Geotrichum flavo-brunneum*. III. Biological activity. J. Antibiotics 28: 112~117, 1975
8) GORDEE, R. S. & T. R. MATTHEWS: Systemic antifungal activity of pyrrolnitrin. Appl. Microbiol. 17: 690~694, 1969