ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1987, p. 1406–1413
0066-4804/87/091406-08$02.00/0

Vol. 31, No. 9

Synergism between 9-Deazainosine and DL-α-Difluoromethylornithine in Treatment of Experimental African Trypanosomiasis

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Received 9 February 1987/Accepted 10 June 1987

Kinetoplastid hemoflagellates are sensitive to growth inhibition by various purine analogs. In this study the activities of 9-deazainosine (9-DINO), formycin B, and sinefungin were compared in experimental murine *Trypanosoma brucei* subsp. *brucei* infections, both singly and in combination with the ornithine decarboxylase inhibitor DL-α-difluoromethylornithine (DFMO, eflornithine). Used singly, all of the purine analogs were able to suppress an acute *T. brucei* subsp. *brucei* infection. 9-DINO and formycin B were the most active. None of the purine analogs was curative when used singly against a strain causing chronic central nervous system infection. 9-DINO was highly effective when used in combination with DFMO in curing this central nervous system infection and another more stringent experimental infection. Neither sinefungin nor formycin B was active in combination with DFMO in curing the central nervous system experimental infection. 9-DINO was metabolized to phosphorylated derivatives of 9-deazaadenosine and 9-deazaguanosine by bloodstream trypomastigotes, but not by murine erythrocyte suspensions or kidney or liver homogenates—a potential rationale for the selectivity of the analog. These studies indicate that 9-DINO is a potent, nontoxic purine analog which, in combination with DFMO, is capable of late-stage cures of African trypanosomiasis.

African trypanosomiasis occurs in a broad area across equatorial Africa and strikes both animals and humans. The disease is initially confined to the blood and lymphatic system, but eventually involves the central nervous system (CNS) and produces the classic symptoms of sleeping sickness in its victims. Chemotherapy of late-stage CNS disease has depended on one drug, the arsenical melarsoprol (Mel B), for over 40 years. Although this drug has saved many lives, it is neurotoxic, and therefore there is a need for less toxic agents for treating CNS disease (26).

Recent studies with purine analogs such as sinefungin, formycin B, and 9-deazainosine (9-DINO) (Fig. 1) have indicated the potential for efficacy of these agents against subspecies of *Trypanosoma brucei*, the agent of African sleeping sickness (4, 12, 14). The activities of these analogs have been analyzed by using culture systems of procyclic or bloodstream trypomastigotes (4, 14) or short-term (acute) experimental infections (12). Although these agents were active in blocking growth or curing acute infections, their efficacy in treatment of central nervous system trypanosomiasis models has not been examined.

The ornithine decarboxylase inhibitor DL-α-difluoromethylornithine (MDL 71782; eflornithine; DFMO) (Fig. 1) cured an acute experimental infection of *T. brucei* subsp. *brucei* (3) and was synergistic with bleomycin (10) and suramin (11) in curing CNS model infections. It is currently undergoing clinical trials in Africa and, used singly, has cured over 100 cases of CNS trypanosomiasis (24, 31–33, 35). In view of the dose regimen and duration of monotherapy with DFMO (400 mg/kg per day for 6 weeks), it may be clinically advantageous to use DFMO in combination with other agents which act synergistically and result in a shorter treatment period. Since organisms of the *T. brucei* subgroup are apparently susceptible to ornithine decarboxylase inhibitors such as DFMO (1a, 25) and to purine analogs (14), we have studied both types of agents to determine whether they can act synergistically.

MATERIALS AND METHODS

Materials. 9-DINO, 9-deazaadenosine, and 9-deazaguanosine were synthesized and purified as described previously (21). 9-Deazaxanthosine was synthesized by a procedure developed by K. V. B. Rao and R. S. Klein (personal communication). DFMO and α-monofluoromethyldehydroornithine methyl ester (ΔMFMO · CH_3; MDL 72403) were from the Merrell Dow Research Institute, Cincinnati, Ohio. Allopurinol and allopurinol riboside were gifts of Burroughs Wellcome Co., Research Triangle Park, N.C. Suramin (Mobay Chemical Corp., New York, N.Y.), sinefungin, Berenil (diminazene aceturate; Calbiochem Biochemicals, San Diego, Calif.), and formycin B (Sigma Chemical Co., St. Louis, Mo.) were purchased. The monophosphates of 9-deazadenosine and 9-DINO were gifts from R. L. Miller, Burroughs Wellcome Co. We purchased radioactive [2,8-^{3}H]9-DINO from Moravek Biochemicals, Brea, Calif., and verified its purity to be >99% by using reverse-phase high-performance liquid chromatography (RP-HPLC). Calf alkaline phosphatase was obtained from Calbiochem, and the culture medium components were purchased from Irvine Scientific, Santa Ana, Calif. All other chemicals were from commercial sources and were of analytical grade or better.

Trypanosome strains. Swiss Webster mice (25 to 30 g) were used throughout. The short-term *T. brucei* subsp. *brucei* EATRO 110 (from the East African Trypanosomiasis

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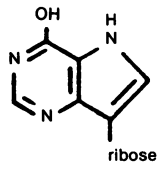
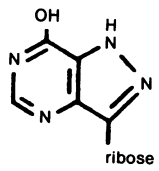
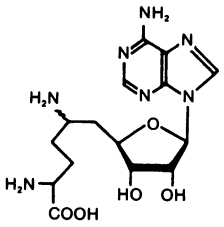
a) 9-DEAZAINOSINE



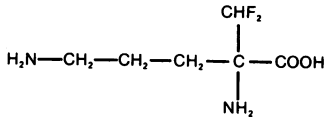
b) FORMYCIN B



c) SINEFUNGIN



d) α-DIFLUOROMETHYLORNITHINE (DFMO)



e) DEHYDROMONOFLUOROMETHYLORNITHINE METHYL ESTER (ΔMFMO.CH_3)

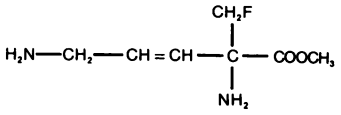


FIG. 1. Structures of purine and ornithine analogs used in this study.

Research Organization) isolate was maintained as described previously (3). It produces an acute, rapidly fatal parasitemia which is lethal to mice 3 to 5 days after inoculation.

Two *T. brucei* subsp. *brucei* strains, TREU 667 (from Trypanosomiasis Research Edinburgh University) and LUMP 1001 (from London University Medical Parasitology), developed by Jennings et al. (17, 18) were used as CNS models. Both produce infections which invade the CNS within 21 days after inoculation. At this stage the trypanocide Berenil (diminazene aceturate) will clear the blood parasites, but the infection will redevelop from the CNS and parasites will repopulate the blood. These infections are not usually fatal until >40 days after inoculation. LUMP 1001 causes a more virulent CNS infection than TREU 667 and is regarded as more difficult to cure (17).

Short-term EATRO 110 model. In the EATRO 110 infection, groups of 100 mice were infected (10^5 trypanosomes per mouse) and the infection was allowed to develop for 24 h before therapy was begun. Animals were divided into groups of five before treatment. Cured animals survived for >30 days beyond the deaths of controls, and tail vein blood smears showed no evidence of parasites. Previous work with this model demonstrated that the blood of animals kept for >60 days after the disappearance of parasites was noninfective to healthy mice (3). DFMO and the analog (ΔMFMO · CH_3) were administered continuously in the drinking water for 3 days (2, 3). Mice consumed 5 ml of a 2% DFMO solution per day, equivalent to a total dose of 100 mg/day per mouse; ΔMFMO · CH_3 at 0.5% was consumed at the rate of 4 ml/day (20 mg/day per mouse). All other compounds were administered by intraperitoneal (i.p.) injection.

CNS models. The TREU 667 and LUMP 1001 strains were stored frozen and passaged once in rats before being used to infect mice. Maintenance and use of these screens were as described previously (2, 10). Briefly, groups of 100 to 120 mice were infected with 10^5 trypanosomes, and the infections were allowed to develop until day 21, when animals were separated into groups of 10 and therapy was begun. Analogs were administered i.p.; DFMO and ΔMFMO · CH_3 were given in the drinking water. Controls were treated with Berenil (40 mg/kg) on day 21. This trypanocide, used to treat animal trypanosomiasis, always clears the blood of trypanosomes, but the animals relapse 40 to 70 days after treatment, indicating the presence of a CNS infection (10). Parasitemia was monitored by examining tail vein blood smears once weekly. Animals which had relapsed were removed from cages. TREU 667 infections were monitored for >250 days after inoculation, and LUMP 1001 infections were monitored for >280 days, as determined by the relapse pattern in previous work (2). "Cured" animals had no evidence of parasitemia, and their brain homogenates failed to produce parasitemia >2 weeks after injection into uninfected control animals.

HPLC analysis. The HPLC system consisted of the following instruments (Varian, Sunnyvale, Calif.): a 5060 liquid chromatograph system, a UV-1 UV (254 nm) detector connected in series with a UV-50 variable-wavelength detector set at 295 nm, and a Vista CDS-401 data system. Nucleotides were separated by strong anion-exchange (SAX) HPLC on a Whatman 10 SAX column (Whatman, Inc., Clifton, N.J.). Samples were eluted at a flow rate of 0.5 ml with a gradient of 0.015 M to 1 M potassium phosphate buffer (pH 3.5). The gradient was isocratic (stable at 0.015 M) for 15 min and then linear for 75 min. Fractions (0.5 ml) of the eluate were collected, 5 ml of scintillation cocktail was added to each, and their radioactivity was determined by liquid scintillation spectrophotometry in a liquid scintillation system (LS-1001; Beckman Instruments, Inc., Fullerton, Calif.). Bases and nucleosides were separated by RP-HPLC on a column (HS201; VYDAC, Hesperia, Calif.) by using a linear gradient starting with 1.2% acetonitrile in 4.8 mM potassium acetate buffer (adjusted to pH 5.0 with trifluoroacetic acid) and finishing with 25% acetonitrile in H_2O over 40 min at a flow rate of 1 ml/min. Fractions (1 ml) were collected, and their radioactivity was determined as described above. Radioactive metabolites of 9-DINO were identified by retention time, UV A_{254}/A_{295} ratio (20), or coelution with authentic compound. When authentic nucleotide was not available, the acid-soluble extract was treated with calf intestine alkaline phosphatase, and the resulting nucleosides were verified by coelution with the respective authentic riboside when analyzed by RP-HPLC.

Mouse metabolism of 9-DINO. Groups of three mice were given 9-DINO by i.p. injection at dosages of 200, 100, 50, and 25 mg/kg of body weight once daily for 3 days. In addition to the required amounts of 9-DINO, each injection contained 1 μCi of [2,8-^{3}H]9-deazainosine. The specific activity of the radiolabeled 9-DINO (11 Ci/mmol) was such that it caused no significant change in the dosage concentration. On day 3, mice from each group were exsanguinated by cardiac puncture approximately 4 h after injection of drug. Serum was acid extracted at 4°C by the following procedure.

Blood was allowed to clot, and the serum was removed after centrifugation. Sera from each dosage group were pooled, and 0.5 ml of each was extracted with 2 ml of 10% perchloric acid. The acid-insoluble fraction was pelleted by centrifugation, and the acid-soluble fraction was decanted. The latter fraction was then neutralized with 3.3 N KOH, the precipitate was pelleted, and the supernatant was decanted for HPLC analysis.

In addition to the serum, the liver was removed from each mouse in the group receiving 200 mg/kg of body weight. After a saline wash, the livers were pooled and homogenized in 25 ml of 5% perchloric acid with a Ten Broeck tissue grinder. The acid-soluble fraction was isolated for HPLC and liquid scintillation spectrometry analysis as described above. The pooled kidneys were acid extracted in a like manner. These procedures were carried out at 4°C.

TABLE 1. Activity of purine analogs and DFMO on the acute *T. brucei* subsp. *brucei* EATRO 110 model

Treatment	Dose	No. cured/total no. (% cured)
Allopurinol	300 mg/kg	0/5 (0)
Allopurinol riboside	250 mg/kg	0/5 (0)
9-DINO	200 mg/kg	5/5 (100)
	100 mg/kg	5/5 (100)
	50 mg/kg	4/5 (80)
	25 mg/kg	15/20 (75)
	10 mg/kg	2/30 (7)
	5 mg/kg	0/5 (0)
Formycin B	50 mg/kg	5/5 (100)
	25 mg/kg	9/10 (90)
	10 mg/kg	5/5 (100)
	5 mg/kg	4/5 (80)
	2.5 mg/kg	5/5 (100)
	0.5 mg/kg	3/5 (60)
	0.25 mg/kg	1/10 (10)
	0.1 mg/kg	0/5 (0)
	0.05 mg/kg	0/5 (0)
Sinefungin	25 mg/kg	9/10 (90)
	10 mg/kg	12/20 (60)
	5 mg/kg	11/20 (55)
	2.5 mg/kg	0/5 (0)
	1 mg/kg	0/20 (0)
	0.5 mg/kg	0/15 (0)
DFMO	2%	10/10 (100)
	0.5%	0/5 (0)
	0.25%	0/5 (0)
DFMO + 9-DINO	0.5% + 25 mg/kg	5/5 (100)
	0.5% + 10 mg/kg	6/10 (60)
	0.5% + 5 mg/kg	1/5 (20)
	0.25% + 25 mg/kg	5/5 (100)
	0.25% + 10 mg/kg	1/5 (20)
DFMO + formycin B	0.5% + 0.5 mg/kg	5/5 (100)
	0.5% + 0.25 mg/kg	2/5 (40)
	0.5% + 0.1 mg/kg	2/5 (40)
	0.5% + 0.05 mg/kg	0/5 (0)
DFMO + sinefungin	0.5% + 25 mg/kg	5/5 (100)
	0.5% + 10 mg/kg	2/5 (40)
	0.5% + 5 mg/kg	5/10 (50)
	0.25% + 25 mg/kg	2/5 (40)
	0.25% + 10 mg/kg	4/5 (80)
	0.25% + 5 mg/kg	1/5 (20)
	0.25% + 1 mg/kg	5/10 (50)
	0.25% + 0.5 mg/kg	0/10 (0)
	0.25% + 0.25 mg/kg	0/10 (0)

TABLE 2. Effects of delayed administration of 9-DINO on the EATRO 110 model[a]

Treatment delay	Dose (mg/kg)	No. cured/total no. (% cured)
48 h	100	5/5 (100)
	50	5/5 (100)
	25	5/5 (100)
72 h	100	6/9[b] (67)
	50	6/10 (60)
	25	6/10 (60)

[a] 9-DINO administration was begun 48 or 72 h postinfection and continued for 3 days as single daily injections.

[b] Only animals receiving full treatment (three i.p. doses) were included in data tabulation.

Bloodstream trypomastigote metabolism of 9-DINO. Bloodstream forms of the EATRO 110 strain of *T. brucei* subsp. *brucei* were isolated on Percoll gradients as previously described by Fish et al. (13). Parasites were incubated in a medium of defined purine composition for 3 h at 37°C in 20 ml of PDM-79 (13) containing 0.5% bovine serum albumin and 40 μM [2,8-^{3}H]9-deazainosine (specific activity, 31.2 μCi/μmol). At the end of incubation, the acid-soluble nucleotides were extracted from the parasites as described previously (23). Before SAX-HPLC analysis, the acid-soluble extract was concentrated 10-fold by lyophilization and then reconstituted in 1/10 the original volume. As the metabolic control, an equivalent number of mouse erythrocytes (RBCs) were incubated and extracted under identical conditions.

Animals. Swiss Webster mice (25 to 30 g) were purchased from Royal Hart Laboratory Animals, New Hampton, N.Y.

RESULTS

Short-term EATRO 110 model. Several purine analogs were compared for activity in the EATRO 110 model (Table 1). Those having the highest activity were formycin B and 9-DINO. Formycin B was the most active and was curative over a range of 5 to 50 mg/kg. 9-DINO was also highly effective, with an activity range of 25 to 200 mg/kg. Comparison of the activity ranges for both compounds indicated that formycin B was >10 times more active than 9-DINO (100% cures at 2.5 mg/kg versus 75% cures at 25 mg/kg, respectively).

Sinefungin, which had cured laboratory infections by strains of *T. brucei* subsp. *brucei*, *T. congolense*, and *T. vivax* (12) when administered in multiple daily doses, was only ca. 50% effective in our experiments in single doses of 5 or 10 mg/kg and 80% active at 25 mg/kg (Table 1). Allopurinol and its riboside were completely inactive at 300 mg/kg.

9-DINO was also active in the short-term EATRO 110 model even if treatment was delayed to 48 or 72 h postinfection (Table 2). At 48 h, mice had a blood parasitemia of 2.5×10^7 to 6.2×10^7/ml. 9-DINO treatment commencing at this time was completely effective at dose ranges of 25 to 100 mg/kg. At 72 h postinfection, mice were clearly ill and had a blood parasitemia of $>1 \times 10^8$/ml. One animal died within 24 h of the initial dose. Of the animals surviving to receive the complete treatment regimen (three doses), a 60% cure rate

TABLE 3. Activity of purine nucleoside analogs singly and in combination with polyamine antagonists in *T. brucei* subsp. *brucei* TREU 667 CNS model

Treatment	Dose[a]	Duration (days)[a]	Avg day of relapse (range)	No. cured/total no. (% cured)
DFMO	4%	14	77 (35–143)	0/10 (0)
	2%	14	56 (43–64)	0/10 (0)
9-DINO	250 mg/kg	7	68 (45–86)	3/10 (30)
	100 mg/kg	7	49 (35–58)	0/20 (0)
	25 mg/kg	7	60 (41–135)	1/5 (20)
	10 mg/kg	7	41	0/5 (0)
	5 mg/kg	7	41	0/5 (0)
DFMO + 9-DINO	2% + 250 mg/kg	14, 7	77	9/10 (90)
	2% + 100 mg/kg	14, 7		18/18 (100)
	2% + 50 mg/kg	14, 7		10/10 (100)
	2% + 25 mg/kg	14, 7	54 (41–76)	11/20 (55)
	2% + 10 mg/kg	14, 7	51 (49–68)	0/10 (0)
	2% + 5 mg/kg	14, 7	51 (49–58)	0/10 (0)
ΔMFMO · CH_3	0.5%	14	96 (55–113)	1/10 (10)
ΔMFMO · CH_3 + 9-DINO	0.5% + 100 mg/kg	14, 7		18/18 (100)
	0.5% + 50 mg/kg	14, 7		10/10 (100)
Sinefungin	10 mg/kg	7	124 (35–209)	6/10 (60)
	10 mg/kg	3	38 (35–46)	0/10 (0)
	5 mg/kg	4	41 (35–46)	0/10 (0)
	2.5 mg/kg	4	39 (35–56)	0/18 (0)
DFMO + sinefungin	2% + 2.5 mg/kg	14, 4	81 (35–147)	11/20 (55)
	2% + 1.0 mg/kg	14, 4	76 (54–92)	3/10 (30)
	2% + 0.5 mg/kg	14, 4	70 (54–92)	0/10 (0)
Formycin B	100 mg/kg	6	35	0/9 (0)
	50 mg/kg	6	34 (23–35)	0/10 (0)
	25 mg/kg	7	40 (37–68)	0/10 (0)
	10 mg/kg	7	40 (37–41)	0/9 (0)
	5 mg/kg	7	42 (37–49)	0/8 (0)
DFMO + formycin B	4% + 100 mg/kg	14, 6	64.7 (30–144)	1/10 (10)
	4% + 100 mg/kg	7, 6	41 (24–64)	0/10 (0)
	2% + 100 mg/kg	14, 6	50 (30–64)	0/10 (0)
	2% + 100 mg/kg	7, 6	42 (30–51)	0/9 (0)
	4% + 50 mg/kg	14, 6	37 (23–64)	2/9 (22)
	4% + 50 mg/kg	7, 6	42 (23–64)	0/9 (0)
	2% + 50 mg/kg	14, 6	55 (23–64)	1/10 (10)
	2% + 10 mg/kg	14, 7	45 (41–58)	2/10 (20)

[a] For combinations, the first and second numbers refer to the first and second drug listed, respectively.

was obtained at 25 and 50 mg/kg and a 67% rate was obtained at 100 mg/kg. 9-DINO was completely effective at 25 mg/kg (48-h dose initiation), although it was only 75% curative in the standard screen at this dose (Table 1). The reason for this difference is unclear.

9-DINO was synergistic with DFMO against the EATRO 110 model. Used singly, 50 to 100 mg of 9-DINO or 2% DFMO per kg was needed to obtain 100% cure rates (Table 1). In combination with 25 mg of 9-DINO per kg, 0.5 or 0.25% DFMO was sufficient for complete cures (Table 1). This represents reductions in curative doses of twofold for 9-DINO and four- to eightfold for DFMO. In addition, the cure rate for 9-DINO at 10 mg/kg rose from 7% when used singly to 60% when used in combination with 0.5% DFMO. Sinefungin was less active in this context, and results were not consistent when it was used in combination with DFMO: a 50% cure rate was obtained with 1 mg of sinefungin per kg plus 0.25% DFMO, but the percentage of animals cured did not increase with sinefungin levels up to 25 mg/kg. Formycin B at 0.5 mg/kg was 60% curative when used singly and 100% curative when used in combination with 0.5% DFMO. At lower doses (0.25 and 0.1 mg/kg), formycin B plus 0.5% DFMO cured 40% of the mice.

CNS infection models. Purine nucleoside analogs were also tested in two CNS screens. Initial trials were done with the TREU 667 model, which produces a less virulent CNS infection than the more stringent LUMP 1001 model. In the TREU 667 model (Table 3), none of the analogs were effective when used singly. 9-DINO acted in synergism with DFMO or the ornithine analog ΔMFMO · CH_3. When administered at 25 or 50 mg/kg for 7 days, 9-DINO was completely effective if used with 2% DFMO. The length of DFMO administration, 2 weeks, has been found to be optimal in combination studies with other agents such as suramin (11) and bleomycin (10). ΔMFMO · CH_3 is 4 to 12 times more potent than DFMO against acute (8) and CNS (2)

TABLE 4. Activity of 9-DINO plus DFMO against *T. brucei* subsp. *brucei* LUMP 1001 isolate

Treatment	Dose[a]	Duration (days)	Avg day of relapse (range)	No. cured/total no. (% cured)
9-DINO	250 mg/kg	7	49 (35–63)	0/10 (0)
	100 mg/kg	7	38 (35–44)	0/9 (0)
	50 mg/kg	7	36 (35–44)	0/10 (0)
DFMO	2%	14	55 (51–62)	0/10 (0)
DFMO + 9-DINO	2% + 250 mg/kg	14, 7	38 (35–41)	15/17 (88)
	2% + 100 mg/kg	14, 7	103 (35–196)	29/33 (88)
	2% + 50 mg/kg	14, 7	91 (35–155)	19/24 (79)
	2% + 25 mg/kg	14, 7	95 (54–135)	6/19 (32)
Sinefungin	10 mg/kg	3	39 (35–71)	0/10 (0)
	5 mg/kg	3	35	0/10 (0)
	2.5 mg/kg	3	35	0/10 (0)
DFMO + sinefungin	2% + 10 mg/kg[b]	14, 4	125 (58–218)	4/10 (40)
	4% + 10 mg/kg	14, 1	66 (35–99)	3/10 (30)
	2% + 10 mg/kg	14, 3	77 (35–246)	0/10 (0)
	2% + 5 mg/kg	14, 3	67 (58–80)	5/10 (50)
	2% + 2.5 mg/kg	14, 3	70 (35–91)	0/10 (0)

[a] For combinations, the first and second numbers refer to the first and second drug listed, respectively.
[b] Started on day 35 postinfection.

infections. This relationship extends to synergism with 9-DINO, since at 0.5% in drinking water, ΔMFMO · CH_3 was completely effective against the TREU 667 screen (Table 3) when combined with 50 or 100 mg 9-DINO per kg and administered for 7 days.

Sinefungin, used singly at 10 mg/kg for seven doses, was 60% curative. At 2.5 mg/kg for four doses in combination with 2% DFMO, it protected 55% of the animals (Table 3). Formycin B, used at 100 mg/kg for six doses, was not curative for TREU 667-infected mice, and when it was used in combination with 4% DFMO (14-day regimen), only 10% of the treatment group were cured. Other combinations of DFMO plus formycin B were equally ineffective, producing only random cures (Table 3).

The LUMP 1001 model was also sensitive to combinations of DFMO and 9-DINO: 80 to 90% cure rates were obtained with 2% DFMO (for 14 days) plus 50, 100, or 250 mg of 9-DINO per kg (for 7 days) (Table 4). Animals relapsing in these groups did so soon after treatment ended (day 35), indicating that some animals may not have taken up sufficient DFMO. Sinefungin varied in its activity against the LUMP 1001 strain: in combination with DFMO it was 50% curative at 5 mg/kg (for 3 days), but extended the average period of aparasitemia only to 77 days if given at 10 mg/kg (days 21 to 24 postinfection) with 2% DFMO. If the same dose of sinefungin (10 mg/kg for 4 days) was delayed until the end of DFMO administration (day 35), 40% cure rates were obtained.

TABLE 5. 9-DINO levels in serum at various dosage concentrations[a]

Dosage (mg/kg)	9-DINO concn (μg/ml of serum)	% Serum radiolabel as 9-DINO	% Radiolabel as water
200	9.7	25	75
100	2.3	14	86
50	0.4	11	89
25	0.2	11	89

[a] Groups of 3 animals were given (i.p.) the appropriate dose of 9-DINO, each containing 1 μCi of [2,8-^{3}H]9-DINO. Mice were exsanguinated 4 h after the last dose, and the total drug concentration and total label were determined by HPLC and scintillation spectroscopy as described in Materials and Methods.

Metabolic studies. Since 9-DINO proved to be very effective against these organisms, both alone and in combination with DFMO, experiments were carried out to determine the approximate levels in serum and the metabolites formed at the various dosages. When acid-soluble serum extracts were analyzed by RP-HPLC, two radioactive peaks were found. The first peak, which contained the majority of the radioactivity (85 to 90%), was recovered in the void volume eluate, while the second peak coeluted with 9-DINO. When these samples were separated by SAX-HPLC, all the radioactivity was recovered in the void volume, showing that no nucleotide metabolites of 9-DINO were present. Further investigation showed that the major radioactive peak was lost and the 9-DINO peak was not affected when the samples were lyophilized and reconstituted before RP-HPLC analysis. The lost radioactivity could be quantitatively recovered in the water frozen in the cold trap. Overnight treatment of serum extracts with activated charcoal before HPLC separation removed only the 9-DINO peak, indicating that the other peak is not a purine-type ring structure. These results are all consistent with loss of the tritium from both the C-2 and C-8 positions of 9-DINO and the subsequent formation of tritiated water. On the basis of the specific activity used to dose the mice and the radioactivity recovered as 9-DINO after RP-HPLC separation, it was found that a dosage of 200 mg/kg resulted in drug levels in serum of 9.7 μg/ml at 4 h after the last injection, while the lowest dosage (25 mg/kg) resulted in a drug level of 0.2 μg/ml in serum at 4 h postinjection (Table 5). Analysis of the acid-soluble extracts of the pooled livers and kidneys from mice treated with 200 mg of [^{3}H]9-DINO per kg by SAX-HPLC indicated no conversion of the drug to nucleotide metabolites, since the only radioactive peak coeluted with the void volume.

Previous work with unlabeled drug has shown that bloodstream forms of *T. brucei* subsp. *gambiense* convert 9-DINO to 9-deazaadenosine triphosphate (15). More recent metabolic studies with *Leishmania donovani* and radiolabeled

TABLE 6. Nucleotide metabolites formed after incubation of cells for 3 h in the presence of 40 μM [2,8-^{3}H]9-DINO

Metabolic product[a]	Amt of product (pmol/10^6 cells) in:	
	EATRO 110	RBCs
9-DINO-MP	0.027	0.64
9-DINO-DP	ND[b]	0.43
9-DINO-TP	ND	0.63
9-DADO-MP	0.139	ND
9-DADO-DP	1.46	ND
9-DADO-TP	2.72	ND
9-DGAO-MP	ND	ND
9-DGAO-DP	0.011	ND
9-DGAO-TP	0.021	ND

[a] 9-DADO, 9-Deazaadenosine metabolites; 9-DGAO, 9-deazaguanosine metabolites.
[b] ND, No metabolites detected (<0.005 pmol/10^6 cells).

drug have shown that 9-DINO is converted not only to the 9-deazaadenosine analogs of AMP, ADP, and ATP, but also to the 9-deazaguanosine analogs of GMP, GDP, and GTP (20). In this study, similar work was done with the bloodstream forms of the EATRO 110 strain to see how this strain metabolizes this compound (Table 6). As was found for *L. donovani*, bloodstream forms of the EATRO 110 strain convert 9-DINO to the 9-deaza analogs of both AMP, ADP, and ATP and GMP, GDP, and GTP. RP-HPLC analysis of the acid-soluble extract of mouse RBCs incubated under the same conditions as the EATRO 110 bloodstream forms resulted in the elution of three radioactive peaks (Table 6). One peak in the monophosphate region of the chromatograph was identified as 9-DINO monophosphate by its A_{254}/A_{295} UV ratio and its coelution with the authentic compound. The other two peaks could not be identified by their A_{254}/A_{295} ratios, since they coeluted with ADP and ATP. These peaks have tentatively been identified as the respective 9-DINO analogs of IDP and ITP on the basis of the relative shifts in retention times between ADP and ATP and their respective 9-deaza analogs and the retention times of IDP and ITP compared with the two radioactive peaks. In addition, when the RBC acid-soluble extract was treated overnight with alkaline phosphatase and then subjected to RP-HPLC analysis, only radioactive 9-DINO was recovered.

DISCUSSION

The data presented in this study indicate that 9-DINO is active against both acute and CNS models of *T. brucei* subsp. *brucei* and that it is highly synergistic with ornithine decarboxylase inhibitors. There was no evidence of toxicity when 9-DINO was used in prolonged (i.e., 1-week) dose regimens, and the agent was more effective than sinefungin, allopurinol, and allopurinol riboside. Formycin B, although active against the acute model in low doses, was ineffective in the CNS model, and appeared toxic at higher doses. The reason for the synergism of 9-DINO with DFMO is unknown, but presumably may be that both compounds act to limit nucleic acid metabolism of trypanosomes. DFMO causes cessation of polyamine synthesis and of nucleic acid synthesis within 12 h of in vivo administration (1), while 9-DINO is converted into analogs of both ATP and GTP within 3 h in bloodstream trypomastigotes as reported here. This appears to be the basis for differential effects of 9-DINO on parasite and host cells.

The impetus for the present study on the trypanocidal activity of purine analogs against *T. brucei* subsp. *brucei* model infections stems from the activities of these purine analogs in vitro and in some in vivo systems against *Leishmania* spp. (6, 27), *T. cruzi* (22), and African trypanosomes (12).

9-DINO is a C-C nucleoside analog which is metabolized to 9-deaza-ATP by *T. brucei* subsp. *gambiense* and *T. brucei* subsp. *rhodesiense* (14). It is neither toxic for mouse L cells nor metabolized to adenosine nucleotide analogs by these cells (20). It had not previously been studied in vivo in trypanosome infections but was recently proven active against an experimental *L. donovani* infection (5). Previous studies have shown that 9-DINO is more active than allopurinol, thiopurinol, and their respective nucleosides against *T. cruzi*, *Leishmania* spp., and African trypanosomes (22).

Both the TREU 667 and LUMP 1001 CNS models were cured with 9-DINO plus DFMO or 9-DINO plus ΔMFMO · CH_3. In general, the degree of synergism found with these combinations parallels that found with DFMO plus bleomycin or DFMO plus suramin: a two- to eightfold reduction in dose levels of both agents (10, 11). ΔMFMO · CH_3 was 4- to 12-fold more active than DFMO in curing acute and, in combination with suramin, CNS infections (2) and appeared here to be at least 4 times more potent than DFMO.

Sinefungin was less active than 9-DINO and formycin B in the short-term EATRO 110 model and did not produce complete cures when used either singly or in combination with DFMO against the TREU 667 and LUMP 1001 CNS models. The low activity of sinefungin both singly and in combination with DFMO is disappointing in light of previous work in which this compound cured a chronic infection by a strain of *T. congolense* as well as acute infections by *T. brucei* subsp. *brucei* and *T. vivax* strains (12). In the previous study sinefungin was administered as multiple daily doses at 2- or 8-h intervals and at lower total doses than in the present work; e.g., the *T. brucei* subsp. *brucei* infection was 100% curable with three 5-mg/kg doses administered at 8-h intervals. *T. congolense* responded to a total dose of 0.45 mg, given as nine injections 2 h apart. These findings indicate that there are significant differences in the susceptibility of strains to sinefungin.

Although formycin B was extremely active when used singly on the EATRO 110 model, it was not dramatically synergistic with DFMO (Table 1). In the TREU 667 (CNS) model, formycin B failed to protect at 100 mg/kg for 6 days, while in combination with DFMO at 4%, formycin B was not effective. Thus formycin B may also exhibit species specificity. Indeed, it is completely inactive against one *T. brucei* subsp. *rhodesiense* strain which responds to 9-DINO (C. J. Bacchi and H. C. Nathan, unpublished data).

In several instances, one or several animals per group recrudesced after being given seemingly curative combinations of DFMO plus 9-DINO (e.g., Table 3, 2% DFMO plus 250 mg of 9-DINO per kg; Table 4, 2% DFMO plus 250 or 100 mg of 9-DINO per kg). Although this could be attributed to normal individual variation in response to treatment, there may be an alternative explanation. A recent study has assessed the pharmacokinetics of oral DFMO administration in mice (30). The conclusions reached indicate that since most of the drug is consumed at night, these DFMO levels in serum are 10- to 15-fold greater than levels in serum during the day. In addition, the average variation in consumption between individual animals was 23%. Although we measured the consumption of DFMO and ΔMFMO · CH_3 in groups of animals and found it to be relatively constant (2), underdos-

ing could easily have occurred in some individuals within the group.

9-DINO appears to be maintained in serum at levels sufficient for significant activity in vivo. The levels of 9-DINO in the plasma samples taken approximately 4 h postinjection on day 3 of a 3-day treatment schedule ranged from 9.7 to 0.2 μg/ml for dosages between 200 and 25 mg of 9-DINO per kg of body weight, respectively (Table 5). Previous evidence from drug tests with a tissue culture system infected with *T. brucei* subsp. *gambiense* suggests that concentrations in serum as low as 0.25 μg/ml could be effective against these parasites (14). The finding presented here that a dosage of 25 mg/kg cured 75% of the mice treated and, when combined with DFMO, cured 100% of the treated population supports this (Table 1). The unexpected finding from these serum studies was the rapid loss of the tritium from both the C-2 and C-8 carbons of 9-DINO. It is believed that this loss of label to water is due to the oxidation of 9-DINO by the aldehyde oxidase of the liver to the 9-deaza analog of uric acid riboside (9-deaza-2,4,8-trioxopurine riboside). This conclusion is based on the ability of liver aldehyde oxidase to use 9-DINO as a substrate (16) and our findings that the DBA/2 mice, which have relatively low levels of this enzyme (19), had much higher levels of 9-DINO in serum than Swiss Webster mice did when treated at the same dosage (data not shown). However, since no new UV peaks were found on the RP-HPLC tracings and we had no authentic compound, we could not verify this.

There was no evidence for the presence in the serum of the 9-deaza analog of xanthosine (the compound formed if only the C-2 position is oxidized), since no radioactivity coeluted with authentic 9-deazaxanthosine during RP-HPLC separations. This compound should have been detectable as a radioactive peak, since there is at least some tritium at the C-8 position because labeled 9-deazaguanosine nucleotides are present in the parasite extracts. In any case, the presence or absence of this compound in the serum should not have had any effect on the parasite, since concentrations as high as 25 μg/ml caused no inhibition in the growth of procyclic culture forms (data not shown).

The metabolites formed by freshly isolated EATRO 110 bloodstream forms during incubation with 9-DINO were found to be identical to those previously reported for *L. donovani* (22). A similar incubation of mouse RBCs resulted only in the formation of 9-DINO monophosphate as well as its di- and triphosphates. There was no further metabolism of 9-DINO to analogs of either adenosine or guanosine nucleotides in these cells. The phosphorylation of 9-DINO monophosphate to its di- and triphosphates has been previously reported for two human cell lines and appears to have no toxicity associated with it (J. J. Marr and R. L. Berens, Fed. Proc. **45**:1528, 1986). The formation of the adenosine and guanosine nucleotide analogs by these parasites and their lack of formation in mammalian cells seem to be necessary in the mechanism of action for the differential toxicity of this compound.

Formycin B, another C-C nucleoside derivative, was active against *Leishmania* spp. and *T. cruzi* in vitro (9, 28, 29), as well as bloodstream and culture forms of African trypanosomes (14). Both formycin B and 9-DINO are C-nucleoside purine analogs which are not cleaved into ribose and purine analog moieties by mammalian cells. Both compounds can be aminated by *T. brucei* subspecies and converted to analogs of ATP (14). A major difference between the two, however, is that formycin B is metabolized by mammalian cells to its aminated analog, formycin A, which can then be incorporated into nucleic acids. The conversion of formycin B to formycin A of adenine nucleotides in mammalian systems is well known. Mouse L cells can convert formycin B to the analogs of adenine nucleotides and incorporate the ATP analog into RNA (34). This raises the likely possibility that this can occur in humans. Berman et al. (6) showed that this occurred in human macrophages and that the toxicity of a series of formycin analogs to *L. tropica* was paralleled by their toxicity for these macrophages (7). Glazer and Lloyd (15) demonstrated that formycin A nucleotides can be incorporated into DNA of human colon carcinoma cells. 9-DINO is not metabolized to adenine nucleotide analogs in mouse L cells (20), U937, or VA-13 human cells (Marr and Berens, Fed. Proc., 1986), the only systems which have been studied thus far. In the present study, some animals dosed at 100 mg of formycin B per kg experienced partial hair loss before the parasitemia recrudesced (Table 3). This was not observed in 9-DINO-treated animals.

Overall, these studies demonstrate the superior in vivo activity of 9-DINO against trypanosome infections and significant synergism with DFMO in curing CNS infections in *T. brucei* subsp. *brucei*. Further in vivo studies will center on the use of these agents in experimental infections with the human pathogens *T. brucei* subsp. *rhodesiense* and *T. brucei* subsp. *gambiense*.

ACKNOWLEDGMENTS

This investigation was funded by Public Health Service grants AI 17340 (C.J.B.), AI 121909 (R.L.B.), AI 19781-01 (J.J.M), N.C.I.-D.D.H.S.CA-24634 (R.S.K.) from the National Institutes of Health, by funds from the United Nations Development Program/World Health Organization/World Bank (C.J.B., J.J.M., and R.L.B.), by grant CH-305 (R.S.K.) from the American Cancer Society, and by an award from the Pace University Scholarly Research Committee. I.A.E. is the recipient of a United Nations Development Program/World Health Organization Research Training Grant.

We are grateful to R. L. Miller for synthesis of the monophosphates of the 9-deaza purine analogs. Prima Barnes, Beverly K. Raab, and Glenn Valadares provided excellent technical assistance.

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