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SHORT COMMUNICATIONS

Prevention by polyamines of the curative effect of amicarbalide and imidocarb for *Trypanosoma brucei* infections in mice*

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Prevention of chemotherapeutic action of a drug by co-administration of nutrients or metabolites is relatively rare. Whenever demonstrable, such an interrelationship deserves examination with regard to the mode of action of the drug. We have found that two known babesicides [1], amicarbalide (3,3′-diaminocarbanilide) and imidocarb [(3,3′-bis-2-imidazolin-2-yl)carbanilide], are curative for a virulent *Trypanosoma brucei* (EATRO 110) infection in mice [2]. We report here that the curative action of these drugs can be prevented by co-administration of spermidine and spermine, but not by putrescine (1,4-diaminobutane).

Bovine and human trypanosomiases continue to be serious afflictions in sub-Saharan regions of Africa. Drug resistance and severe toxicity limit the use of the few drugs now available [3, 5]. Unfortunately, there is little information on the primary binding sites of the available drugs that might lead to development of new agents [6]. We have found that prevention of therapy with amicarbalide and imidocarb by spermidine and spermine also applies to several well-known, widely used antitrypanosomal agents.

Materials and Methods

Organism. The EATRO 110 isolate of *T. b. brucei* was obtained from Dr. A. B. Clarkson (New York University Medical Center) and serially syringe-passaged in mice. This pleomorphic strain produces a rapidly fatal (3–6 days) infection.

Inoculum and drug administration. This was done as in Nathan *et al.* [2]. Briefly, blood from a mouse with a 72-hr infection was obtained by cardiac puncture, and was diluted with sterile buffer (0.09 M Tris, 5 mM saline, 2% glucose, pH 7.8) to a final concentration of $\sim 8\,10^5$ organisms/ml. Groups of five (20–30 g) animals, mixed males and females, were inoculated i.p. with 0.25 ml ($2\,10^5$ organisms). After 24 hr, drug treatment was begun and consisted of three daily doses at 24-hr intervals. Polyamines were administered concurrently in separate i.p. or subcutaneous injections.

Experiments consisted of eight to eleven groups of animals, infected with the same inoculum. Each drug/polyamine combination experiment contained a control (uninfected) group, as well as groups dosed singly with polyamine or drug. Survival was measured as the average survival in days beyond death of control animals.

Cell counts. These were done on tail-vein blood with Becton–Dickinson Unopettes (BD No. 5855) and improved Neubauer hemocytometers.

Results and Discussion

Imidocarb and amicarbalide are effective babesicides [1]. Imidocarb has a broader therapeutic range as indicated by its effectiveness against bovine anaplasmosis [7]. When administered daily to mice for 3 days, starting 24 hr after infection with *T. b. brucei*, both drugs cured at doses between 10 and 25 mg/kg (Table 1). At lower doses, the survival time was prolonged. Spermidine, 30–300 mg/kg in saline, given i.p. for 3 days with either drug, blocked cures by amicarbalide (10 and 25 mg/kg) and imidocarb (5 and 10 mg/kg). At doses of 30 mg/kg and higher, spermidine decreased survival time and at 300 mg/kg completely prevented the curative effect. Spermine acted similarly, blocking cures at a dose of 100 mg/kg. Putrescine, however, even at 500 mg/kg, was inactive.

To evaluate toxicity of the polyamines or their interaction with the drugs, uninfected mice were treated with higher doses of the drugs and polyamines. Animals, usually groups of ten, were dosed separately or in combination with either amicarbalide (25 mg/kg) or imidocarb (10 mg/kg), along with either spermidine (300 mg/kg) or spermine (100 mg/kg), for 3 successive days. Some deaths occurred in groups that received amicarbalide (25 mg/kg) plus spermine (200 mg/kg) (four deaths/eleven animals) or spermidine (300 mg/kg) (two deaths/ten animals). At these high doses, however, toxicity did not become manifest until 10 days after initiation of therapy.

Trypanosomes in the peripheral circulation of the treated animals were counted daily, and the counts were compared with those of untreated infected mice. In mice in which cures by both drugs were negated by spermine or spermidine, the number of trypanosomes at the time of death closely paralleled the numbers in infected, untreated control animals. Although survival in separate experiments varied from 3 to 6 days, control infected and polyamine-treated infected mice died within 24 hr of each other (Fig. 1). To detect any drug–polyamine interaction, drugs were given i.p. and polyamines s.c. and vice versa. The route of administration did not affect the outcome of the experiment.

Dave *et al.* [8] observed that Trypanosomatidae have ornithine decarboxylase (ODC: EC 4.1.1.17) and putrescine-activated *S*-adenosylmethionine (SAM) decarboxylase (EC 4.1.1.50). Since the evidence suggested that polyamine syntheses may be inhibited, amicarbalide and imidocarb were tested for activity as inhibitors of ODC and SAM decarboxylase in *T. b. brucei* homogenates. ODC catalyzes formation of putrescine from ornithine, and SAM decarboxylase is involved in the transfer of an aminopropyl group from SAM to putrescine to form spermidine. ODC was assayed according to a liquid cation exchange procedure† and SAM decarboxylase according to Pegg [9]. Under these conditions, the average specific activity of the trypanosome enzyme was 11.4 $\text{nmoles} \cdot \text{hr}^{-1} \cdot (\text{mg protein})^{-1}$ for ODC and 5.7 $\text{nmoles} \cdot \text{hr}^{-1} \cdot (\text{mg protein})^{-1}$ for SAM decarboxylase. Although both drugs inhibited rat liver SAM decarboxylase, neither drug inhibited the trypanosomal enzyme. Moreover, there was no inhibition of trypanosomal ODC by either drug even at $1\ 10^{-3}$ M.

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† D. S. Duch, unpublished observations.

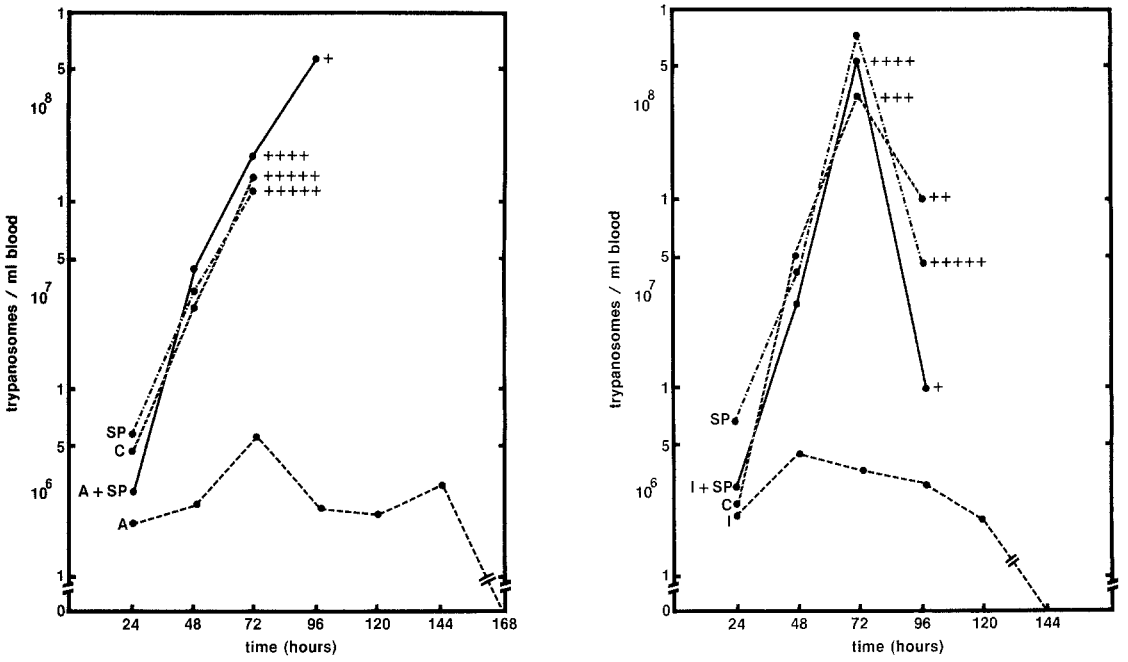

Fig. 1. Panel A: Effects of spermine (100 mg/kg) on amicarbalide (25 mg/kg) treatment of *T. b. brucei* infection. Groups of five animals were infected (5 10^5 trypanosomes/animal). After 24 hr, animals were dosed three times at 24-hr intervals. Key: a plus sign (+) marks the death of the animal. Animals were examined at 24-hr intervals: a (+) at 72 hr indicates death between 72 and 96 hr. Abbreviations: (A) amicarbalide; (SP) spermine; and (C) control. Panel B: Effects of spermine (100 mg/kg) on imidocarb treatment of *T. b. brucei* infection. Conditions are the same as those given in the legend for Panel A. Abbreviations: (SP) spermine; (I) imidocarb; and (C) control.

To study the effect of these drugs on biosynthesis of polyamines, intact trypanosomes from rat blood [10] were washed and then incubated for 1 hr with imidocarb and amicarbalide (25–250 μM) in the presence of [^{3}H]ornithine and unlabeled methionine, or with unlabeled L-ornithine and [^{14}C]methionine [11]. Polyamines, as dansyl derivatives, were separated from cell extracts by thin-layer chromatography, and radioactivity was estimated as described [11]. Incorporation of label into putrescine or spermidine was unaffected at the drug concentrations (25–250 μM) tested.

Effects of both drugs on polyamine uptake were also examined on intact trypanosomes from rat blood ethidium, pentamidine, and the SAM decarboxylase inhibitor methylglyoxal-bis(guanylhydrazone) (MGBG) interfere with uptake of putrescine and polyamine synthesis by sev-

Table 1. Effect of co-administration of polyamines and drugs on survival time of *T. b. brucei*-infected mice*

Treatment		Increased lifespan (days) in presence of drug				
		Imidocarb		Amicarbalide		
Polyamine	Dose/day (mg/kg)	(5 mg/kg)	(10 mg/kg)	(5 mg/kg)	(10 mg/kg)	(25 mg/kg)
None		24.9	>30	25.1	>30	>30
Putrescine	300			>30	>30	>30
	500		>30	>30	>30	
Spermidine	15	>30		28.6	>30	
	30		>30	>30	26.8	
	100	20.6		18.8	19.7	17.0
	150	15.0		15.1	14.3	16.5
	250	3.9	5.1			
	300		3.6			2.4
	500	1.9	3.0			
Spermine	25			15.9	4.3	>30
	50			11.4	19.1	>30
	100		0	0	0	0

* Groups of five animals (20–30 g Swiss–Webster mice) were infected with trypanosomes (2 10^5 organisms/animal) as described by Nathan *et al.* [2]. After 24 hr, drugs and/or polyamines were administered daily (i.p.) for 3 days. Results were scored as increased lifespan beyond death of controls; the survival time of infected untreated animals was 3–6 days. Animals surviving >30 days with no parasites in peripheral blood smears were considered cured. Results are the average of two or more trials (five mice/group); controls consisting of untreated, drug-treated, and polyamine-treated groups were always included in each combination experiment.

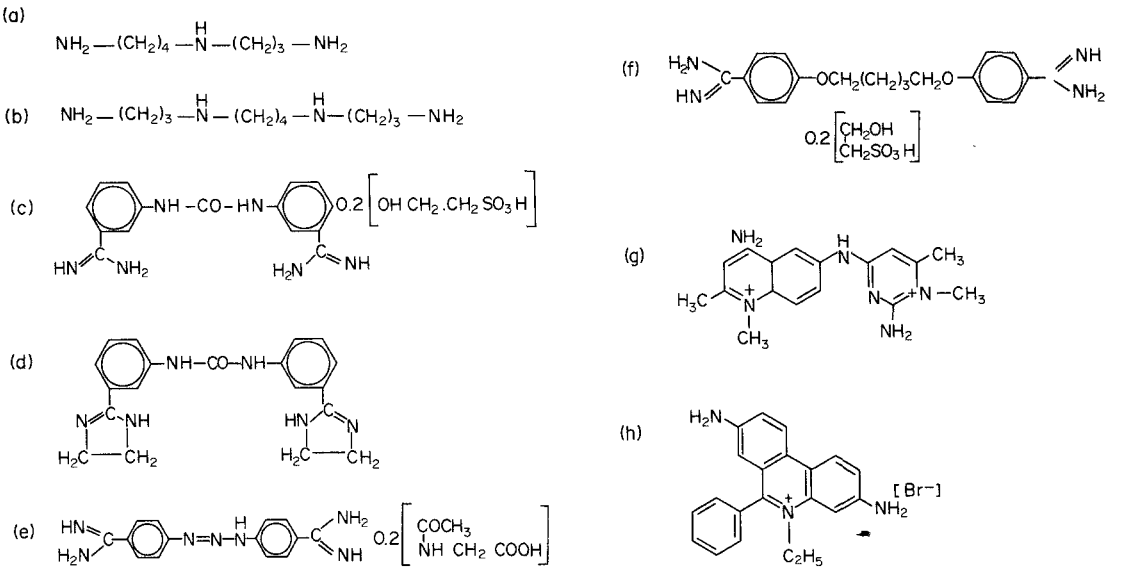

Fig. 2. Structures of: (a) spermidine; (b) spermine; (c) amicarbalide; (d) imidocarb; (e) Berenil; (f) pentamidine; (g) Antrycide; and (h) Ethidium bromide.

eral *Leishmania* spp. [12]. Imidocarb and amicarbalide at 250 μM inhibited uptake of [^{3}H]spermidine by 26 and 29 per cent respectively. Cell respiration, determined polarographically, was only slightly inhibited (10 per cent) under the same conditions. Inasmuch as polyamine uptake by *T. b. brucei* is low compared with that of other organisms [11], this partial inhibition of uptake is unlikely to account for prevention of therapy by polyamines.

Does this drug–polyamine interaction have implications for future therapy? Polyamines are ubiquitous in nature and influence many metabolic functions. They are essential growth factors for various bacteria and a *Neurospora* mutant [13]. Elevated levels of polyamines are associated with rapid growth in many mammalian cells [14, 15]. Of low molecular weight and strongly cationic, they can bind to many anionic sites on nucleic acids and other macromolecules. Their syntheses and functions are intricately regulated [16, 17]. Amicarbalide and imidocarb are also cationic compounds that were developed from phthalanilides with antileukemic, tuberculostatic, and antitrypanosomal (*T. b. brucei* and *T. congolense*) activities [18, 19]. Both drugs structually resemble diamidine drugs that are cationic at neutral or blood pH, e.g. diminazene (Berenil) (Fig. 2). Diamidines can inhibit nucleic acid and protein synthesis, disrupt ribosomal structure, and inhibit thymidylate synthetase [20–22]. The activity of diamidines, as well as of other cationic trypanocides, may relate to their cationic nature and in some instances appears to be directed against cellular polyamine function. Newton [23, 24] found that quinapyramine released putrescine from *Crithidia* ribosomal preparations. Wallis [21] also observed that pentamidine, propamidine, or stilbamidine released Mg^{2+} and polyamines from trypanosomatid ribosomal preparations. Miller and Peters [25] have observed a polyamine–diamidine interaction in which spermidine and spermine prevented bacteriostasis by propamidine. We found that the cationic trypanocides Ethidium (homidium) and Antrycide (quinapyramine) interfered with polyamine activation of the critical trypanosomatid respiratory enzyme NAD-linked α-glycerophosphate dehydrogenase [26, 27].

The structures of cationic trypanocides and of the polyamines spermidine and spermine were compared by assembling space-filling molecular models. The free rotation about the C—M, C—N or N—N bonds in the atoms bridging the phenyls of amicarbalide and imidocarb allows terminal amidino and imidazole nitrogen atoms to match the spacing between the first and third amine groups in spermidine or spermine (Fig. 2). Structural relationships of the amine groups of polyamines to those of quinapyramine and Ethidium are also clearly evident. Indeed, Sakai *et al.* [28] have demonstrated that Ethidium displaces spermidine from the tRNA molecule. We are extending studies of the polyamine–drug interaction to several standard cationic trypanocides. In preliminary work, spermine, but not putrescine, negated the trypanocidal activity of Antrycide and prothidium in the mouse *T. b. brucei* system [29].

The relative importance of polymines in the metabolism of pathogenic trypanosomatids is not yet clear, but, significantly, the SAM decarboxylase inhibitor MGBG induced temporary remission of *T. b. brucei* laboratory infections [2, 30]. It was also effective in blocking *in vitro* multiplication of *T. b. brucei* culture trypomastigotes; the latter effect, however, could not be reversed by co-administration of, or preloading, cells with spermidine or spermine [30]. Recently, another polyamine antagonist α-difluoromethylornithine (RMI 71,782), a specific, irreversible inhibitor of ODC [31], cured *T. b. brucei* laboratory infections [32]. This work strongly indicates that enzymes essential to polyamine synthesis may be useful in guiding drug design.

In this work we have demonstrated that two babesicidal agents, amicarbalide and imidocarb, are active in curing *T. b. brucei* infections and that the polyamines spermidine and spermine, but not putrescine, blocked the curative effect of both drugs. Polyamines have such diverse functions that one cannot presently attribute critical amicarbalide/imidocarb–polyamine interaction to a single receptor site or polyamine-dependent enzyme. These experiments, however, indicate that the cationic trypanocides can be used as metabolic probes to elucidate the manner in which the growth of these and other parasites is polyamine dependent, and that biosynthesis and function of polyamines in trypanosomes are attractive targets for new antiparastitic drugs.

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