Comparative Study of Trichothecin, Amphotericin B, and 5-Fluorocytosine Against *Cryptococcus neoformans* In Vitro and In Vivo

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Received for publication 7 March 1977

Trichothecin (T-cin), amphotericin B (AB), and 5-fluorocytosine (FC) were compared singly and in combination for capacities to inhibit growth of *Cryptococcus neoformans* in culture and to protect mice bearing infections with this yeast. The minimum inhibitory concentrations for T-cin, AB, and FC were found to be 0.5, 0.2, and 5.0 $\mu\text{g/ml}$, respectively. In vitro viability studies demonstrated a marked reduction in colony counts with the AB-FC combination and additive effects with the AB-T-cin and FC-T-cin combinations for a 3-day period. In mice infected intravenously with *C. neoformans*, the mean effective dose for AB was 0.38 mg/kg, and for FC it was 100 mg/kg for a 30-day treatment period. No mean effective dose could be ascertained when T-cin was tested at doses of 0.1 to 50 mg/kg. Despite this, marked beneficial effects were noted in vivo with the AB-T-cin combination, whereas additive effects and indifference were observed for AB-FC and FC-T-cin combinations, respectively. High-dose T-cin controls survived despite having received a cumulative dosage of more than twice the reported (LD_{50}) mean lethal dose value.

Trichothecin (T-cin) is a trichothecene mycotoxin produced by the saprophytic mold *Trichothecium roseum* and inhibits protein synthesis in eucaryotic cells (5,19,24). Freeman reported the LD_{50} (mean lethal dose) of T-cin in mice to be over 250 mg/kg (6) and toxic to a number of pathogenic fungi in vitro, including *Cryptococcus neoformans* (*Torulopsis neoformans*). Preliminary in vitro experiments in our laboratory demonstrated that *C. neoformans* was susceptible to this drug at $<1 \mu\text{g/ml}$ under more defined conditions. The large ratio between the reported mean lethal dose in mice and the in vitro effectiveness against *C. neoformans* suggested that T-cin might have useful therapeutic properties.

The studies reported here were designed to evaluate the capacities of T-cin to control the growth of *C. neoformans* in culture and in mice, compare these capabilities with those of amphotericin B (AB) and 5-fluorocytosine (FC), and determine the benefits of administering T-cin in combination with AB or FC.

The investigation was therefore divided into two primary phases: (i) in vitro viability studies to determine relative inhibitory properties of T-cin, AB, and FC against *C. neoformans* for a 3-

day period when used singly and in combination (similar to the work of Medoff et al. [16]; and (ii) a parallel study on mice by using an animal model system similar to that of Block and Bennett (3).

MATERIALS AND METHODS

Medium. A modified Wickerham growth medium, designated M9A, as reported previously (22) was employed in all experiments.

Strain. *C. neoformans* 184 was used in all in vitro and in vivo experiments. This strain was provided by L. Friedman, Charity Hospital, New Orleans, La.

Drugs. T-cin was prepared as previously described (22). For in vitro experiments, T-cin stock solutions and dilutions were prepared in distilled water. For animal inoculations, T-cin was dissolved in light mineral oil.

Crystalline FC was purchased from Hoffman-La Roche, Inc., Nutley, N.J., and was prepared in distilled water for in vitro studies and in physiological saline for in vivo experiments.

AB (Fungizone, E.R. Squibb & Sons, Princeton, N.J.) was reconstituted with distilled water and diluted with 5% dextrose in water.

All drug stock solutions were sterilized by filtration and stored in dark containers at 4°C when not in use.

In vitro. For minimum inhibitory concentration (MIC) studies, cells were inoculated by the loop method directly from Sabouraud dextrose agar slants ca. 7 days old. They were grown for 24 h in

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M9A medium on a rotating shaker at 37°C, diluted with fresh medium to 10^6 cells per ml, and allowed to grow overnight until a concentration of ca. 10^6 cells per ml was obtained. The cells were then transferred, in 50-ml portions, to sterile Erlenmeyer flasks to which drugs were added singly at various concentrations. Ten milliliters from each flask was transferred to each of four test tubes (125 by 15 mm), which were placed on a New Brunswick tube platform (New Brunswick Scientific Co., New Brunswick, N.J.) and shaken at a 15° angle for 3 days at 37°C. Duplicate tubes were removed after 24 and 72 h for viability platings. After an initial screening, twofold serial dilutions of each drug were prepared to determine the MIC. In subsequent experiments, drugs were added singly or in combination at fractional MIC levels. Cell counts were made after dilutions by spread plating in triplicate on Sabouraud dextrose agar to determine the number of colony-forming units. All experiments were duplicated, and the results were averaged.

In vivo. (i) Cells. Cells were grown for 24 h at 37°C in M9A medium, washed three times in physiological saline, and diluted to 10^6 cells per ml in physiological saline.

In all experiments, initial viable counts were also determined to confirm hemacytometer counts.

(ii) Mice. Six- to eight-week-old BALB/c mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.), 25 to 30 g, were used in each experiment. All mice were weighed at the time of treatment. On day 1, mice were inoculated intravenously via a lateral tail vein with 0.1 ml of a suspension (10^6 cells per ml) of *C. neoformans*. Drug administration was started on day 4 and repeated on alternate days for a total of 14 doses when mice survived to the end of the 30-day treatment period. All drugs were administered intraperitoneally. Injections varied between 0.1 and 0.4 ml, depending upon the drug and its concentration. Ten mice, five males and five females, were assigned to each dosage regimen. Mice were housed in groups of five. They were provided water and food (Purina Lab Chow) ad libitum and were maintained on a 12-h day-night cycle. The mice were examined three times daily for signs of illness or impending death. Dead mice were refrigerated (4°C) upon discovery and autopsied within 24 h. Surviving mice of the combined-drug experiment were killed and autopsied immediately. The spleen and brain were ground with a tissue homogenizer and plated on Sabouraud dextrose agar containing 100 U of penicillin and 100 µg of streptomycin per ml. Growth of yeastlike colonies at 37°C and the presence of an encapsulated yeastlike organism in random colonies examined by India ink wet mount constituted positive identification criteria for *C. neoformans*. Plates classified as negative contained less than four colonies, whereas plates classified as positive produced confluent growth.

(iii) Experiments. Three separate experiments were carried out. In the first, T-cin was evaluated at doses of 1.0, 2.5, 5.0, 7.5, and 10 mg/kg only. In the second experiment, the activities of AB, FC, and T-cin were compared. AB was administered at doses of 0.1, 0.25, 0.50, 0.75, 1.0, and 1.5 mg/kg, and FC was

administered at doses of 10, 25, 50, 100, and 150 mg/kg. Because of the large quantities of T-cin required for the study and the desire to maintain uniform numbers of mice for each dosage regimen, T-cin was administered to groups of five mice only in doses of 0.1, 1.0, 10, 25, and 50 mg/kg. Control groups for this experiment included uninfected and untreated mice, uninfected mice treated with maximal doses of each drug, infected and untreated mice, and infected mice treated with solvent alone.

The third experiment dealt with the activities of two-drug combinations. The three agents were paired at the mean effective dose (ED_{50}) and one-half ED_{50} ($ED_{50}/2$), as determined in the second experiment. Controls consisted of T-cin at 1.0 mg/kg and AB and FC at ED_{50} levels, uninfected and infected mice.

(iv) Assessment of drug activity. The MIC was defined as the lowest concentration of each compound that completely inhibited multiplication of *C. neoformans* 184 over a 3-day experimental period. The exception to this was FC, for which a 2-day period was chosen, since overgrowth could not be prevented with this strain after 2 days, even with drug concentrations up to 160 µg/ml.

An additive effect was defined as the effect achieved by two drugs in combination when equal to the effect of a single drug at a concentration equal to that of the mixture (11, 12).

In the viability studies, antifungal synergy was defined as a decrease of 10-fold or more in colony counts after 3 days when drugs were used in combination as compared to the counts when a single drug was used alone (16). In vivo protective effects were any effect greater than additive.

RESULTS

In vitro. Results of the in vitro studies are summarized in Table 1. The MIC of T-cin was only about twice that of AB. The MIC of FC was 5 µg/ml for a 2-day period or >160 µg/ml for a 3-day period, since all concentrations tested up to 160 µg/ml were unable to maintain viable counts at or below 10^6 colony-forming units per ml for the longer period. The most effective MIC/2 combination (each drug at one-half MIC) after 3 days was AB-FC, which exhibited synergy by the criterion of Medoff et al. (16). The AB-T-cin MIC/2 combination approximated an additive effect based upon the comparative value of its colony-forming units with the MIC value of 10^6 cells per ml. For the FC-T-cin MIC/2 combination, the effect appeared to be additive, based on a comparison with the T-cin MIC; however, since FC was inactive at 3 days, there appeared to be a greater than additive effect. Other combinations tested and their colony-forming units are presented in Table 1.

In vivo. (i) Single-drug experiments. The curative effect of various concentrations of AB, FC, and T-cin on *C. neoformans*-infected mice

is represented in Fig. 1A through C. The ED_{50} of AB at 30 days was 0.38 mg/kg, which was determined by inspection on the basis of percent survivors. On the same basis, the ED_{50} of FC was established at 100 mg/kg. No ED_{50} for T-cin could be obtained at 30 days, since all concentrations tested fluctuated around control values (Fig. 1C). All of the high-dose, T-cin control mice (uninfected) suffered a severe weight loss of up to 11 g/mouse, and their coats were unkempt and ruffled after receiving a total of 250 to 350 mg of T-cin per kg. Minimum weight was reached after cumulative dosage with 500 mg of T-cin per kg. Thereafter, recovery and weight gain were rapid. At the end of the experiment, the mice weighed only 4.6 g

less than their initial weight, even though they had received a total of 700 mg of T-cin per kg, more than twice the estimated mean lethal dose value.

(ii) Combined-drug experiment. The activities of various drug combinations are compared in Table 2, with results expressed as percent survivors at 30 days. The percent survival of the AB-FC ED_{50} and $ED_{50}/2$ groups approximated additive effects when compared with observed or expected values of controls. The value of 1.0 mg of T-cin per kg was chosen for conservation purposes and because this concentration permitted relatively greater survival time (Fig. 1C). Both AB-T-cin combinations exhibited marked beneficial effects, since a greater than

TABLE 1. *In vitro* effects of T-cin, AB, and FC, used singly and in combination against *C. neoformans*

μg of drug/ml of medium			CFU ^a /ml		Reduction of CFU ^a	
T-cin	AB	FC	Day 1	Day 3	Day 1	Day 3
			2.2×10^7	9.2×10^7		
0.5 ^b			9.8×10^6	9.0×10^6	2.2×10^1	1.0×10^2
0.25			5.1×10^6	6.7×10^6	4.3×10^0	1.4×10^1
0.125			1.6×10^7	5.6×10^7	1.4×10^0	1.6×10^0
	0.2 ^b		5.9×10^6	5.8×10^6	3.7×10^1	1.6×10^2
	0.1		1.9×10^6	9.2×10^7	1.2×10^2	
	0.05		7.0×10^6	8.0×10^7	3.1×10^0	1.2×10^0
		5.0 ^c	4.7×10^6	4.4×10^7	4.7×10^1	2.1×10^0
		2.5	8.9×10^6	3.5×10^7	2.5×10^1	2.6×10^0
		1.25	1.2×10^6	5.6×10^7	1.8×10^1	1.6×10^0
0.25	0.1		6.3×10^4	4.3×10^5	3.5×10^2	2.1×10^2
0.125	0.05		1.1×10^6	1.2×10^7	2.0×10^1	7.7×10^0
0.25		2.5	9.4×10^6	9.5×10^6	2.3×10^1	9.7×10^1
0.125		1.25	8.8×10^6	9.9×10^6	2.5×10^1	9.3×10^1
	0.1	2.5	5.8×10^3	6.3×10^3	3.8×10^3	1.5×10^4
	0.05	1.25	2.3×10^5	4.0×10^6	9.6×10^1	2.3×10^1

^a Colony-forming units.

^b MIC.

^c MIC for 2 days.

FIG. 1. Mortality curves for AB (A), FC (B), and T-cin (C) compared with those of untreated controls. The ED_{50} values for AB and FC were established at 0.38 and 100 mg/kg, respectively. No ED_{50} value could be established for T-cin, since an 80 to 100% mortality occurred in all cases.

TABLE 2. *In vivo* effects of T-cin, AB, and FC, used singly and in combination against *C. neoformans*

Daily dose (mg/kg of body wt)			30-Day survivors ^a (%)
T-cin	AB	FC	
1.0			10
	0.38		10
		100.0	60
			40
1.0	0.38		90
0.5	0.19		50
1.0		100.0	50
0.5		50.0	30
	0.38	100.0	90
	0.19	50.0	60

^a Ten mice per group.

expected percentage of mice survived. The FC-T-cin pair showed effects approximating those of FC alone, suggesting that T-cin did not act in combination with this drug. Similar relationships were observed when shifts in the mortality curves or the cumulative total weight loss of survivors of the groups were compared (data not shown).

All mice that died during the experiments were classified as positive for the presence of *C. neoformans* in the organ cultures. Negative spleen cultures were obtained in two of nine AB-FC and AB-T-cin ED₅₀ survivors and in two of five FC-T-cin ED₅₀ survivors. Negative brain cultures were obtained in two of nine of the FC ED₅₀ and AB-FC ED₅₀ survivors.

DISCUSSION

The MIC of AB against *C. neoformans* 184 was 0.2 µg/ml. The MIC of FC was 5 µg/ml after 48 h of incubation. These values agree with those reported by Shadomy et al. (21) for AB and Shadomy (20) and Block et al. (4) for FC. We report the MIC of T-cin to be 0.5 µg/ml against *C. neoformans* 184.

The ED₅₀ of AB against *C. neoformans* was 0.38 mg/kg, injected intraperitoneally. Although this value differs from 0.86 mg/kg, injected intravenously, reported by Block and Bennett (3), the differences could be due to a number of variables (2). The ED₅₀ of FC was 100 mg/kg, injected intraperitoneally. This is equivalent to the daily dosage administered by Block and Bennett (3).

In the *in vivo* drug combination studies (experiment 3), the AB-FC ED₅₀ group permitted a 90% survival in mice; however, total drug concentrations were twice as much as individual ED₅₀ controls. Based upon the AB and FC mortality curves, higher drug concentrations would be expected to increase survival.

The AB-FC ED₅₀/2 group demonstrated addi-

tive effects *in vivo* similar to the results obtained by Block and Bennett (3).

Antibiotic properties of the trichothecenes against pathogenic yeasts have been investigated previously with various results (1, 7, 8, 18; Loevens Kemiske Fabrik produktionsaktieselskab, Netherlands, Patent 320,547). Although there have been numerous recent investigations into the concurrent use of AB and FC against pathogenic yeasts (3, 9, 17, 21, 23; Program Abstr. Intersci. Conf. Antimicrob Agents Chemother. 13th, Washington, D.C., Abstr. no. 239, 1973; P.D. Hoepflich, and P.D. Finn, Abstr. Annu. Meet. Arn. Soc. Microbiol. 1972, Mm42, p. 135), to date, there have been no reports on the activity of T-cin when combined with AB or FC *in vitro* or *in vivo*. One possible explanation for the difference between *in vitro* and *in vivo* results for the AB-T-cin drug pair is that T-cin may be biologically modified in mice. The new compound might also be active against *C. neoformans* but could be less permeable to yeast cells than T-cin unless assisted by AB. The data presented in Fig. 1C and the survival of high-dose T-cin controls suggest that T-cin is inactivated in mice. This may also explain why an effect approximating that of FC when used alone is achieved in the FC-T-cin combinations *in vivo* but not *in vitro*. Supportive evidence for the degradation or inactivation of T-cin and other trichothecenes has been reported previously (8, 10, 14, 15).

High-dose T-cin control mice demonstrated clinical signs only when they were given near-lethal doses, similar to the results of Kosuri et al. (13) who studied T-2 toxin in rats. The gradual loss of weight suggested an initial lack of tolerance to the drug concentrations used.

In conclusion, although T-cin is active *in vitro* against *C. neoformans*, it may be rapidly degraded in mice and is ineffective when used alone in the treatment of systemic cryptococcosis. *In vitro*, the AB-T-cin combination demonstrated an additive effect but afforded marked protection *in vivo*. A more comprehensive study of this drug combination with various concentrations of T-cin and AB against a number of *C. neoformans* strains in mice might reveal more concerning the relative toxicity of this drug pair compared with either drug alone, and, hence, better describe the chemotherapeutic efficacy of T-cin. Studies in these areas are presently being conducted.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service training grants 5 TO1 AIOO 123-AID and AI-05022 from the National Institute of Allergy and Infectious Diseases and by Faculty Research Committee and Arts and

Sciences Faculty Research grants from the University of Oklahoma.

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