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Feeder Layer-Free In Vitro Assay for Screening Antitrypanosomal Compounds against *Trypanosoma brucei brucei* and *T. b. evansi*†

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A drug-susceptible *Trypanosoma brucei brucei* stock, a multidrug-resistant *T. b. brucei* stock, and a *T. b. evansi* stock resistant to two commercial trypanocides were adapted to a feeder layer-free culture system. Bloodstream forms were grown continuously in a liquid medium at 37°C in 4% CO_2 in air. Samples of trypanosome populations in the logarithmic growth phase were incubated with various concentrations of commercial and experimental compounds. Growth inhibition was monitored after a 24-h incubation and quantified by comparing the number of generations between control and drug-treated cultures. Some of the experimental compounds [taxol, formicin B, thioridazine, Ro 15-0216, and DL-α-(difluoromethyl)ornithine hydrochloride monohydrate] showed activity against both drug-susceptible and drug-resistant trypanosomes. Other compounds [sinefungin, 1,3,5-triacetylbenzene tris(guanylhydrazone)trimethanesulfonate hydrate, and 9-deazainosine] which inhibited the growth of drug-susceptible trypanosomes showed little or no effect upon drug-resistant parasites. Gossypol, however, had no antitrypanosomal effect on either trypanosome stock. The results obtained in this study correlate with observations obtained from drug screening in mice. The main advantages of the described in vitro screening assay are as follows: (i) lower amounts of drugs are required, (ii) results are obtained more rapidly, (iii) animals are not necessary, and (iv) the method is less labor intensive. These advantages result in an economical and rapid assay for primary drug screening.

Humans and livestock are exposed to the risk of infection with trypanosomes in an area of approximately 10×10^6 km^2 in sub-Saharan Africa infested with tsetse flies. Trypanosomes transmitted mechanically by other biting flies are also a threat to livestock in broad areas across South America, Asia, and Africa. Today, chemotherapy and chemoprophylaxis are the main methods of trypanosomiasis control. However, the increasing appearance of pathogenic trypanosomes resistant to the limited number of commercial trypanocides calls for the development of new drugs (16, 21).

At present, screening for trypanocides is done in rodents. This, however, is time-consuming and expensive, and in vitro techniques offer alternatives. An in vitro assay for screening drugs effective against *Trypanosoma brucei brucei* bloodstream form parasites was described by Borowy et al. (6). However, the use of mammalian feeder layer cells for the in vitro propagation of the parasites and the tedious method of counting with a hemacytometer were drawbacks of this assay.

The introduction of feeder layer-free culture systems for the propagation of *T. brucei* subspp. by Baltz et al. (2) opened possibilities for the development of simpler in vitro screening systems. We describe herein a rapid in vitro assay which does not require feeder layer cells. Employing this assay, we were able to monitor the effects of various compounds not only against drug-susceptible trypanosomes but also against trypanosome stocks resistant to commercial compounds. For an evaluation of the efficacy of this screening assay, experimental compounds which have been reported to be effective against various protozoa were investigated along with four commercial drugs presently in use.

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MATERIALS AND METHODS

Trypanosome stocks. *T. b. brucei* ILTat (IL = International Laboratory for Research on Animal Diseases) 1.4 is a monomorphic clone of a derivative of EATRO (East African Trypanosomiasis Research Organization) 795, which was isolated in 1964 from a bovine in Uhembo, Kenya (17). The clone was adapted to a feeder layer-free culture system. ILTat 1.4, which is highly virulent in mice, is susceptible to diminazene (Berenil), isometamidium (Samorin), suramin (Naganol), and pentamidine (14a). The pleomorphic *T. b. brucei* stock CP (Chemotryp Project) 547 was isolated in 1985 from a naturally infected cow in Jilib, Somalia. This stock causes relapsing parasitemia in mice. Stock CP 547 is resistant to diminazene, isometamidium, quinapyramine, and melarsoprol but is susceptible to suramin (14a, 24a). *T. b. evansi* CP 893 was isolated in 1982 from a camel in Rumuruti, Laikipia District, Kenya. This stock is resistant to suramin and quinapyramine. Stock CP 893 causes fatal parasitemia in mice (24).

Culture media. Two different media were used for the cultivation of the parasites. Iscoves medium (Flow Laboratories, Inc., Irvine, United Kingdom) with albumin, transferrin, soybean lipid, glutamine, and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) was supplemented with 3.024 mg of sodium bicarbonate per ml and the pH was adjusted to 7.0. The medium was stored at 4°C for up to 8 weeks. Shortly before use the medium was supplemented according to Baltz et al. (2). However, some modifications were made, and the final concentrations were as follows: hypoxanthine, 0.1 mM; adenosine, 0.075 mM; sodium pyruvate, 2 mM; L-glutamine, 2 mM; 2-mercaptoethanol, 0.2 mM; gentamicin, 50 μg/ml; and heat-inactivated fetal bovine serum, 12% (vol/vol).

The second medium consisted of liquid minimal essential medium (GIBCO Laboratories, Paisley, United Kingdom) supplemented with 1% (vol/vol) nonessential amino acids

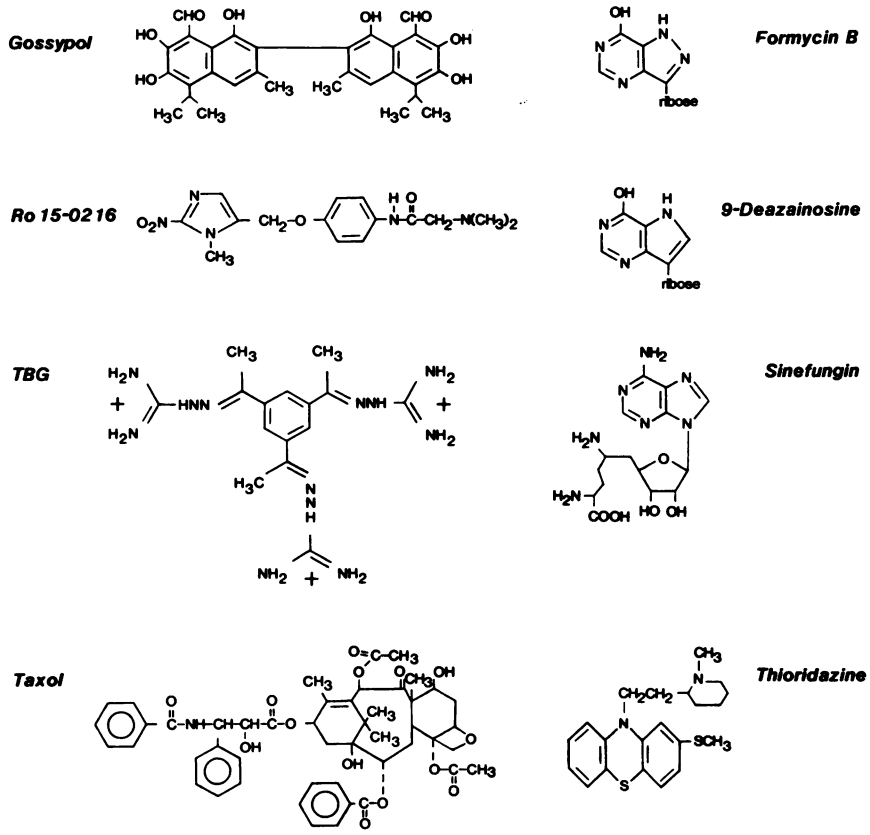

FIG. 1. Structures of experimental compounds tested for antitrypanosomal activity.

(GIBCO), 0.1 mM hypoxanthine, 0.075 mM adenosine, 2 mM L-glutamine, 0.2 mM 2-mercaptoethanol, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 20% (vol/vol) heat-inactivated horse serum.

Adaptation of trypanosomes to in vitro cultures and continued maintenance. All trypanosome stocks used were harvested aseptically from mice by cardiac puncture and transferred to culture flasks (surface area, 25 cm^2; Costar, Cambridge, Mass., or Falcon, Becton Dickinson Labware, Oxnard, Calif.) containing feeder layer cells. Trypanosome cultures were maintained for a period of 2 to 6 weeks on feeder layer cells as previously described (8, 14) but employing the media as described above. After this adaptation period, samples of trypanosome suspensions were transferred into wells of a 24-well culture plate (Costar) which did not contain feeder layer cells. In approximately 2 weeks the trypanosome populations were completely adapted to the feeder layer-free culture system and showed constant growth characteristics throughout the whole period of experimentation. All except 0.1 to 0.2 ml of a 1-ml culture in a 24-well plate was removed and replaced with fresh medium daily.

Drugs. Diminazene aceturate (Berenil, Hoechst AG, Frankfurt, Federal Republic of Germany), isometamidium chloride (Samorin, May & Baker, United Kingdom), quinapyramine sulfate (Trypacide, May & Baker), pentamidine isethionate (May & Baker), and suramin (Naganol, Bayer AG, Leverkusen, Federal Republic of Germany) were purchased commercially.

The following experimental compounds were tested: Ro 15-0216, 2-(dimethylamino)-4′-[(1-methyl-2-nitroimidazole-5-yl)methoxy]acetoanilide (Hoffmann-La Roche & Co. AG, Basel, Switzerland); DL-α-(difluoromethyl)ornithine hydrochloride monohydrate (DFMO; Merrell Dow Research Institute, Cincinnati, Ohio); sinefungin (Eli Lilly & Co., Indianapolis, Ind.); 1,3,5-triacetylbenzene tris (guanylhydrazone)trimethanesulfonate hydrate (TBG; P. Ulrich, Rockefeller University, New York, N.Y.); taxol (M. Suffness, National Cancer Institute, Bethesda, Md.); 9-deazainosine (J. J. Marr, University of Colorado Health Science Center, Denver); formicin B (Sigma Chemical Co., United Kingdom); thioridazine hydrochloride (Aldrich Chemical Co., Inc., United Kingdom); and gossypol (Sigma). The structures are shown in Fig. 1.

Twenty-four-hour growth inhibition test. Samples from continuously growing trypanosome cultures were diluted in medium to a density of 1×10^5 to 4×10^5 trypanosomes per ml. Except for taxol and gossypol, which were dissolved in dimethyl sulfoxide, drugs were dissolved in triple-distilled water (1 mg/ml). Drugs were diluted to appropriate concentrations, so that it was necessary to add only 1% (vol/vol) of the drug dilution to 1 ml of trypanosome suspension to achieve the final drug concentrations. All drugs were tested in 10-fold dilutions covering final concentrations from 1 ng/ml to 10 μg/ml; suramin and DFMO were tested from 1 ng/ml to 100 μg/ml. The cultures were incubated at 37°C in 4% CO_2 in air for 24 h. Control cultures (without drugs) were incubated under the same conditions but with 1% (vol/vol) distilled water or 1% (vol/vol) dimethyl sulfoxide (in the case of taxol and gossypol) added. All experiments were carried out in 24-well culture plates (Costar). After incubation a 500-μl sample was removed from each well, transferred into Coulter Counter bottles (Coulter Electronics, Inc., Hialeah, Fla.), fixed with 6 μl of Formalin (37%), diluted with Isoton,

TABLE 1. EC_{50}s determined by the 24-h growth inhibition assay

Drug	EC_{50} (μg/ml)[a]		
	T. b. brucei ILTat 1.4	*T. b. brucei* CP 547	*T. b. evansi* CP 893
Isometamidium	0.113 (S)	0.178 (R)	0.005 (S)
Diminazene	0.046 (S)	5.263 (R)	0.030 (S)
Suramin	0.097 (S)	0.025 (S)	21.80 (R)
Pentamidine	0.007	0.005	0.006
Sinefungin	0.002	2.445	0.005
DFMO	19.2	62.7	41.6
Formicin B	0.311	0.347	0.218
TBG	0.309	3.599	0.466
Taxol	0.027	0.034	0.033
Thioridazine	0.032	0.031	0.043
Ro 15-0216	0.022	0.012	0.022
9-Deazainosine	0.293	1.459	0.263
Gossypol	>10	>10	>10

[a] S, Drug susceptible in mice; R, drug resistant in mice (see also Materials and Methods).

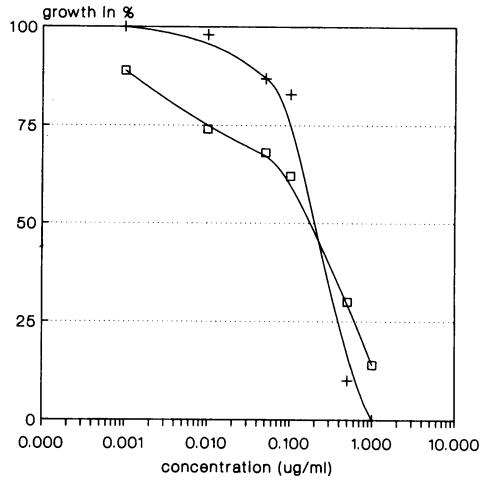

FIG. 2. Growth of *T. b. brucei* ILTat 1.4 (□) and CP 547 (+) bloodstream forms after incubation for 24 h in the presence of various concentrations of isometamidium chloride.

and counted in a model ZB1 Coulter Counter (70-μm aperture). The number of generations which occurred in drug-treated cultures was calculated for each well (number of generations = $\log n_{24} - \log n_0/\log 2$, where n_{24} is the number of trypanosomes after 24 h and n_0 is the number of trypanosomes at the start), and the relative growth of trypanosome populations was determined by comparison with the number of generations (100%) occurring in control cultures. The average generation time in control cultures was 10.5 h for CP 547 (~2.3 generations per 24 h), 9.0 h for ILTat 1.4 (~2.6 generations per 24 h), and 16.7 h for CP 893 (~1.4 generations per 24 h). The data were first analyzed graphically by plotting growth inhibition versus the drug concentration (Harvard Graphics Program). The effective concentration which inhibited the growth of trypanosome populations by 50% (EC_{50}) was determined using the minimum chi-square method; all values of ≥100 and ≤0% growth were rejected.

A second method was used in dissolving and diluting the drugs, except gossypol and taxol, in the culture medium to appropriate concentrations, so that a 50% (vol/vol) drug dilution was added to 0.5 ml of trypanosome suspension. The two different methods of preparing the drug dilutions did not alter the results.

RESULTS

Drug-resistant trypanosome stocks CP 547 and CP 893 were both successfully adapted to feeder layer-free culture systems. *T. b. brucei* CP 547 could be grown in both culture media described above. However, *T. b. evansi* CP 893 could only be initiated in minimal essential medium with 20% horse serum, but once established, cultures could be maintained in either medium.

The results, EC_{50}s, of the 24-h growth inhibition test of four commercial and nine experimental drugs are shown in Table 1. Differences in drug susceptibility between the two *T. b. brucei* isolates were particularly high for diminazene (>100-fold) and sinefungin (>1,200-fold). A comparison of *T. b. brucei* CP 547 with *T. b. evansi* CP 893 also showed a pronounced dissimilarity in the EC_{50}s for diminazene (~175-fold) and sinefungin (~500-fold). Differences in drug susceptibility were also detected for TBG and 9-deazainosine. Compared with the susceptible stock ILTat 1.4, a fivefold increase in the concentration of 9-deazainosine was necessary to inhibit the growth of the resistant stock CP 547. Pentamidine, formicin B, taxol, thioridazine, DFMO, and Ro 15-0216 had similar antitrypanosomal effects on all species. No antitrypanosomal effect was detected for gossypol. The EC_{50}s for isometamidium differed among all of the stocks tested, but the difference was only minor among the susceptible and resistant *T. b. brucei* isolates. Curiously, differences in growth inhibition were more pronounced when the trypanosome populations were incubated in the presence of lower isometamidium concentrations (1 and 10 ng/ml) (Fig. 2).

DISCUSSION

The results obtained in this study demonstrate that drug-resistant *T. brucei* subspp. stocks can be adapted to feeder layer-free culture systems. Furthermore, the growth inhibition tests with commercial compounds showed that drug resistance is expressed in vitro. Isometamidium and diminazene inhibited growth of the susceptible stock ILTat 1.4 more than that of the resistant stock CP 547. Although the EC_{50}s of isometamidium did not differ significantly between susceptible and resistant stocks, the differences were more marked when trypanosomes were incubated with drug concentrations below the EC_{50}s. This is noteworthy since isometamidium is used as a prophylactic drug and levels in serum are <10 ng/ml (15).

Stock CP 547 is highly resistant to sinefungin and diminazene when compared with the susceptible stock ILTat 1.4. Diminazene inhibits the *S*-adenosyl-L-methionine decarboxylase (4), and sinefungin can be considered to be an analog to *S*-adenosyl-L-methionine (5). Therefore, both drugs may interfere, at the same point, with polyamine metabolism in trypanosomes. The resistance of stock CP 547 to both drugs suggests that in this stock the specific steps affected by the drug are less susceptible to interdiction. However, detailed biochemical investigations are necessary to provide evidence in support of this speculation. Although sinefungin has been reported to have activity against susceptible trypanosomes both in mice (11) and in vitro (6), it shows a lack of efficacy against diminazene-resistant *T. b. brucei* both in vitro (this paper) and in mice (24a). Furthermore, sinefungin is severely nephrotoxic in goats (25). Thus, sinefungin in its

present form does not appear to be a good candidate for further development to a new antitrypanosomal drug, unless analogs, which are unavailable, prove to be less toxic.

The EC_{50}s for 9-deazainosine and formicin B obtained in this study were 4- to 25-fold (9-deazainosine) and 11- to 17-fold (formicin B) higher than those found by Fish et al. (13) for a *T. b. gambiense* stock. However, these workers used a culture system with feeder layer cells and monitored growth over a period of 5 days. Differences in susceptibilities might be related to effects of the drugs on the feeder layer cells or to differences in trypanosome species used, and the longer time of exposure to the drug may possibly account for the lower EC_{50}s.

DFMO showed activity against *T. b. brucei* in vivo (1) and in vitro. However, different trypanosome stocks show various susceptibilities to DFMO (E. Zweygarth and R. Kaminsky, manuscript in preparation).

The fact that no significant differences were detected in the drug susceptibility of both *T. b. brucei* stocks toward formicin B, taxol, thioridazine, and Ro 15-0216 suggests a different mode of action of these drugs compared with the commercial compounds tested. However, thioridazine, which inhibited the growth of procyclic forms of *T. b. brucei* (22), had no antitrypanosomal effect in mice infected with *T. b. brucei*, unless it was used in combination with suramin (19).

Compound Ro 15-0216 is a 5-substituted 2-nitroimidazole which is chemically unrelated to the other compounds tested and currently used only experimentally. Ro 15-0216 has shown activity in vivo against *T. brucei* subspp. (20), *T. congolense*, and *T. vivax* (24). Ro 15-0216 was tested in vitro against African trypanosomes by Borowy et al. (7), using a culture system which required feeder layer cells. Nevertheless, the EC_{50}s determined for *T. brucei* subspp. (7) were 0.03 to 0.09 μg/ml, similar to the values obtained in this study. However, Ro 15-0216 is less effective against resistant stock CP 547 in mice; a 13-fold-higher concentration was necessary to cure mice infected with stock CP 547 when compared with a susceptible *T. b. brucei* stock (24a). At present we can offer no satisfactory explanation of why the in vitro susceptibility of stock CP 547 was the same as that of the susceptible stock ILTat 1.4. Suramin-resistant *T. b. evansi* was very susceptible to Ro 15-0216 in vitro, which corresponds to results obtained in vivo (24). In addition, Ro 15-0216, even at high doses, is very well tolerated by rodents (24). Thus, this compound might be of future value as a chemotherapeutic drug against suramin-resistant and, to a lesser degree, diminazene-resistant trypanosomes.

Taxol, an experimental antitumor agent and stabilizer of microtubules (3), is capable of blocking the replication of *T. cruzi* (3). We found that taxol also inhibits the growth of both susceptible and resistant *T. brucei* subspp. Therefore, it would be worthwhile to investigate taxol in vivo to evaluate its toxicity and its potential antitrypanosomal activity.

Gossypol, an antispermatogenic drug, has been reported to have antitrypanosomal activity at a concentration of 20 μM (12, 23). However, in our hands, gossypol showed no antitrypanosomal activity, although we used an incubation time (24 h) longer than that previously employed by Eid et al. (12). These workers observed a reversible loss of motility after incubation for 3 min at 5 μM (2.59 μg/ml); an irreversible loss of motility was apparent after only 5 min at 20 μM (10.36-μg/ml) gossypol (12). The fact that Eid et al. (12) did not include animal serum of any kind in their test system, while our medium contained 12% fetal bovine serum, may account for the two different observations. The role of serum seems to be essential for the activity of gossypol, which has been shown to bind to serum albumin (D. L. Vander Jagt, J. E. Heidrich, R. E. Royer, and L. A. Hunsaker, Fed. Proc. **41**:1428, 1982), and thus the concentration of free gossypol available to enter the parasites is lowered (9). Our findings confirm the results of Croft et al. (10), who found no activity of gossypol against *T. cruzi*, using a screening assay containing heat-inactivated newborn calf serum. In addition, the levels of gossypol in plasma after 14 daily oral doses of 10 mg/kg were less than 10 μg/ml (18), a concentration at which, in our screening assay, gossypol had no effect on trypanosome growth.

Care must be taken when evaluating drugs for potency with an in vitro system because drugs might be inactive in the in vitro system but still be efficacious in vivo. However, all drugs which showed trypanocidal activity in vivo (24a) also inhibited in vitro growth of the susceptible trypanosome stocks. Furthermore, resistance of *T. b. brucei* CP 547 and *T. b. evansi* CP 893 to all commercial and experimental compounds tested was similar in vitro, as was found in mice, except for Ro 15-0216. Thus, the in vitro screening assay can effectually substitute for drug screening in mice, which could spare large numbers of laboratory animals. Another feature of the in vitro drug screening assay presented herein is that results can be obtained in only 24 h, whereas drug screening in mice requires at least 30 days. Besides, only minute amounts of drugs are required for the in vitro assay. These advantages result in an economical and rapid assay for primary drug screening.

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