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Susceptibility of an Insect *Leptomonas* and *Crithidia fasciculata* to Several Established Antitrypanosomatid Agents

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Growth inhibition of the lower trypanosomatids *Crithidia fasciculata* and a *Leptomonas* from a hemipteron by several established trypanocides and leishmanicides were compared in four complex and one defined media. The *Leptomonas* was more susceptible than *C. fasciculata* in all media, especially to phenanthridines (ethidium, prothidium, isometamidium) and diamidines (pentamidine, diminazene diaceturate [Berenil], hydroxystilbamidine, stilbamidine); concentrations of these drugs required for 50% inhibition of the *Leptomonas* were <5 μg/ml. In contrast, *C. fasciculata* was uninhibited by <20 μg of diamidines per ml and was three- to sixfold less susceptible than the *Leptomonas* to isometamidium and prothidium. Both trypanosomatids were susceptible to nucleoside antibiotics, e.g., nucleocidin. Neither was inhibited by suramin, melarsen, melarsen oxide, or tryparsamide. The *Leptomonas* was more susceptible to standard trypanocides than five other insect trypanosomatids in a complex medium; it was the only one inhibited by <20 μg of stilbamidine and hydroxystilbamidine per ml.

No novel drugs have been introduced in the past 25 years against trypanosomiasis and leishmaniasis (5), yet the incidence of African trypanosomiasis is not declining (8). Drug resistance is spreading in human and animal infections (12, 13) and is likewise increasing in leishmanial infections (13). The drugs available for use against the various leishmaniases are occasionally quite toxic at therapeutic dosages (16). Treatment of Chagas' disease with the one drug available (nifurtimox) is protracted and poorly tolerated by many adults (20); naturally resistant strains are reported from Chile (15).

Present means for the in vivo evaluation of candidate drugs depends upon the existence of lead compounds of known structure or upon recognition of high-activity natural products. Such tests are costly since they require much material, animals, and labor. In vitro tests with the pathogens require culture media generally containing unheated blood, a source of contaminants, especially mycoplasmas (6). Cross and Manning (4) have developed a defined medium for *Trypanosoma brucei*, but growth was variable with different isolates and the essentiality of some of its rather unstable and expensive components is yet to be demonstrated.

In view of these considerations, we believe that a rapid, sensitive screening procedure is necessary for detection of novel trypanocides; there is need for a method suitable for assaying antitrypanosomatid activity in antibiotic beers. In preliminary testing, an insect *Leptomonas* sp. appeared highly susceptible to several standard trypanocides (7). In this report, we present evidence that the *Leptomonas* is far more susceptible than five other insect trypanosomatids to standard trypanocides and that lower trypanosomatids merit further scrutiny as surrogates (models) for detecting novel trypanocides and leishmanicides.

MATERIALS AND METHODS

Organisms and media. The *Leptomonas* sp. ATCC 30250 was isolated (from a hemipteron) and provided by F. G. Wallace, Univ. of Minnesota, as was a symbiont-bearing *Blastocrithidia culicis* (ATCC 14806). *Crithidia fasciculata* was obtained from the American Type Culture Collection (ATCC 11745). *Crithidia oncopelti* (not the strain listed in the American Type Culture Collection as "*C. oncopelti*") was obtained from B. A. Newton, Molteno Institute, Cambridge; it is to be deposited in the collection. *Leptomonas pessoai* (ATCC 30252) was from I. Roitman, Univ. Brasília and *Crithidia acanthocephali* (ATCC 30251) was from R. B. McGhee, Univ. of Georgia.

The stock-culture medium contained (g/liter): liver infusion (Oxoid), 5; proteose peptone, 7.5; brain heart

infusion (BBL), 7.5; NaCl, 5; KCl, 2; ascorbic acid, 0.2; NaH_2PO_4 (anhydrous), 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; sucrose, 2.5; morpholinopropane sulfonic acid, 2.5; and hemin (Sigma "equine, type III"), 14 mg in 50% aqueous (vol/vol) 1,1',1'',1'''(ethylenedinitrilo)-tetra-2-propanol (J. T. Baker Chemical Co., Quadrol). The components were dissolved, filtered through Whatman no. 1 paper, and brought to pH 7.4 with Quadrol; the solution was then brought to a boil and Ionagar no. 2 (Oxoid; 6 g/liter) was added. The medium was dispensed (~14 ml per Kimble screw-capped tubes [100 by 25 mm]) and autoclaved slanted for 20 min at 121 C. Before transferring cultures, the slants were flooded with 5 ml of sterile suspension fluid containing (g/liter): NaCl, 3; KCl, 2.5; NaH_2PO_4 (anhydrous), 0.1; $MgSO_4 \cdot 7H_2O$, 0.4; calcium gluconate, 0.2; morpholinopropane sulfonic acid, 0.2; tricine [*N*-tris(hydroxymethyl)aminomethane-methyl glycine], 0.2; and Quadrol, 0.4, to pH 7.4. Two drops were inoculated onto each slant. After 2 to 3 days of incubation (the *Leptomonas* and *L. pessoai* at 29 to 30 C; all others at 26 C), the wet slants of the *Leptomonas* and *L. pessoai* were stored at 13 to 15 C, the others at 5 to 6 C. Transfers were made at intervals not over 3 weeks.

Drug susceptibilities were determined in one defined and four complex media. The *Leptomonas* and *C. fasciculata* grew well in the defined medium (Table 1). The cane sugar medium (CS) grew the *Leptomonas* and *C. fasciculata* well. The mannitol (MM) and high-yield (HY) media supported exceptionally heavy growth of the *Leptomonas* alone, yet permitted sensitive responses to drugs. CS medium contained (g/liter): yeast hydrolysate (ICN Nutritional Biochemicals Corp.), 5; N-Z Amine (type AS, Sheffield Chemical Co.), 3; and $MgSO_4 \cdot 7H_2O$, 0.1 MM medium contained (g/liter): yeast hydrolysate, 5; N-Z Amine, 3; mannitol, 10; and $MgSO_4 \cdot 7H_2O$, 0.1. HY medium contained (g/liter): yeast hydrolysate, 7; N-Z Amine, 3; mannitol, 10; corn hydrolysate (ICN Nutritional Biochemicals Corp.), 4; and $MgSO_4 \cdot 7H_2O$, 0.1. All three complex media had 8 mg of hemin per liter added as in Table 1; pH was brought to 7.4 with 5 M KOH.

TBM medium, used to compare all six flagellates, contained (g/liter): NaCl, 2.15; KCl, 2.15; $MgSO_4 \cdot 7H_2O$, 0.17; morpholinopropane sulfonic acid, 4.7; cane sugar, 4.7; L-proline, 1.17; L-histidine (base), 9.4; N-Z Amine, 8.5; yeast hydrolyzate, 1.3; Na_2 DL-α,β-glycerophosphate (Sigma Chemical Co., 75% β-isomer), 7.5; hemin in milligrams (added as in Table 1) and Quadrol to pH 7.4. TBM also grew 11 other *Crithidia* isolates, several *Herpetomonas* spp., and *Trypanosoma mega*.

Growth-inhibition tests. Test media were in 50-ml microfernbach flasks (Bellco) containing 10 ml final volume. Thermostable solutions and media were autoclaved for 20 min at 121 C. Growth-curves were run in triplicate and included pH controls for the "no drug" flasks and flasks with the highest drug concentration. Flasks were inoculated with 1 drop (0.04 to 0.06 ml) of 72-h log-phase cultures grown in screw-capped test tubes (20 by 125 mm); flasks in any one

TABLE 1. *Defined medium for the Leptomonas sp. and C. fasciculata*

Medium	Weight (per 100 ml)[a]
Nitrilotriacetic acid	0.03 g
Cyclo acid[b]	0.25 g
KH_2PO_4	0.04 g
$MgCO_3$	0.06 g
$CaCO_3$	0.02 g
Metals mix[c]	12.0 mg
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	7.0 mg
KCl	0.2 g
Nicotinamide	1.0 mg
Calcium pantothenate	1.0 mg
Sodium riboflavin phosphate	0.2 mg
Thiamine-hydrochloride	0.06 mg
Pyridoxamine-dihydrochloride	0.05 mg
Folic acid	0.1 mg
Biopterin[d]	1.0 μg
DL-Carnitine-hydrochloride	0.1 mg
Choline hydrogen citrate	5.0 mg
Cystamine-dihydrochloride	0.04 mg
Thiamine nitrate	0.06 mg
Biotin	0.4 μg
Ferulic acid	0.04 mg
Na_2 fumarate $\cdot H_2O$	0.3 g
Na acetate $\cdot 3H_2O$	0.075 g
Ethanolamine-hydrochloride	0.1 g
Betaine-hydrochloride	0.05 g
Adenine	6.0 mg
Guanine	2.0 mg
Orotic acid	0.6 mg
Uracil	0.3 mg
Thymine	0.1 mg
L-Valine	0.1 g
L-Alanine	0.05 g
L-Arginine (base)	0.03 g
L-Glutamic acid	0.05 g
Glycine	0.1 g
L-Histidine (base)	0.03 g
L-Isoleucine	0.025 g
L-Leucine	0.05 g
L-Lysine-hydrochloride	0.05 g
L-Methionine	0.02 g
L-Phenylalanine	0.05 g
L-Proline	0.3 g
L-Serine	0.1 g
L-Threonine	0.1 g
L-Tryptophan	0.04 g
L-Tyrosine	0.016 g
NaCl	0.2 g
$MgSO_4 \cdot 7H_2O$	0.05 g
Tween 80 (vol/vol)	0.2 g
Hemin[e]	1.0 mg
Sucrose	0.25 g

[a] Final pH was adjusted to 7.4 with 50% (vol/vol) aqueous Quadrol.

[b] 1,2,3,4-Cyclopentanetetracarboxylic acid (Eastman Organic Chemicals).

[c] To yield (in milligrams per 100 ml): Fe, 0.6; Mn, 0.5; Zn, 0.5; Mo, 0.2; Cu, 0.04; V, 0.02; Co, 0.01; B,

TABLE 1—*Continued*

0.01; Ni, 0.01; Cr, 0.01.
[d] Crude (~6%); Sigma Chemical Co.
[e] As an aqueous solution in 50% Quadrol.

experiment were inoculated with one pipette. Inocula were grown in the same medium as for the test. To minimize evaporation and ease handling, the flasks were put in Pyrex utility trays, taped after inoculation to inverted trays.

Incubation temperatures were as for stock cultures. Incubation of assays varied with organism and medium: *Leptomonas* was incubated 6 days in CS, MM, and HY, 5 days in TBM, and 4 days in MM; *C. fasciculata* was incubated 3 days in CS and MM and 4 days in TBM; *L. pessoai* and *B. culicis* were incubated 5 days in TBM; *C. oncopelti* and *C. acanthocephali* were incubated 4 days in TBM.

Drug solutions were prepared just before use. The drug (20 mg) was added to 20 ml of sterile water, and aseptic serial dilutions were made. The volume of drug additions was not more than 2.5 ml. Most drugs were water soluble except for agaricin, 4-cumylphenol, and melarsen oxide; these were solubilized with several drops of 5 M KOH. Cordycepin stock solutions had to be filter-sterilized (Kreuger A-400 H.A.K.-1 pad, prewashed) because of initially high contamination rates.

Microscopic checks for contamination were made in all assays; random samples from flasks were cultured on brain heart infusion agar (BBL) and thioglycolate broth (Difco) both at 30 and 38 C. Contamination was <1 per 250 flasks.

Growth was read as absorbance on a Bausch & Lomb Spectronic 20 spectrophotometer at 750 nm after subtraction of appropriate blanks. Growth inhibition was plotted as percent inhibition, based on absorbances lying between 20 and 80% inhibition, to arrive at the 50% inhibition concentrations (mean infective dose [ID_{50}]). Wide ranging response curves were initially employed, then a curve of closely spaced values. Such curves were derived independently at least twice (see Tables 2, 3, and 4).

Drugs. The drugs were obtained as follows: acridine orange from Harleco; acriflavine hydrochloride, cordycepin, ethidium bromide, and primaquine diphosphate from Sigma Chemical Co.; agaricin and tryparsamide from K & K Laboratories; quinapyramine (Antrycide methylsulfate) from Imperial Chemical Industries, Ltd. (gift); diminazene aceturate (Berenil) from Farbwerke Hoechst AG. (gift); chloroquine diphosphate and quinacrine hydrochloride from Sterling-Winthrop Laboratories (gift); 4-cumylphenol from Aldrich Chemical Co.; hydroxystilbamidine diisethionate from Merrell-National Laboratories, Div. of Richardson-Merrell, Inc. (gift); potassium antimony tartrate from Fisher Scientific Co.; isometamidium, melarsen sodium, melarsen oxide, pentamidine isethionate, and stilbamidine isethionate from May and Baker Ltd. (gift); nucleocidin from Lederle Laboratories Div., American Cyanamid Co. (gift); oxophenarsine from Parke, Davis & Co. (gift); prothidium bromide from The Boots Co. Ltd. (gift); rhodamine B from National Aniline Co.; and crystal violet from Eastman Organic Chemicals.

RESULTS

The CS, MM, and HY media were used initially in this study. CS medium was used for comparative studies on the *Leptomonas* and *C. fasciculata*. The MM medium permitted the lowest ID_{50} for the *Leptomonas*. Large quantities (3.5 g [wet weight] per liter) of "no drug" cells as well as drug-inhibited cells were obtained in HY medium for biochemical studies on the *Leptomonas* (to be reported elsewhere). Except for *C. oncopelti*, all flagellates used grew in the defined medium, but their absorbances were <0.50.

The drugs chosen are in practical use as trypanocides or leishmanicides or else are progenitors of useful drugs. The antimalarials primaquine, quinacrine, and chloroquine served as controls of specificity. Agaricin and 4-cumylphenol, experimental antitumor agents, are potent α-glycerophosphate dehydrogenase inhibitors (1). Crystal violet is commonly added to banked blood in South America to kill *T. cruzi*; the dye so used has negligible toxicity (3).

Table 2 compares the *Leptomonas* sp. and *C. fasciculata* in complex media; most trypanocides tested, aside from the organo-arsenicals and suramin, gave ID_{50} >50 μg/ml for the *Leptomonas*. *C. fasciculata* grown in CS medium was susceptible to several drugs, yet was relatively refractory to the diamidines. The *Leptomonas* was most susceptible in the MM medium, particularly to the diamidines, nucleoside antibiotics, Antrycide, and the acridines.

Responses of *C. fasciculata* and the *Leptomonas* to some practical antityrpanosomatid agents were compared in the defined medium (Table 3). The observed susceptibilities were similar to those observed with the CS medium: unsusceptibility of *C. fasiculata* to the diamidines and low ID_{50} for the *Leptomonas* with ethidium, prothidium, crystal violet, and acriflavine. Interestingly, *C. fasciculata* was unsusceptible to Antrycide in the defined medium. *Leptomonas* was ~fivefold less susceptible to hydroxystilbamidine in the defined medium than in CS or MM media.

Susceptibilities of other insect trypanosomatids to antitrypanosomatid agents were tested on several *Crithidia* spp., *L. pessoai*, and *B. culicis* (Table 4). The *Leptomonas* was generally more susceptible than the other trypanosomatids grown on TBM medium. Antrycide was less

TABLE 2. *ID_{50} values for C. fasciculata and Leptomonas sp. in complex media*[a]

Drugs	*Leptomonas* sp.			*C. fasciculata* (*CS medium*)
	CS medium	MM medium	HY medium	
Acridine orange	7.2	4.3	4.9	0.30
Acriflavine hydrochloride	0.12	0.064	0.168	0.35
Agaricin	0.39	0.30	0.505	1.25
Antrycide methyl sulfate	0.83	0.23	0.29	2.45
Berenil	1.7	0.12	0.11	17
Chloroquine diphosphate	[b]	[b]	[b]	[b]
Cordycepin	5.0	0.38	0.20	[b]
Crystal violet	0.095	0.062	0.072	0.48
4-Cumylphenol	3.40	2.00	2.50	0.40
Ethidium bromide	0.009	0.008	0.014	0.010
Hydroxystilbamidine diisethionate	1.20	0.825	2.05	20[c]
Isometamidium chloride	0.068	0.17	0.345	0.28
Melarsen, sodium	[b]	[b]	[b]	[b]
Melarsen oxide	[b]	[b]	[b]	[b]
Nucleocidin	2.18	1.48	1.00	0.31
Pentamidine isethionate	1.48	0.61	1.18	20[c]
Primaquine phosphate	[b]	[b]	[b]	[b]
Prothidium bromide	0.81	0.98	1.60	2.80
Quinacrine hydrochloride	[b]	3.90	[b]	[b]
Rhodamine B	[b]	8.50	13	[b]
Stilbamidine isethionate	1.10	0.235	1.075	20[c]
Suramin	[b]	[b]	[b]	[b]
Tryparsamide	[b]	[b]	[b]	[b]

[a] Values are shown in micrograms per milliliter. *C. fasciculata* was cultured in CS medium only. Control absorbance values were as follows. *Leptomonas* sp.: CS, 0.40; MM, 0.60 to 0.70; and HY, 0.85 to 1.0. *C. fasciculata*: CS, 0.40.

[b] No inhibition at 20 μg/ml.

[c] Less than 35% inhibition at 20 μg/ml.

inhibitory for the *Leptomonas*: its ID_{50} was the highest obtained in five media. Both *C. oncopelti* and *C. acanthocephali* were more susceptible to the drugs than *L. pessoai*.

DISCUSSION

The most recently developed trypanocides, e.g., metamidium and prothidium, are not novel, having been synthesized from the active portions of existing drugs. Resistance has indeed already developed (12) to their progenitors. One way to uncover new lead compounds is to develop more sensitive yet specific in vitro model systems for initial detection of antitrypanosomatid activity in antibiotic beers and other natural products. As a case in point, metronidazole, effective in amebiasis and trichomoniasis, evolved from the antibiotic azomycin which contains a novel nitroimidazole group (9).

The *Leptomonas* was clearly more susceptible to standard antitrypanosomatid agents than *C. fasciculata*. Reports of inhibition of trypanosomatids by drugs (17, 18) support this conclusion. Such comparisons, however, are only inferential since media and culture conditions differed from ours. The relative unsusceptibility of *L. pessoai* underscores the heterogeneity of the genus *Leptomonas*.

The variation in ID_{50} with Antrycide and hydroxystilbamidine in the defined medium as compared with complex media suggests that at least in part they act as antimetabolites. A minimal defined medium may be necessary to make this system more practical for initial screening, especially for detecting agents with antimetabolite activity. All modes of antitrypanosomatid activity are not detected by the present in vitro system for several clinically active compounds; e.g., the trivalent arsenical melarsen oxide and the pentavalant tryparsamide and melarsen did not inhibit *Leptomonas* or *C. fasciculata*. Arsenicals must be metabolized by the host to the trivalent state (21) along with other modifications in some instances. Preliminary results with the trivalent oxophe-

TABLE 3. *ID_{50} values for Leptomonas sp. and C. fasciculata in defined medium*[a]

Drugs	*Leptomonas* sp.	*C. fasciculata*
Acriflavine hydrochloride	0.30	1.95
Antrycide methyl sulfate	0.34	[b]
Berenil	1.8	[b]
Crystal violet	0.04	4.5
Ethidium bromide	0.03	0.54
Hydroxystilbamidine diisethionate	6.25	[b]
Isometamidium chloride	0.09	0.26
Potassium antimony tartrate	[b]	[b]
Pentamidine isethionate	1.12	[b]
Prothidium bromide	0.62	4.25
Stilbamidine isethionate	1.68	[b]

[a] Values are expressed in micrograms per milliliter. Control absorbance values were: *Leptomonas* sp., 0.50 to 0.70; *C. fasciculata*, 0.60 to 0.70.

[b] No effect at 20 μg/ml.

TABLE 4. ID_{50} values for trypanosomatids in TBM medium[a]

Drugs	Leptomonas sp.	C. fasciculata	C. oncopelti	L. pessoai	B. culicis	C. acanthocephali
Acriflavin hydrochloride	0.065	2.65	0.45	1.15	0.6	4.0
Antrycide methyl sulfate	9.0	[b]	2.25	[b]	7.0	[b]
Berenil	5.2	20[c]	12	[b]	7.2	20[c]
Crystal violet	0.05	1.0	0.5	0.05	0.1	1.0
Ethidium bromide	0.06	0.48	0.47	0.23	0.9	0.98
Hydroxy stilbamidine diisethionate	2.85	[b]	[b]	[b]	[b]	[b]
Isometamidium chloride	0.35	6.8	0.51	1.2	1.2	10.25
Potassium antimony tartrate	20[c]	[b]	[b]	[b]	[b]	[b]
Pentamidine isethionate	0.60	20[c]	7.9	[b]	4.5	15.0
Prothidium bromide	2.0	[b]	7.7	[b]	5.7	20[c]
Stilbamidine isethionate	1.07	[b]	[b]	[b]	[b]	[b]

[a] Values are expressed in micrograms per milliliter. Control absorbance were: *Leptomonas* sp., 0.70 to 0.80; *C. fasciculata*, 0.60 to 0.70; *C. oncopelti*, 0.90 to 1.10; *L. pessoai*, 0.70 to 0.80; *B. culicis*, 0.85 to 1.0; *C. acanthocephali*, 0.40 to 0.55.

[b] No effect at 20 μg/ml.

[c] Less than 50% inhibition at 20 μg/ml.

narsine (Mapharsen) indicate the *Leptomonas* is susceptible below 20 μg/ml. Tryparsamide and melarsen here were used as controls, also suramin, whose mode of metabolic activation and trypanocidal action remain unknown (2). The disparate chemotherapeutic patterns in trypanosomatidae argues that, correspondingly, additional lower trypanosomatids should be investigated.

Hardy lower trypanosomatids are useful for mode-of-action studies. *Crithidia* systems were used to study subcellular effects of pentamidine and its uptake (10, 19), and Newton (11) used *C. oncopelti* to study the action of Antrycide. Extension of such studies to the *Leptomonas* now seems warranted since it is appreciably more susceptible than those *Crithidia* isolates tested.

Another advantage of in vitro procedures is the ease of distinguishing between novel and known agents by comparing wild-type and induced resistant strains of an appropriately susceptible organism. This "finger-printing" technique now finds use in screening for novel antitumor agents as well as antimicrobial antibiotics (14). The ease of preparation of drug-resistant *Leptomonas* clones indicate that it may be useful for this purpose.

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