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Selective and Potent *in Vitro* Antitrypanosomal Activities of Ten Microbial Metabolites

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Abstract More than 400 compounds isolated from soil microorganisms, and catalogued in the antibiotic library of the Kitasato Institute for Life Sciences, were screened against African trypanosomes. Ten compounds were found to have selective and potent antitrypanosomal activity *in vitro*: aureothin, cellocidin, destomycin A, echinomycin, hedamycin, irumamycin, LL-Z 1272*β*, *O*-methylnanaomycin A, venturicidin A and virustomycin A. Results of the *in vitro* assays using the GUTat 3.1 strain of *Trypanosomal brucei brucei* and the STIB900 strain of *T. b. rhodesiense* are presented. Cytotoxicity was determined using a human MRC-5 cell line. This is the first report of antitrypanosomal activities of the 10 microbial metabolites listed above.

Keywords screening, *in vitro*, antitrypanosomal antibiotics, *Trypanosomal brucei brucei*, *T. b. rhodesiense*, HAT

Introduction

African trypanosomes, protozoan parasites of the genus *Trypanosoma*, transmitted through the bite of bloodfeeding tsetse flies (*Glossina* spp.), cause disease in cattle and in people. Human African trypanosomiasis (HAT), also known as sleeping sickness, is caused by infection with *Trypanosoma brucei rhodesiense* or *T. b. gambiense* and is a major threat to communities throughout sub-Saharan Africa. A third subspecies, *T. b. brucei* is responsible for the cattle disease, N'gana, but does not infect humans. Accurate statistics for HAT are difficult to estimate the World Health Organization (WHO) estimated that, in 2000, some 300,000 individuals were affected by the disease, a figure far in excess of the 27,000 cases reportedly diagnosed and treated that year. With increased surveillance activities in the last 7 years, recent estimates indicate that there are 70,000 cases, causing 25,000 deaths annually [1, 2].

Currently, only four drugs are registered for the treatment of HAT: pentamidine, suramin, melarsoprol and eflornithine (Fig. 1). Pentamidine and suramin are used in the first or early stage of *T. b.gambiense* and *T. b. rhodesiense* infections. Melarsoprol is used in the advanced stage of both forms of the disease, while eflornithine is only used in the advanced stage of *T. b. gambiense* infections and is not effective against *T. b. rhodesiense*. Today, increasing numbers of patients, 20~25% in certain foci, are no longer responding to melarsoprol treatment, probably due to evolving drug resistance. Although expensive, eflornithine is an effective alternative drug for the treatment of *T. b. gambiense* patients who do not respond to melarsoprol [3].

All four drugs used to treat HAT are unsatisfactory, since they cannot be given orally and all are hampered by severe toxicity and increasing resistance of the parasites.

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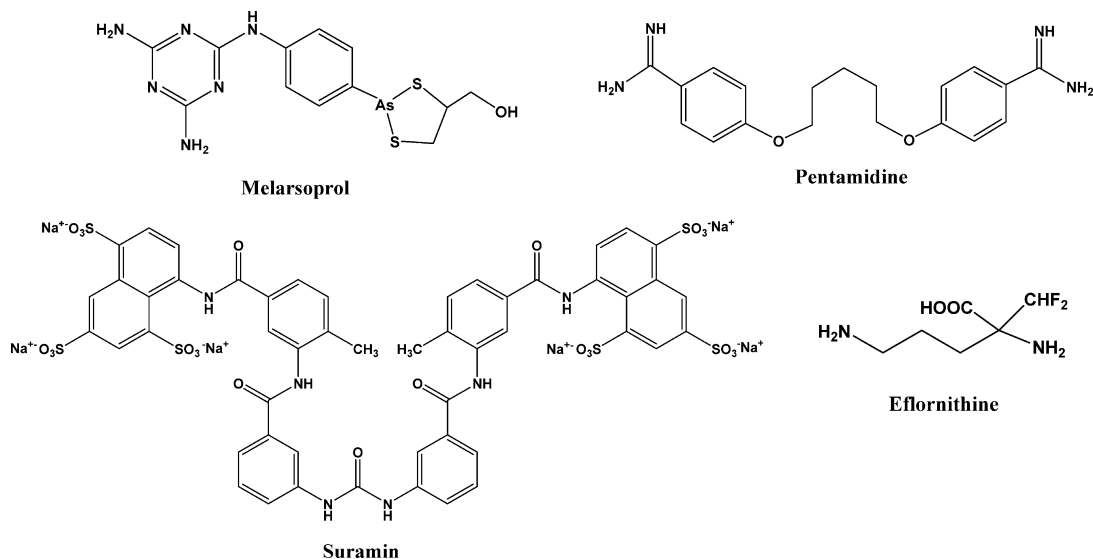
Fig. 1 Structures of the currently used antitrypanosomal drugs.

Melarsoprol for example, the only drug for 2nd-stage infections caused by *T. b. rhodesiense*, causes around 5.0% fatalities due to severe adverse effects [4]. Consequently, there is an urgent need for new antitrypanosomal drugs which have novel structures and mechanisms of action and which are both safe and effective.

In the course of our screening program to discover new antitrypanosomal antibiotics, by screening metabolites from soil microorganisms, we have discovered 10 compounds that display potent antitrypanosomal activity *in vitro*. We report the results of sensitivity tests on the GUTat 3.1 strain of *T. b. brucei* and the STIB900 strain of *T. b. rhodesiense* using the four commonly used antitrypanosomal drugs as well as detailing the novel antitrypanosomal activity and cytotoxicity of the ten antibiotics *in vitro*.

Materials and Methods

Parasites and History

Trypanosoma brucei brucei strain GUTat 3.1 (Glasgow University, *Trypanozoon* antigenic type 3.1) was donated Dr. Y. Yabu (Nagoya City University, Japan). It is a cloned derivative of a stock EVE (Edinburgh Veterinary Expedition) 10 that was originally isolated in 1996 from a naturally infected bovine in Uganda. The original clone was provided by Dr. P. R. Gardiner, International Livestock Research Institute (ILRI), Nairobi, Kenya. A HAT parasite, *T. b. rhodesiense* strain STIB900, was supplied by Prof. R. Brun (Swiss Tropical Institute, Switzerland). It is a clone of

a parasite, isolated in 1982 from a patient in Tanzania, which is known to be susceptible to all currently used drugs.

Chemicals

Test compounds were obtained from the antibiotic library of the Kitasato Institute for Life Sciences. Melarsoprol, suramin and eflornithine (DFMO) were provided by Prof. R. Brun (Swiss Tropical Institute, Switzerland). Pentamidine isothionate salt was obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Iscove's Modified Dulbecco's Medium (IMDM, with L-glutamine and HEPES, without $NaHCO_3$), Minimum Essential Medium (MEM) with Earle's salts, MEM non-essential amino acids solution and Penicillin-Streptomycin solution were obtained from Gibco Laboratories Life Technologies (Grand Island, NY, USA). Fetal Bovine Serum (FBS) was obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA) and Horse Serum (HS) was obtained from Gibco Laboratores Life Technologies (Grand Island, NY, USA). Alamar Blue reagent was obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Other chemicals were commercially available and of analytical grade.

In Vitro Antitrypanosomal Assay against *T. brucei* Species

T. b. brucei GUTat 3.1 strain was cultured in IMDM with 3.024 g/liter $NaHCO_3$, 100 μM hypoxanthine, 30 μM thymidine, 40 μM adenosine, 1.0 mM sodium pyruvate, 50 μM L-glutamine, 100 μM 2-mercaptoethanol and 50 Unit/ml of penicillin and 50 μg/ml of streptomycin

containing 10% heat-inactivated FBS at 37°C, under 5.0% CO_2 - 95% air, according to the method of Yabu *et al.* [5]. *T. b. rhodesiense* STIB900 strain was cultured in MEM with Earle's salts supplemented with 25 mM HEPES, 1.0 g/liter additional glucose, 2.2 g/liter $NaHCO_3$, 10 ml/liter MEM non-essential amino acids (100×), 0.2 mM 2-mercaptoethanol, 1.0 mM sodium pyruvate, 0.1 mM hypoxanthine 50 Unit/ml of penicillin and 50 μg/ml of streptomycin containing 15% heat-inactivated HS at 37°C, under 5.0% CO_2 - 95% air, according to the method of Baltz *et al.* [6]. *In vitro* antitrypanosomal activity of test compounds was determined by a dose response curve using Alamar Blue according to the method of Räz *et al.* and Tasdemir *et al.* [7, 8] with some modification. Ninety five μl of the trypanosomes suspension (2.0~2.5×10^4 trypanosomes/ml for GUTat 3.1 strain or 2.0~3.0×10^4 trypanosomes/ml for STIB 900 strain) of bloodstream forms was seeded in a 96-well microplate, and 5.0 μl of a test compound solution (dissolved in 5.0% dimethylsulfoxide) was added. After the incubation for 72 hours at 37°C under 5.0% CO_2 - 95% air, 10 μl of the fluorescent dye Alamar Blue was added to each well. After a further incubation for 3~6 hours at 37°C under 5.0% CO_2 - 95% air, the plate was read at 528/20 nm excitation wavelength and 590/35 nm emission wavelength by a FLx800 fluorescent plate reader (Bio-Tek Instrument, Inc. Vermont, USA). Data were transferred into a graphic program (Excel) and the IC$_{50}$ values were determined by using the fluorescent plate reader software (KC-4, Bio-Tek).

Stock cultures were kept in 24-well tissue culture plates at 37°C under 5.0% CO_2 - 95% air.

Cytotoxicity Tests on MRC-5 Cells
Cytotoxicity was assayed against a human diploid embryonic cell line MRC-5 as described previously [9].

Antitrypanosomal Activity and Cytotoxicity of Currently Used Drugs
The drug sensitivity of the GUTat 3.1 strain is not known. Conversely, the STIB900 strain is known to be susceptible to all existing antitrypanosomal drugs [10]. We therefore first established the *in vitro* drug sensitivity of the GUTat 3.1 and the STIB900 strains against the four common trypanocidal drugs. Suramin, pentamidine, melarsoprol and eflornithine were tested against the two *T. brucei* strains and MRC-5 cells and IC$_{50}$ values were determined (Table 1). Melarsoprol showed the highest antitrypanosomal activity against both parasite strains. The GUTat 3.1 strain was 85-fold more sensitive to the drug than STIB900. The sensitivity to pentamidine was the same for both strains. For suramin, STIB900 was 30-fold more sensitive than GUTat 3.1 while for eflornithine the sensitivity was only double. The cytotoxicity of the existing drug was also determined using MRC-5 cells. Melarsoprol and pentamidine showed IC$_{50}$ values in the low μg/ml range whereas for suramin and eflornithine, the IC$_{50}$ values were >100 μg/ml.

To compare the antitrypanosomal activities and cytotoxicities, we calculated the selectivity index (SI: [IC$_{50}$ for MRC-5 cells]/[IC$_{50}$ for trypanosome strain]) (Table 1). The SIs were in a range >44 to 127,273 for GUTat 3.1 and in a range >96 to 3,807 for STIB900. Melarsoprol and pentamidine present SI values >1,000, peaking at >100,000 for melarsoprol and the GUTat 3.1 strain. For eflornithine it is difficult to determine a reasonable SI value because very high drug concentrations are tolerated by mammalian cells.

Table 1 *In vitro* antitrypanosomal activity and cytotoxicity of drugs used to treat human African trypanosomiasis

| Compound | IC$_{50}$ (ng/ml) | | | Selectivity index (SI) | |
| | Antitrypanosomal activity | | Cytotoxicity MRC-5 | | |
	GUTat 3.1	STIB900		MRC/GUTat	MRC/STIB
Melarsoprol	0.011	0.94	1,400	127,273	1,489
Pentamidine	1.6	1.5	5,710	3,569	3,807
Suramin	1,580	52.0	>100,000	>63	>1,923
Eflornithine	2,270	1,040	>100,000	>44	>96

Table 2 *In vitro* antitrypanosomal activity and cytotoxicity of 10 microbial metabolites

| Compound | IC$_{50}$ (ng/ml) | | | | |
| | Antitrypanosomal activity | | Cytotoxicity MRC-5 | Selectivity index (SI) | |
	GUTat 3.1	STIB900		M/GUTat	M/STIB
Aureothin	1.4	1.1	>25,000	>17,857	>22,727
Cellocidin	150	30	5,910	39	179
Destomycin A	330	210	21,140	64	101
Echinomycin	20	14	6,310	316	451
Hedamycin	14	18	>25,000	>1,786	>1,389
Irumamycin	20	31	>12,500	>625	>403
LL-Z 1272β	49	59	13,620	278	231
O-methylnanaomycin A	210	16	4,890	23	306
Venturicidin A	120	540	>25,000	>208	>185
Virustomycin A	0.45	480	80	178	0.2

In Vitro **Antitrypanosomal Activity and Cytotoxicity of 10 Antibiotics**

The *in vitro* antitrypanosomal activity of the 10 microbial metabolites was determined using GUTat 3.1 (*T. b. brucei*) and STIB900 (*T. b. rhodesiense*). The IC$_{50}$ values are presented in Table 2. Virustomycin A and aureothin showed the highest antitrypanosomal activity, with IC$_{50}$ values around 1.0 ng/ml which is comparable to pentamidine (Tables 1 and 2). Echinomycin, hedamycin, irumamycin and LL-Z 1272β were 10-fold less active than virustomycin A and aureothin, with IC$_{50}$ values of 14~59 ng/ml for both trypanosome strains. Cellocidin, destomycin A, *O*-methylnanaomycin A and venturicidin A were the least active compounds against GUTat 3.1 but their IC$_{50}$ values can still be considered as active. Interestingly, cellocidin and *O*-methylnanaomycin A resulted in much lower IC$_{50}$ values for STIB900 as compared to the GUTat 3.1 strain.

The cytotoxicity of the 10 compounds were evaluated against a human diploid embryonic cell line (MRC-5). The IC$_{50}$ values are listed in Table 2. Virustomycin A was the only compound with a pronounced cytotoxicity, having an IC$_{50}$ value of 80 ng/ml. Cellocidin, echinomycin and *O*-methylnanaomycin A were revealed to be slightly cytotoxic, demonstrating IC$_{50}$ values of 5~6 μg/ml, while the remaining six compounds had IC$_{50}$ values of >12.5 μg/ml and do not seem to be cytotoxic.

Among the tested compounds, aureothin showed the highest SI with values of >17,857 for GUTat 3.1 and >22,727 for STIB900. It exhibited a significantly better SI value for *T. b. rhodesiense* than any other compound. Echinomycin, hedamycin, irumamycin, LL-Z 1272β,

venturicidin A and virustomycin A showed SIs >100 for GUTat 3.1. The SI values for cellocidin and destomycin A were in a lower range of 40~180, based on the lower antitrypanosomal activity of these compounds.

Discussion

Aureothin is a γ-pyrone antibiotic, and is reported to have antitumor, antifungal and pesticide activity [11~13], and we previously reported that it showed potent nematocidal activity against the pine wood nematode [14]. The mode of action of aureothin has been reported as a non-specific inhibition of NADH: ubiquinone oxidoreductase (complex I) on bovine heart, fungal and bacterial cells [15].

Cellocidin is an amide antibiotic, and is reported to have antitumor and antibacterial activity [16, 17]. The mode of action of cellocidin is *via* inhibition of the α-ketoglutarate-succinate system in the Krebs' cycle in *Xanthomonas oryzae* [18] and inhibition of nucleic acid synthesis in tumor cells [19].

Destomycin A is an aminoglycoside antibiotic and is reported to have antibacterial and anthelmintic activity [20~22]. The mode of action of destomycin A is the inhibition of polypeptide synthesis in cells of *Escherichia coli* and stimulation of adenylate cyclase in several animal tissues [23, 24].

Echinomycin is a cyclic depsipeptide antibiotic containing two quinoxaline moieties and is reported to have antibacterial and antitumor activity [25, 26]. The mode of action of echinomycin is reported to be intercalation with
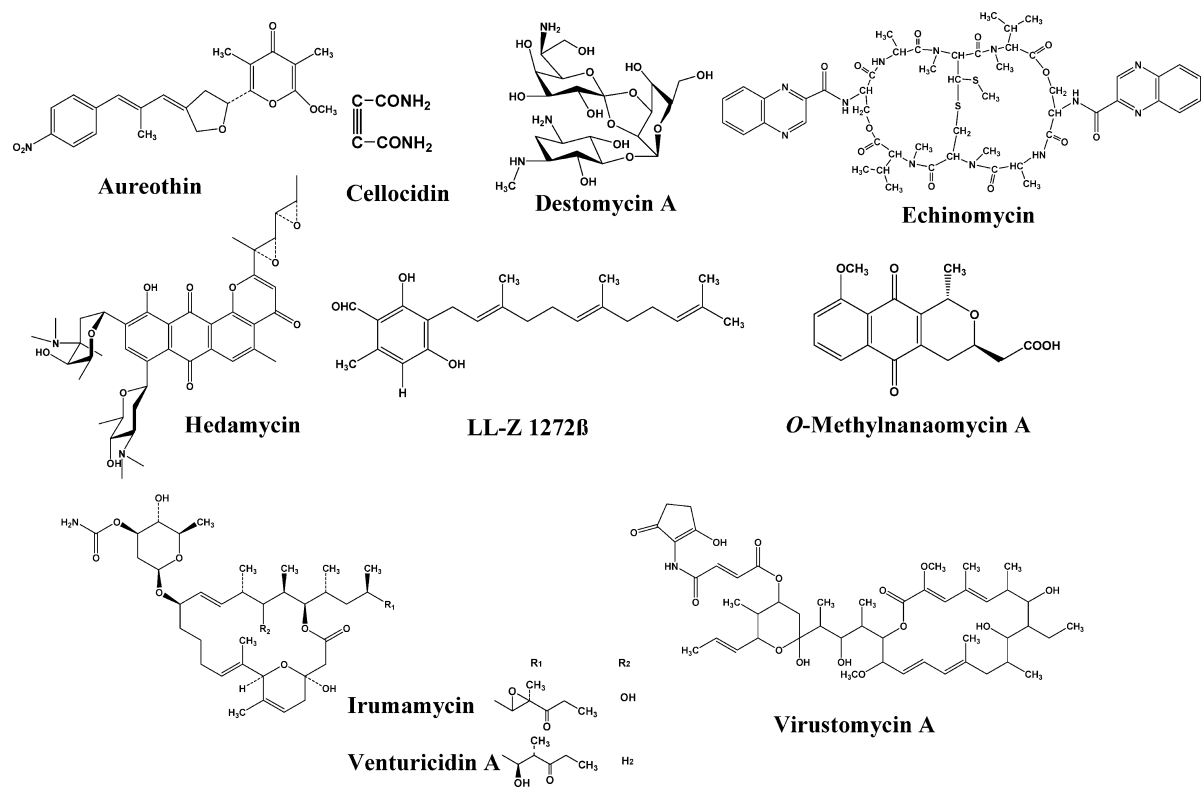
Fig. 2 Structures of antitrypanosomal antibiotics.

DNA [25, 26].

Hedamycin is an anthraquinone antibiotic exhibiting antitumor, antibacterial and anti-*Tetrahymena pyriformis* (anti-protozoal) activity [27]. Recently, we reported that it also possesses selective and potent antimalarial activity [28]. The mode of action of anthraquinone antibiotics is inhibition of nucleic acid synthesis [27].

LL-Z 1272β is a terpenoid antibiotic and is reported to have anti-*T. pyriformis* (anti-protozoal) and antitumor activity [29, 30], and we reported that it showed inhibition of testosterone 5α-reductase from rat prostate [31]. A related antibiotic, ascofuranone, is reported to have *in vitro* and *in vivo* anti-trypanosomal activities [32, 33]. The mode of action of ascofuranone is inhibition of the mitochondrial electron-transport system in trypanosomes [32].

O-Methylnanaomycin A is a semi-synthetic analogue of nanaomycin A, a naphtoquinone antibiotic, and *O*-methylnanaomycin A is reported to exhibit antifungal activity [34]. The mode of action of nanaomycin A is *via* inhibition of the respiratory chain-linked flavin dehydrogenase in bacterial cells [35].

Irumamycin and venturicidin A are similar to the 20-membered ring macrolide antibiotics and both antibiotics are reported to have antifungal activity [36, 37]. For venturicidin A, antimalarial activity has also identified [38]. The mode of action of venturicidin A is inhibition of phosphoryl transfer reactions (in rat liver mitochondria) and inhibition of mitochondrial adenosine triphosphatase of *T. pyriformis* [39, 40].

Virustomycin A is an 18-membered ring macrolide antibiotic, reportedly having antiviral and anti-*Trichomonas foetus* (anti-protozoal) activities [41, 42]. The mode of action of virustomycin A is through interference with the formation of the phosphate donor(s) in the ATP-forming system (in *T. foetus*) [42].

Recently, Berriman *et al.*, proposed several molecular targets in *T. brucei* determined from the available sequence of the *T. brucei* genome. One of them is mitochondorial electron transport and oxidative phosphorylation, including ATP synthase [43]. Some of the antitrypanosomal antibiotics we describe in this article (aureothin, cellocidin, the ascofuranone related LL-Z 1272β, *O*-methylnanaomycin A, irumamycin, venturicidin A and virusomycin A) may actually inhibit *T. brucei* by inhibition of mitochondrial functions.

The mode of action of aminoglycoside antibiotics, such as destomycin A, is *via* inhibition of polypeptide synthesis, that of cyclic depsipeptide antibiotics (such as

echinomycin) *via* intercalation with DNA, and anthraquinone antibiotics (such as hedamycin) through inhibition of nucleic acid synthesis are all well documented [23, 25~27]. Destomycin A, echinomycin and hedamycin may also inhibit *T. brucei* in the same manner and detailed studies of the mode of action need to be undertaken to provide further evidence.

Further studies, including *in vivo* tests in animal models of trypanosomal infection and characterization of other biological activities of aureothin, cellocidin, destomycin A, echinomycin, hedamycin, irumamycin, LL-Z 1272β, *O*-methylnanaomycin A, venturicidin A and virustomycin A are in progress.

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