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INHIBITION OF *TRYPANOSOMA BRUCEI BRUCEI* PEPTIDYL TRANSFERASE ACTIVITY BY SPARSOMYCIN ANALOGS AND EFFECTS ON TRYPANOSOME PROTEIN SYNTHESIS AND PROLIFERATION

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Abstract—Peptidyl transferase activity of *Trypanosoma brucei brucei* polyribosomes was competitively inhibited by analogs of sparsomycin (K_i = 1–100 μM). The analogs were also potent inhibitors of [^{3}H]-leucine and [^{3}H]mannose incorporation into the proteins of intact trypanosomes with little or no effect on overall respiratory rate, suggesting a specific site of action for these analogs on protein synthesis. The peptidyl transferase inhibitors were effective at low concentrations at limiting the proliferation of trypanosomes both *in vitro* and *in vivo*. The potency of the compounds as inhibitors of cell proliferation was positively correlated with their efficacy as inhibitors of peptidyl transferase activity. One compound, MDL 20828 (1 mg/kg), increased the survival time of *T. b. brucei*-infected mice 4-fold in the absence of any overt drug toxicity to the hosts.

African trypanosomiasis remains a major world health problem, and new chemotherapeutic agents for the treatment of the disease are needed since few new drugs have been developed over the last 40 years [1, 2]. Past studies demonstrated that the antibiotic puromycin, which inhibits protein synthesis by prematurely terminating the synthesis of polypeptide chains on the ribosome through interference with peptidyl transferase activity [3, 4], was an effective agent against experimental trypanosomiasis [5–7]. Recently, it was demonstrated that 5′-chloro-puromycin, a nontoxic analog of puromycin, effectively cured *Trypanosoma rhodesiense* infections in mice [8]. A number of compounds with a mechanism of action similar to puromycin have been synthesized in our laboratories as potential chemotherapeutic agents [9, 10]. These compounds are analogs of the highly toxic antibiotic sparsomycin but do not appear to possess the toxicity of sparsomycin [11]. The apparent lack of toxicity of our analogs against mammalian cells led us to test them for trypanocidal activity. We detail here the effects of these compounds on the protein synthesis and proliferation of *Trypanosoma brucei brucei in vitro* and *in vivo*.

MATERIALS AND METHODS

Organisms. T. b. brucei (EATRO 110), used in all experiments, were obtained from Dr. Cyrus Bacchi, Haskins Laboratories, New York, and were maintained in our laboratory by syringe passage through male Sprague–Dawley rats.

Preparation of polyribosomes. Purification of polyribosomes was carried out using a modification of an existing procedure [12]. Trypanosomes were harvested from blood obtained by cardiac puncture of 72-hr-infected rats and separated from blood elements on DEAE cellulose columns [13]. All subsequent steps were carried out at 4°. Purified trypanosomes were sedimented by centrifugation and washed twice with a solution of 90 mM Tris·HCl (pH 7.8) containing 50 mM NaCl and 2% (w/v) glucose. After the second wash, trypanosomes were suspended in 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (pH 7.5) containing 25 mM KCl, 40 mM $MgCl_2$, 5% (w/v) sucrose, 50 μg/ml heparin, 100 μg/ml cycloheximide and 0.5% Nonidet P-40 at a concentration of 2.5×10^9 trypanosomes/ml and stirred on ice for 15 min. Lysis of the cells was completed by homogenization in a Dounce homogenizer. The broken cell suspension was diluted with the lysis buffer to a concentration of 1.5×10^9 cells/ml and then centrifuged at 12,000 rpm in a Sorvall SS-34 rotor for 15 min. The supernatant fraction was decanted and layered on a discontinuous sucrose gradient which consisted of 3 ml of 2 M sucrose containing 150 mM KCl, 40 mM $MgCl_2$ and 50 mM HEPES (pH 7.5) overlaid with 2.5 ml of 0.5 M sucrose containing 150 mM KCl, 40 mM $MgCl_2$, 50 mM HEPES (pH 7.5) and 0.1% Nonidet P-40 in a 25-ml tube. The gradient was centrifuged at 50,000 rpm for 3.5 hr in a Beckman Ti 50.2 rotor, and the clear polyribosome pellet was dispersed into a small volume of 25 mM Hepes (pH 7.5) containing 25 mM KCl and 1 mM $MgCl_2$ and frozen in liquid N_2. A typical yield was 0.1 to 0.2 mg polyribosomes/10^9 trypanosomes when the concentration of polyribosomal RNA was determined by measurement of $A_{260\,nm}$ or by the orcinol procedure [14].

Peptidyl transferase activity. Peptidyl transferase activity of polyribosomes was determined by meas-

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uring the incorporation of [^{3}H]puromycin into nascent peptide chains essentially as described [15] with minor alterations to optimize conditions for trypanosome polyribosomes. The standard assay mix contained 25 mM Tris·HCl (pH 8.5), 400 mM KCl, 2.5 mM $MgCl_2$, 2.5 μM or 5 μM [^{3}H]puromycin (1–2 $\times$ 10^6 cpm/assay), as indicated, and 150–200 μg polyribosomal RNA in a total volume of 50 μl.

Reactions were initiated by the addition of polyribosomes, run for 2 min at 24° and terminated by the addition of 2 ml of cold 10% trichloroacetic acid. The precipitated polyribosomes were then collected by filtration onto Millipore type BD filters (0.6 μm). The filters were washed twice with 3 ml of 5% trichloroacetic acid, eight times with 3 ml of absolute ethanol, and then air dried. Radioactive puromycin was determined by liquid scintillation counting in 10 ml Aquasol.

Trypanosome cultures. Bloodstream form trypanosomes were maintained in culture according to the method of Brun *et al.* [16]. Feeder layers consisted of embryonic bovine tracheal cells (ATCC No. CCL-44) as described by Hill [17] grown in Eagle's MEM supplemented with nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10% fetal bovine serum and 20 mM HEPES (pH 7.3) (Medium A). The feeder layer cells were maintained in 25 cm^2 flasks with 10 ml of Medium A. When trypanosomes were to be added to cultures they were isolated aseptically from rat blood by repeated centrifugation of the blood at 1200 *g*. The trypanosomes sedimented in the buffy coat and could be almost completely separated from red blood cells by the centrifugation procedure. Trypanosomes were washed twice with Medium B which was identical to Medium A but contained additionally 30 mM HEPES (pH 7.5), 0.2% glucose and 20% fetal bovine serum. Trypanosomes were added to flasks containing confluent feeder layers at a concentration of 1 $\times$ 10^5 trypanosomes per ml Medium B (total 10 ml/flask). The trypanosomes divided at a constant rate for 48 hr without a medium change. Drugs were initially dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted so as to add not more than 0.2% DMSO to the cultures. Up to 0.5% DMSO had no effect on the growth of trypanosomes or feeder layer cells. Trypanosome proliferation was monitored daily by counting aliquots of each culture in a hemacytometer.

Murine trypanosomiasis. Mice (20 g; CD-1) were inoculated intraperitoneally with 2.5 $\times$ 10^5 trypanosomes, and drug treatments were begun 24 hr later. Drugs were injected subcutaneously in 0.2 ml of 25% DMSO containing 5 mM NaOH (pH 8.5). DMSO alone had no effect on the progress of the infection. Untreated mice survived for an average of 4 days after inoculation. Progression of parasitemias was monitored daily by counting trypanosomes in blood samples, diluted 5 to 10-fold with 0.8% NH_4Cl, using a hemacytometer.

Incorporation of [^{3}H]leucine by intact trypanosomes. The incorporation of [^{3}H]leucine into trypanosome macromolecules was determined in a manner similar to that previously described [18]. Trypanosomes were isolated on DEAE-cellulose columns as described above. Purified trypanosomes were washed twice with Medium B, dispersed in the same medium, and then 4 $\times$ 10^6 trypanosomes were added to microtiter wells in a total well volume of 200 μl. Each well also contained 5 μCi of [^{3}H]leucine and some wells contained test drugs. Triplicate wells were done for controls as well as each drug concentration. After 60 min of incubation at 37° the incorporation of [^{3}H]leucine was terminated by filtration of the trypanosomes onto glass fiber filters using an automatic PHD cell harvester (Cambridge Technology). Filters were washed extensively with distilled water, and then radioactivity trapped on the filters was determined by scintillation counting in minivials containing 5 ml Aquasol.

Glycoprotein synthesis. Glycoprotein synthesis was measured in intact trypanosomes by following the incorporation of [^{3}H]mannose into trypansomal proteins. Experiments were conducted as described above for the incorporation of [^{3}H]leucine except that 5 μCi [^{3}H]mannose was substituted for leucine and incubations were allowed to continue for up to 4 hr.

Chemicals. Cycloheximide and puromycin were purchased from Sigma; L-[3,4,5-^{3}H]leucine (140 Ci/mmole) and D-[3,4-^{3}H]mannose (40 Ci/mmole) were from New England Nuclear; [^{3}H]puromycin (8 Ci/mmole) was from Amersham; and all media and cell culture reagents were from GIBCO. Inhibitors of peptidyl transferase activity were synthesized in our laboratories [9, 10]. The only compounds available in quantities sufficient for more extensive testing *in vivo* and *in vitro* were MDL 20828 and MDL 19152.

RESULTS

Inhibition of peptidyl transferase. The kinetics of inhibition of peptidyl-puromycin synthesis by our analogs was determined by assaying enzyme activity at 2.5 μM and 5 μM [^{3}H]puromycin and various inhibitor concentrations at each puromycin concentration. Data from these experiments were used

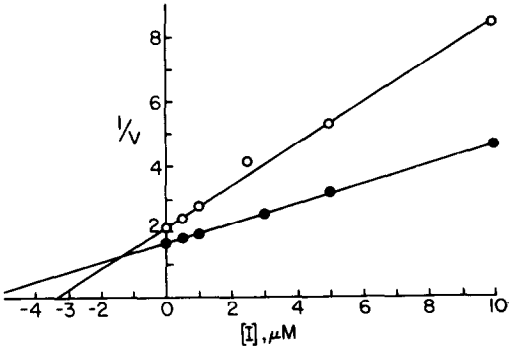


Fig. 1. Inhibition kinetics of MDL 20828 on peptidyl-puromycin synthesis. Peptidyl transferase activity of purified polyribosomes was measured as described in Materials and Methods. [^{3}H]Puromycin was present at concentrations of either 2 μM (●) or 5 μM (○), while MDL 20828 was added to give the indicated concentrations. The data were plotted according to Dixon [19].

to draw Dixon plots [19] (1/V vs [I]) and an apparent K_i was determined graphically from the intersection of the lines obtained at the two concentrations of puromycin. A representative experiment is shown in Fig. 1.

The apparent inhibitory constants (K_i) for the analogs tested can be found in Table 1. The inhibitors all exhibited competitive inhibition of peptidyl transferase activity with respect to puromycin.

Inhibition of protein synthesis. All of the analogs tested were potent inhibitors of [^{3}H]leucine incorporation in intact trypanosomes (Table 1). A good correlation was found between the potency of the compounds as inhibitors of peptidyl transferase activity and the inhibition of [^{3}H]leucine incorporation.

Inhibition of trypanosome growth in vitro. The results of the tests of the analogs on trypanosome growth are presented in Table 1. All the compounds were tested at concentrations of 0.1, 1.0 and 10 μg/ml in the medium. A cytostatic concentration of drug was that concentration which slowed or stopped the increase in cell number whereas a cytocidal concentration was that concentration which caused a decrease in cell numbers over 48 hr. Clearly the analogs with the best activity against peptidyl transferase activity and [^{3}H]leucine incorporation were also the most potent in limiting cell proliferation. None of the analogs tested demonstrated any noticeable toxicity to the feeder layer cells.

Inhibition of glycoprotein synthesis. The incorporation of [^{3}H]mannose into trypanosomal proteins was determined in the presence and absence of MDL 19152. MDL 19152 (10 μg/ml) inhibited the incorporation of [^{3}H]mannose by greater than 60% (Fig. 2). The inhibition appeared to occur almost immediately and was sustained for the full 4-hr incubation period.

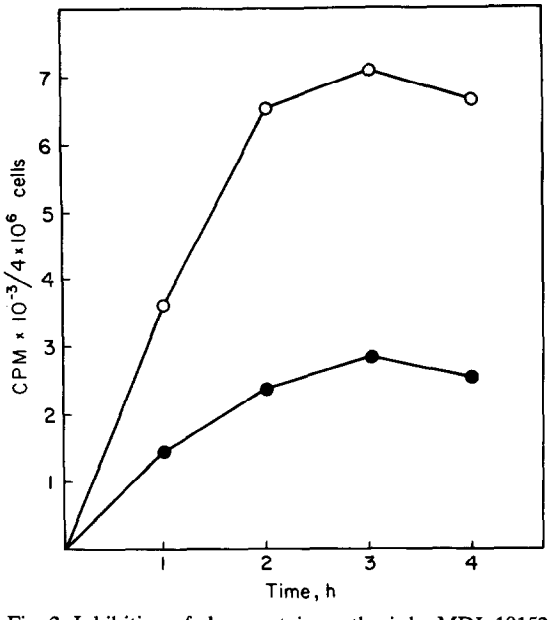


Fig. 2. Inhibition of glycoprotein synthesis by MDL 19152. The incorporation of [^{3}H]mannose into intact trypanosomes was monitored for 4 hr in either the absence (○) or presence (●) of 10 μg/ml MDL 19152 as described in Materials and Methods.

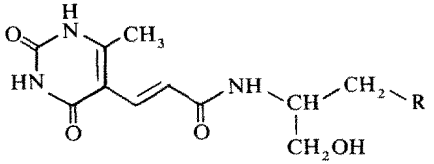
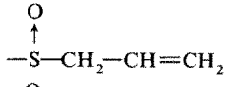
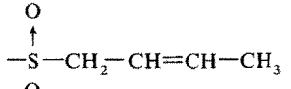
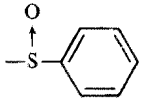
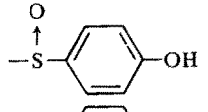
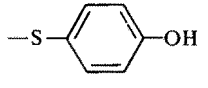
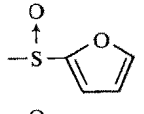
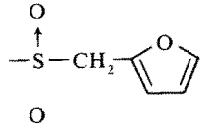
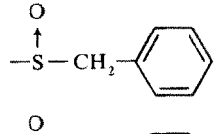
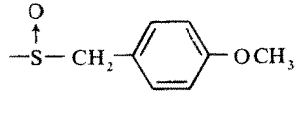
Effects of peptidyl transferase inhibitors on murine trypanosomiasis. The survival time of trypanosome-infected mice was increased by the administration of either MDL 19152 or MDL 20828 (Table 2). Infections were most successfully treated with 1 mg/kg MDL 20828, four times/day; survival time increased 4-fold (17 days for treated mice vs 4 days for controls). Four days post infection (a time when all controls were dead), all of the animals treated with MDL 20828 appeared to be free of blood parasites (limit of detection = 10^4/ml). Infections eventually reappeared in all mice and produced death 17 days post infection. MDL 19152 was toxic at the 500 mg/kg dose, while MDL 20828 exhibited toxicity and two drug-related deaths at 3 mg/kg. MDL 20828 at 1 mg/kg (four times/day) did not appear to be toxic.

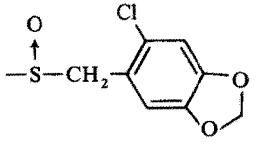
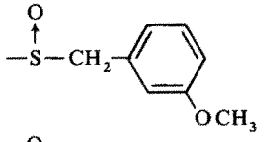
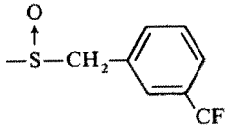
DISCUSSION

In this report, we have shown that potent new inhibitors of mammalian and bacterial peptidyl transferase activity [9–11] were effective at inhibiting protein synthesis in intact *T. b. brucei* as well as proliferation of the growing organisms both *in vitro* and *in vivo*. Furthermore, MDL 19152 and MDL 20828 were found to increase the survival of trypanosome-infected mice by up to 4-fold over controls. This therapeutic effect was obtained using doses that produced no evident toxicity. While possible toxic side effects of our compounds have not been ruled out entirely, early indications from a previous study [11] and the present work are that MDL 19152 and MDL 20828 are less toxic than puromycin and other similar agents. Ash *et al.* [11] demonstrated that the lack of toxicity of our compounds towards mammalian cells is determined by a very slow rate of uptake of the analogs by mammalian cells. The apparent K_i values of our analogs for inhibition of peptidyl-puromycin synthesis by HeLa cell polyribosomes were not much different from the K_i values determined with bacterial or trypanosomal polyribosomes. The lack of effect of the compounds on protein synthesis in intact mammalian cells is probably a general phenomenon. Protein synthesis in the embryonic bovine trachea cells used for feeder layers in our trypanosome cultures was unaffected by concentrations of compounds which inhibited trypanosome protein synthesis and proliferation (data not shown).

It appeared likely that the lethal effect of the drugs against trypanosomes was due to the inhibition of peptidyl transferase and the inhibition of protein synthesis. As shown in Table 1, there was good correlation between reduced cell proliferation or cell death caused by the analogs, the apparent K_i values for inhibition of peptidyl-puromycin synthesis and the extent to which the incorporation of [^{3}H]leucine was inhibited. This correlation demonstrates the specificity of the compounds for their site of action on the trypanosome ribosome. The inhibitors had little or no effect on trypanosome respiration as measured with an O_2 electrode (data not shown), suggesting that the effects of our compounds, unlike the inhibitory effects that some other antibiotics have

Table 1. Properties of peptidyl transferase inhibitors

MDL No.		Peptidyl transferase K_i^* (μM)	[^{3}H]Leucine incorporation† IC_{50} (μg/ml)	Cytostatic conc.‡ (μg/ml)	Cytocidal conc.§ (μg/ml)
19745		1.0	0.25	0.1	1
19823		3.4	0.35	0.1	1
19973		2.8	0.25	0.1	1
20828		1.5	0.10	0.1	1
19944		11	8	1	10
26151		10	3	0.1	1
19851		15	2.2	1	10
19152		12	1	1	10
19477		12	0.8	10	ND‖

19699		23	1.9	>10	ND
19697		40	2.6	>10	ND
19681		120	3.4	>10	ND

* Inhibition constants (K_i) for the listed compounds were determined as described in Materials and Methods using purified polyribosomes for peptidyl-puromycin synthesis. Dixon plots were drawn using velocity data obtained at 2 μM and 5 μM [^{3}H]puromycin and various concentrations of inhibitors, as illustrated in Fig. 1.

† [^{3}H]Leucine incorporation was measured as described in Materials and Methods. Trypanosomes (4×10^6) were incubated for 60 min at 37° with 5 μCi of [^{3}H]leucine. IC_{50} is the concentration of inhibitor which inhibited the incorporation of [^{3}H]leucine by 50%.

‡ Cytostatic concentration was that concentration of inhibitor at which no increase in the number of trypanosomes occurred in 48 hr.

§ Cytocidal concentration was that concentration of inhibitor at which the number of trypanosomes decreased or, in some cases, at which trypanosomes completely disappeared from the medium over 48 hr.

‖ Not determined in these experiments.

Table 2. Effects of peptidyl transferase inhibitors on murine trypanosomiasis*

Drug	Dose (mg/kg)	Repetitions/day	Average survival time (days)	Number of mice
None			4	11
MDL 19152	100	1	6	6
	100	2	7	6
	100	4	12	6
	500	1	7	6
MDL 20828	1	4	17	5
	3	4	13	10†

* Mice were infected with 2.5×10^5 trypanosomes, and the infection was allowed to develop for 24 hr, after which time the drug treatments were begun. Drugs were administered at 9:00 a.m. (one repetition/day), or 9:00 a.m. and 5:00 p.m. (two repetitions/day), or every 6 hr (four repetitions/day) by subcutaneous injection in 0.2 ml of 25% DMSO/5 mM NaOH (approximate pH 8.5). Dosing was continued for 3 consecutive days except for 1 mg/kg MDL 20828 which was given for 5 consecutive days. Trypanosomes were monitored in the blood as described in Materials and Methods.

† Two mice died from drug toxicity before controls died.

on trypanosome protein synthesis [20], were not secondary to a generally depressed metabolism.

In addition to an inhibition of overall protein synthesis, one analog that was tested caused depression of glycoprotein synthesis in *T. b. brucei* as measured by reduced incorporation of [^{3}H]mannose into intact cells. Glycoprotein synthesis is important for the production of the variant specific antigen coat which completely covers the exterior of the trypanosome [21]. Shifts in the antigenicity of the glycoprotein coat allow the trypanosome to escape destruction by the host immune system [21]. Inhibition of this synthetic process may allow the host to mount a more effective immunological response to the parasite as has been suggested may be the case with the ornithine decarboxylase inhibitor α-difluoromethylornithine [22]. It would appear, therefore, from the current studies that treatment of trypanosomiasis by the use of specific nontoxic inhibitors of peptidyl transferase activity may be possible.

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