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Novel combination chemotherapy of experimental trypanosomiasis by using bleomycin and DL-α-difluoromethylornithine; reversal by polyamines*

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Previously we reported that an irreversible inhibitor of polyamine biosynthesis, DL-α-difluoromethylornithine (DFMO), cured mice infected with *Trypanosoma brucei brucei*, a parasite of game and cattle in Africa [1], and also cured similar infections of *Trypanosoma b. rhodesiense*, a human sleeping sickness pathogen [2]. DFMO is a specific inhibitor of ornithine decarboxylase (ODC), the major rate-controlling enzyme of polyamine biosynthesis [3–5], and slows tumor cell replication *in vitro* and *in vivo* [6, 7]. Trypanosome cures effected by DFMO can be blocked by coadministration of the commonly occurring polyamines putrescine, spermidine or spermine [2, 8]. We have found recently that another agent, the potent antitumor antibiotic Blenoxane (a commercial mixture of bleomycinic acid derivatives, hereafter referred to as bleomycin) also cures this infection [9]. Bleomycin inhibits nuclear division and causes malformation of the nucleus and disorders of microtubule morphology in *Trypanosoma b. gambiense* [10], another human sleeping-sickness pathogen. Cures by bleomycin can also be blocked by spermidine and spermine but not putrescine [9]. Thus, two agents which have antitumor properties also seem to restrict parasite growth in infected animals in a manner analogous to their antineoplastic effects.

Modern chemotherapies of neoplastic disease in fact routinely employ drugs in combination [11, 12]. Such regimens in some instances have proven significantly better for prolonging life and even obtaining permanent clinical remission than single-drug therapy [11, 12]. In contrast to the highly developed protocols for neoplastic disease, no such drug-combination regimens have been developed for chemotherapy of hemoflagellate infections. These rapidly growing organisms, including *Leishmania* spp. and *Trypanosoma* spp. cause several debilitating diseases such as visceral leishmaniasis, Chagas' disease and sleeping sickness, and therefore cause vast human suffering and economic loss in Asia, Africa and South America. New drugs or drug combinations are needed to supplant the toxic and often ineffective agents currently in use for these diseases [13–15]. We now report successful combination chemo-

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Table 1. Combination therapy of *Trypanosoma b. brucei* in mice with DFMO and bleomycin*

DFMO (% in drinking water)	Survival (days past the untreated controls)									
	Bleomycin (mg/kg)									
	0	0.1	0.2	0.25	0.3	0.4	0.5	0.75	1.0	3.0
0	0†	0	0	3	12	13.6	16	18.5	27.8	>30
0.1	0	8	10	12.6	10.2	16.3	20.4			
0.2	0	15	22.5	18.6	23.2	28.1				
0.25	0.5	20	21.1	>30	>30		>30			
0.3	1.5	22.1		>30	>30					
0.4	3.1									
0.5	4	28.1					>30			
0.75	28	>30								
1.0	>30									

* Mice with 24-hr infections were given various concentrations of DFMO for 3 days in the drinking water or single daily i.p. injections of bleomycin for 3 days, or both. At least thirty mice were used for each of the data points. Results are scored as average survival in days beyond control death. Animals surviving >30 days were considered cured after routine examination for parasites in tail-vein blood smears.
† Controls.

therapy of *T. b. brucei* in mice through synergistic action of DFMO and bleomycin, and the reversal of cures by polyamines (some of these data have been reported in a preliminary communication: Ref. 16).

Materials and methods

Organism. The EATRO 110 isolate of *T. b. brucei*, maintained and used as described [1, 17, 18], produces a rapidly fatal (3–6 days) parasitemia on inoculation of 2–5 × 10^5 parasites/rat or mouse.

Materials. α-DFMO (RMI 71,782) was obtained from Merrell–Dow Pharmaceuticals Inc., Cincinnati, OH. Bleomycin (as sterile, outdated Blenoxane) was a gift of Bristol Laboratories, Syracuse, NY. Polyamines were obtained from the Sigma Chemical Co., St. Louis, MO.

Experimental procedure. The experimental model of this infection has been described [1, 17, 18]. Groups of mice were inoculated (time 0) with 2–5 × 10^5 rat-passaged trypanosomes. Drug therapy was started 24 hr after infection and was continued for 3 days. DFMO was administered *ad lib.* in the drinking water as described [1, 8]. Sterile bleomycin (aqueous) was injected i.p. once daily, alone, or concurrent with the DFMO. Polyamines were also administered i.p. as a single daily injection. Animals receiving both bleomycin and polyamines were given separate injections. Animals were checked daily throughout the experiments, and were considered cured if surviving >30 days beyond control deaths with no parasites visible in peripheral blood smears. Total DFMO doses may be calculated based on average fluid intake of ~5 ml per animal per day (e.g. 150 mg/day, on intake of 5 ml of a 1% solution). Parasites were counted by hemocytometer in tail-vein blood samples for parasite densities >10^6/ml; below 10^6 organisms/ml, Giemsa-stained blood smears were used to estimate cell numbers.

Results

Table 1 summarizes the course of infection with individual dosage schedules of each drug and with the two drugs in combination. As evident, low (<0.5%) doses of DFMO had little effect on the course of infection. Bleomycin likewise was ineffective up to 0.3 mg per kg per day. Routinely, 3 mg/kg of bleomycin or 1% DFMO was needed to clear the infection. Some combinations of individually subcurative doses of the drug acted synergistically, a feature most conspicuous with 0.25% DFMO + 0.25 mg/kg bleomycin (Table 1). These values represented $\frac{1}{4}$ and $\frac{1}{12}$ of the individual curative levels of DFMO and bleomycin respectively. Further evidence for synergism can be seen in the lower dose ranges in Table 1. Thus, although 0.2% DFMO or 0.2 mg/kg bleomycin alone did not cure, the combination allowed survival of animals for >20 days beyond control deaths. Small increments in the DFMO dose concentration (e.g. from 0.1 to 0.2%, or 0.25%), in combination with 0.25 mg/kg bleomycin, markedly increased survival time (e.g. 12.6 to >30 days) when compared to survival due to increases in bleomycin at constant DFMO levels. For example, increasing the bleomycin dose from 0.1 to 0.25 mg/kg at 0.1% DFMO increased survival time only from 8 to 12.6 days. Hence, DFMO concentration seems to be the most significant variable determining the effectiveness of the drug combinations.

The effect of a DFMO (0.25%) + bleomycin (0.25 mg/kg) drug combination on blood parasitemia was dramatic: animals receiving the combination were cleared of parasites 48 hr after treatment ceased (day 6 post-infection). Animals receiving either 0.25% DFMO or 0.25 mg/kg bleomycin singly developed severe parasitemia (>10^8 parasites/ml blood) and died on the fifth to eighth day (not shown).

As mentioned, we had found that putrescine, spermidine, and spermine antagonized the curative effects of DFMO in *T. b. brucei* infection [2, 8] while bleomycin cures were annulled by spermidine and spermine but not by the diamine, putrescine [9]. Because of the unusual synergism between the drugs, it seemed of interest to study polyamine reversal in drug combination-treated animals. Each of the three polyamines blocked the effect of the lowest curative drug combination (Table 2), and two putrescine analogs not generally present in cells, 1,3-diaminopropane and cadaverine (1,5-diaminopentane), had no effect. The same levels of polyamines also blocked the activity of double the curative dose levels, although the animals survived for a longer time. As shown in Fig. 1, coadministration of polyamines with DFMO/bleomycin resulted in increases in parasite numbers eventually about equal to those found in untreated animals, with subsequent death of the animals. Infected animals, treated with polyamines alone, died at the same time as untreated controls (not shown).

Discussion

These results clearly point to synergism between two chemically unrelated agents, DFMO and bleomycin. While the precise mechanism of the DFMO–bleomycin synergism is not readily apparent, it seems clear that it is based on a polyamine mechanism since the curative effects of the two drugs, whether used alone or in combination, were antagonized by polyamines. We have shown that *T. b. brucei* ornithine decarboxylase is highly sensitive to DFMO

Table 2. Reversal of DFMO/bleomycin cures of *T. b. brucei* by polyamines*

Drug treatment	Polyamine treatment	mg/kg	Average survival (days)
0.25% DFMO + 0.25 mg/kg bleomycin			>30
0.25% DFMO + 0.25 mg/kg bleomycin	Spermine·4HCl	50	16.1
0.25% DFMO + 0.25 mg/kg bleomycin	Spermidine·3HCl	100	13.6
0.25% DFMO + 0.25 mg/kg bleomycin	Putrescine·2HCL	500	9.7
0.25% DFMO + 0.25 mg/kg bleomycin	Cadaverine·2HCl	500	>30
0.25% DFMO + 0.25 mg/kg bleomycin	1,3-Diaminopropane	500	>30
0.5% DFMO + 0.5 mg/kg bleomycin			>30
0.5% DFMO + 0.5 mg/kg bleomycin	Spermine·4HCl	50	24.3
0.5% DFMO + 0.5 mg/kg bleomycin	Spermidine·3HCl	100	19.3
0.5% DFMO + 0.5 mg/kg bleomycin	Putrescine·2HCl	500	20.3

* Animals were inoculated and drugs were administered as described in the legend to Table 1. Polyamines were administered concurrently with drugs as separate i.p. injections. Polyamines, administered singly to infected animals, did not influence the course of infection.

in vitro (50% inhibition at 10^{-6} M; [19]), and parasites in heavily infected rats are rapidly depleted of polyamines after even a 12-hr exposure to the compound [16].

Polyamines decrease bleomycin cytotoxicity in tissue culture [20]. As noted, the biochemical reasons for these nullifications of bleomycin action are not clear, but might relate to drug synergism as reported here. The congeners of bleomycin contained in the commercial preparations are distinguished by the type of amine moiety attached to a terminal bithiazole group. The major components of commercially available bleomycin preparations contain agmatine, spermidine, spermine or aminopropyl-dimethylsulfonium as the amine-containing groups [21]. These positively charged groups are essential for the subcellular activity of the drug, i.e. the scission of single- and double-stranded DNA with the release of free bases and oligonucleotides [22–24]. Thus, it seems reasonable to relate drug synergism mechanism to intracellular polyamine depletion. Such DFMO-induced polyamine depletion might alter the cellular effects of bleomycin by: (a) increased cellular uptake of bleomycin since amine groups are part of the molecule; it is known that polyamine-depleted tumor cells have enhanced transport (uptake) of polyamines and of the polycationic drug, methylglyoxal-bis(guanylhydrazone) (MGBG) [25]; (b) impaired DNA repair mechanisms which would normally allow some parasitic cells to recover from the bleomycin effect [26]; and (c) inhibition of parasites in a specific phase of the cell cycle particularly susceptible to bleomycin as has been suggested from results in tumor cells in culture subjected to a combination of DFMO + Ara-C [27].

Since bleomycin is in current clinical use against certain tumors [21, 28], and DFMO is being similarly tested against leukemia [29] and small-cell lung carcinomas [7], the

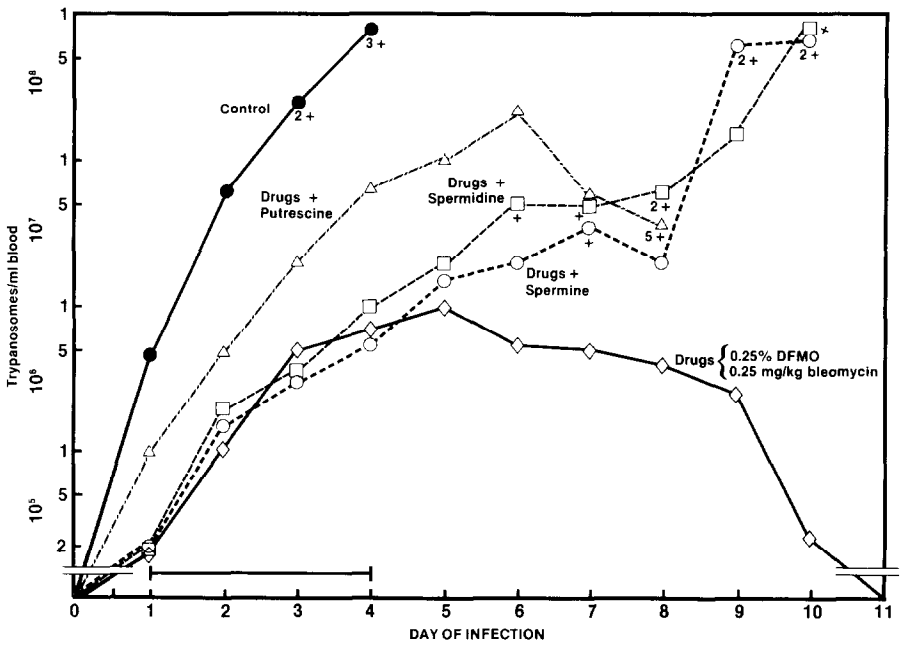

Fig. 1. Negation of the therapeutic effects of a DFMO/bleomycin combination (0.25% + 0.25 mg/kg) by polyamines. Results of a typical experiment in which groups of five animals with 24-hr *T. b. brucei* infections were treated with 0.25% DFMO in the drinking water (3 days) + 0.25 mg/kg bleomycin (single daily i.p. injections for 3 days), and in which polyamines were administered concurrently as single daily i.p. injections (|——|). Doses of polyamines were: putrescine·2HCl, 500 mg/kg; spermidine·2HCl, 100 mg/kg; and spermine·4HCl, 50 mg/kg. Tail-vein blood samples were taken daily, and parasite density was estimated as described in the text. Deaths of animals are indicated by the number of +s (+ = 1 death).

polyamine-related synergism reported here can now be explored as a clinical regimen against hemoflagellate protozoa. With the recent exception of rifampicin and isoniazid [30], no drug combinations have been applied to hemoflagellate chemotherapy although a combination of sulfadiazine + pyrimethamine has long been a standard in malarial chemotherapy [31]. The doses of bleomycin used by us were well below toxic levels—an important consideration with this agent whose repeated use is also constrained because it may cause an often fatal pulmonary fibrosis [32]. The pulmonary toxicity has been related to the amine side chains, since most of the amines alone, including spermidine and spermine, can cause similar fibrosis when instilled intratracheally in mice [33]. We found no evidence of gross bleomycin toxicity in this combination drug study, nor where it was used as a single agent against *T. b. brucei* in mice [9].

In this study, two antitumor agents—one of them a potent inhibitor of polyamine synthesis—acted synergistically to cure a model acute parasitemia by *T. b. brucei*, the veterinary subspecies of African sleeping sickness. Cures were blocked by coadministration of polyamines with the drug. The efficacy of these drugs suggests a trial of such a combination chemotherapy for African trypanosomiases. There is also good reason to believe that this drug combination will be effective in the late (central nervous system) stage of the disease, since a DFMO–bleomycin regimen cures a late-stage model infection (TREU 667 isolate of *T. b. brucei*) which is refractory to the standard trypanocide Berenil (diminazene aceturate) ([16], and A. B. Clarkson, G. Mellow, C. J. Bacchi and P. P. McCann, unpublished observations). That polyamines can annul the activity of the DFMO–bleomycin drug combination as well as that of other trypanocidal agents re-emphasizes the importance of polyamines in trypanosome metabolism [34–36].

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