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In Vitro and *in Vivo* Antitrypanosomal Activitiy of Two Microbial Metabolites, KS-505a and Alazopeptin

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Abstract Our on-going screening program to discover new antitrypanosomal antibiotics has been evaluating compounds isolated from soil microorganisms as well as investigating the antibiotic libraries of the Kitasato Institute for Life Sciences and BioFrontier Laboratories of Kyowa Hakko Kogyo Co., Ltd. We have now discovered two compounds, KS-505a and alazopeptin, which exhibit moderate antitrypanosomal characteristics. We report here the *in vitro* and *in vivo* antitrypanosomal activities and cytotoxicities of KS-505a and alazopeptin, compared with some commonly-used antitrypanosomal drugs. This is the first report of *in vitro* and *in vivo* antitrypanosomal activities of either KS-505a or alazopeptin.

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

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Introduction

Transmitted *via* the bite of tsetse flies (*Glossina* spp.) two sub-species of trypanosomes, *Trypanosoma brucei rhodesiense* and *T. b. gambiense*, infect humans and one sub-species, *T. b. brucei*, infects cattle. Human African Trypanosomiasis (HAT), also known as Sleeping Sickness, is recognized as one of African's most neglected diseases and is a major cause of mortality and morbidity in sub-Sahara Africa. The cattle disease, nagana, devastates livestock production and causes massive economic losses. Accurate statistics for HAT are difficult to obtain. The World Health Organization (WHO) estimated that, in 2000, some 300,000 Africans 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 there are 70,000 cases of HAT annually, causing 25,000 deaths [1, 2].

Currently, only four drugs (pentamidine, suramin, melarsoprol and eflornithine) are registered for the treatment of HAT. Pentamidine and suramin are used in the early stage of *T. b. gambiense* and *T. b. rhodesiense* infections. Melarsoprol is used in the late-stage of both forms of the disease, while eflornithine is only used in the late-stage of *T. b. gambiense* infections. These drugs used to treat HAT are unsatisfactory, since they cannot be given orally and are hampered by severe toxicity. For example, melarsoprol is so poisonous that 5.0～10% of patients die because of toxic side effects [3]. Furthermore, drug resistance in trypanosomes is increasing and treatment failures are becoming more common [4, 5]. Therefore, there is an urgent need for new antitrypanosomal drugs that

have novel structures and mechanisms of action, and which are both more effective and safer for chemotherapeutic control of HAT.

During our screening program to discover new antitrypanosomal antibiotics, we have been evaluating isolates from soil microorganisms and compounds from the antibiotic libraries of the Kitasato Institute for Life Sciences and BioFrontier Laboratories of Kyowa Hakko Kogyo Co., Ltd. We have previously reported on 10 microbial metabolites that exhibit selective and potent antitrypanosomal activities [6]. We have now discovered two compounds, KS-505a and alazopeptin, which show moderate antitrypanosomal characteristics. We report here *in vitro* antitrypanosomal activities, cytotoxicities and *in vivo* antitrypanosomal activities of KS-505a and alazopeptin, in comparison with commonly-used antitrypanosomal drugs.

Materials and Methods

Parasites and History

The history of *T. b. brucei* strain GUTat 3.1 and *T. b. rhodesiense* strain STIB900 were described previously [6]. *T. b. brucei* strain S427, a clone derived from an isolate from a Ugandan sheep [7], was donated by Prof. T. Kinoshita (Research Institute for Microbial Diseases, Osaka University, Japan). The original clone was kindly provided by Dr. G. A. M. Cross, Rockefeller University, USA.

Chemicals

Test antibiotics were obtained from the antibiotic libraries of the Kitasato Institute for Life Sciences and BioFrontier Laboratories of Kyowa Hakko Kogyo Co., Ltd. Suramin and eflornithine 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, N. Y. USA). Fetal Bovine Serum (FBS) was obtained from Sigma-Aldrich Inc. (St. Louis, MO. USA) and horse Serum (HS) was obtained from Gibco Laboratories Life Technologies (Grand Island, N. Y. USA). Alamar Blue reagent was obtained from Sigma-Aldrich Inc. (St. Louis, MO. USA). Other chemicals were commercially available and all of analytical grade.

Animals

Female CD1 mice (ICR), 20～25 g, were obtained from Charles River Japan, Inc. Animals were placed in groups of four per cage, kept in a room under negative pressure with flow of 0.1～0.2 m/second. The animal room was held at a temperature of 25±2°C and 60±10% relative humidity. Animals are maintained on a diet of CE-2 (Clea, Japan Inc.) and water *ad libitum*.

In Vitro Antitrypanosomal Assay against *T. brucei* Species

In vitro antitrypanosomal activities for *T. b. brucei* strain GUTat 3.1 and *T. b. rhodesiense* strain STIB900 were described previously [6]. In brief, *T. b. brucei* strain GUTat 3.1 was cultured in IMDM with various supplements and 10% heat-inactivated FBS at 37°C, under 5.0% CO_2/95% air. *T. b. rhodesiense* strain STIB900 was cultured in MEM with Baltz supplements containing 15% heat-inactivated HS at 37°C, under 5.0% CO_2/95% air, according to the method of Baltz *et al.* [8]. Ninety five μl of the trypanosomes suspension ($2.0～2.5\times10^4$ trypanosomes/ml for strain GUTat 3.1 or $2.0～3.0\times10^4$ trypanosomes/ml for strain STIB 900) was seeded in a 96-well microplate, and 5.0 μl of a test compound solution (dissolved in 5.0% dimethylsulfoxide) was added followed by incubation for 72 hours (long incubation-low inoculation test: LILIT). Ten μl of the fluorescent dye Alamar Blue was added to each well. After incubation for 3～6 hours, the resulting solution 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 spreadsheet program (Excel). The IC_{50} values were determined using a fluorescent plate reader software (KC-4, Bio-Tek). Successive subcultures were done in 24-well tissue culture plates under the same conditions.

Cytotoxicity Tests on Human Diploid Cell Line (MRC-5 Cells)

Cytotoxicity assay against MRC-5 cells was carried out as previously described [9].

In Vivo Antitrypanosomal Assay against *T. brucei* Species

T. brucei from *in vitro* culture were well adapted to repeated infection and passage in ICR mice. Infected blood (about $10^{8～9}$ trypanosomes/ml) was collected with a heparinized syringe from mouse heart under CO_2 anesthesia. Collected blood was mixed gently with cryostabilant solution (1 : 1) consisting of phosphate-buffered saline containing 10% glucose (PSG) and 10%

glycerol [10]. The cryostabilate was transferred to cryocapillary tubes and stored in liquid nitrogen before use.

T. b. brucei S427 Acute Mouse Model

Groups of four female ICR mice were infected intraperitoneally (i.p.) on day 0 (d0) with 10^4 bloodstream forms of *T. b. brucei* strain S427, prepared in cryostabilate dilution with PSG. Infected mice were treated i.p. with each test compound (10% DMSO-Tween 80/EtOH mixture (7 : 3) aqueous solution) on four consecutive days (d1 to d4). A control group remained untreated. Parasitaemia levels of the mice were checked by microscopic examination of tail blood on d4 after final treatment and thereafter twice a week until d30. The date of death of each mouse was recorded to calculate the mean survival day (MSD). Surviving and aparasitaemic mice at d30 were considered cured.

T. b. rhodesiense STIB900 Acute Mouse Model

Groups of four female ICR mice were infected i.p. on d0 with 3×10^4 bloodstream forms of *T. b. rhodesiense* strain STIB900 prepared from cryostabilate dilution with PSG. Infected mice were treated i.p. with each test compound (10% DMSO-Tween 80/EtOH mixture (7 : 3) aqueous solution) on four consecutive days (d3 to d6). A control group remained untreated. Parasitaemia levels of the mice were checked by microscopic examination of tail blood on d6 after final treatment and thereafter twice a week until d60. The date of death of each mouse was recorded to calculate the MSD. Surviving and aparasitaemic mice at d60 were considered cured.

Results

In Vitro Antitrypanosomal Activities and Cytotoxicities of KS-505a and Alazopeptin

In vitro antitrypanosomal activity of KS-505a and alazopeptin were evaluated on both *T. b. brucei* strain GUTat 3.1 and *T. b. rhodesiense* strain STIB900. The IC_{50} values of KS-505a, alazopeptin and three reference drugs (pentamidine, suramin and eflornithine) are listed in Table 1. KS-505a and alazopeptin possessed antitrypanosomal activities against strain GUTat 3.1, with the IC_{50} values of 1.03 and 0.51 μg/ml, respectively. These antibiotics also possessed antitrypanosomal activities against strain STIB900, with IC_{50} values of 1.66 and 1.21 μg/ml, respectively. The antitrypanosomal activities of these antibiotics for strains GUTat 3.1 and STIB900 were lower, by 319～1,107-fold than those of pentamidine for the same strains, whereas, antitrypanosomal activities of these antibiotics for strains GUTat 3.1 and STIB900 were the same or similar to those of suramin and eflornithine for strain GUTat 3.1, and to that of eflornithine for strain STIB900 (Table 1).

We subsequently investigated the cytotoxicities of KS-505a and alazopeptin against MRC-5 cells, with IC_{50} values also listed in Table 1. KS-505a and alazopeptin showed lower cytotoxicities with IC_{50} values of >27.33 and >9.1 μg/ml, respectively, and do not seem to be cytotoxic.

To compare the antitrypanosomal activities and cytotoxicities, we introduced the selectivity index (SI) listed in Table 1. KS-505a and alazopeptin showed moderate and lower SI, with ratios ranging between >17.8～>26.5 and >7.5～>16.5 for MCR-5 cells/strain GUTat 3.1 and MCR-5 cells/strain STIB900, respectively. These SI were lower than the commonly-used antitrypanosomal drugs, pentamidine, suramin and

Table 1 *In vitro* antitrypanosomal activity and cytotoxicity of KS-505a, alazopeptin and drugs used to treat Human African Trypanosomiasis

Compound	IC_{50} (μg/ml)				
	Antitrypanosomal activity		Cytotoxicity	Selectivity index (SI)	
	GUTat 3.1	STIB900	MRC-5	*M/T. b. b.*	*M/T. b. r.*
KS-505a	1.03	1.66	>27.33	>26.5	>16.5
Alazopeptin	0.51	1.21	>9.10	>17.8	>7.5
Pentamidine	0.0016	0.0015	5.71	3,569	3,807
Suramin	1.58	0.052	>100	>63	>1,923
Eflornithine	2.27	1.04	>100	>44	>96

eflornithine.

In Vivo Antitrypanosomal Activities of KS-505a and Alazopeptin

KS-505a, alazopeptin and pentamidine were evaluated using the *T. b. brucei* S427 acute mouse model and *T. b. rhodesiense* STIB900 acute mouse model. Their cure rates and MSD are listed in Table 2. With the *T. b. brucei* strain S427, KS-505a cured all mice infected at a dose of 30 mg/kg. In the case of a lower dose (10 mg/kg), cure was not achieved, but MSD was extended (13.0 days) which represented a 3-fold increase over control MSD (4.4 days). Conversely, alazopeptin (at a dose of 50 mg/kg) did not achieve cure, but it also extended MSD (10.3 days), a 1.8-fold increase over control MSD (5.8 days). Pentamidine, at a dose of 1.0 mg/kg, cured all infected mice in this model. A lower dose (0.2 mg/kg) achieved only 25% cure but also showed an extended MSD (>17.3 days) which was >2.9-fold longer than that of control MSD (6.0 days). Ten mg/kg dosage of KS-505a exhibited similar antitrypanosomal activity to that of pentamidine (0.2 mg/kg) in the *T. b. brucei* S427 acute mouse model.

KS-505a was also tested with the *T. b. rhodesiense* STIB900 acute mouse model. In this model, KS-505a at a dose of 42 mg/kg achieved only 25% cure but did show an extended MSD (>28.5 days), which was >3.6-fold longer than that of control MSD (8.0 days) (Table 2). In the case of pentamidine, at a dose of 20 mg/kg, the drug achieved 50% cure and exhibited an extended MSD (>49.3 days), which was >5.8-fold longer than that of control MSD (8.5 days) in this model compared with the *T. b. brucei* S427 acute mouse (Table 2).

The KS-505a data suggest that it is possibly a new candidate compound for discovering new antitrypanosomal drugs with more potent activity.

Discussion

We discovered the antitrypanosomal activity of two microbial metabolites and evaluated this *in vitro* and *in vivo* using *T. b. brucei* strain GUTat 3.1 and *T. b. rhodesiense* strain STIB900. In the STIB900 acute mouse model, Baliani *et al.*, suggested that parasites appear to leave the vasculature system early so that drugs must work in an extravascular fashion to effect radical cure. Therefore, this *in vivo* test represents a good model for evaluating likely outcomes of drugs in late-stage models [11]. Our results also indicated that the STIB900 acute mouse model was more stringent than the S427 acute mouse model in the case of pentamidine.

KS-505a (Fig. 1) is a tetraterpenoid antibiotic, and is reported to possess biological activities, including inhibition of brain Ca^{2+}/calmodulin-dependent cyclic nucleotide phosphodiesterase (CaM-PDE) and neurite-promoting activity in NG108-15 neuroblastoma×glioma hybrid cells [12, 13], as well, when administered intraperitoneally it exhibits *in vivo* anti-amnesia activity in an electroconvulsive shock-induced amnesia model in the rat [14]. In contrast to the mammalian PDEs, the function of PDEs in parasitic protozoa remains mostly unknown. With regard to *Trypanosoma brucei* species, Zoraghi *et al.* [15], Rascón *et al.* [16], and Zoraghi *et al.* [17] recently reported that *T. brucei* PDEs (TbPDE2 family: TbPDE2A, TbPDE2B and TbPDE2C) were found to be essential for bloodstream form trypanosome proliferation. Furthermore, Oberholzer *et al.*, reported that TbPDE2B and TbPDE2C, as flagellar enzymes, were found to be essential for parasite virulence [18]. They also suggested that the TbPDE2 family might be considered as new molecular targets for chemotherapeutic antitrypanosomal drugs [15～18].

Table 2 *In vivo* antitrypanosomal activity of KS-505a, alazopeptin and pentamidine in two mouse models

Compound	Dosage (mg/kg)	Route	No. of mice cured/ No. of mice infected	MSD	Control MSD
T. b. brucei S427 mouse model					
KS-505a	30×4	i.p.	4/4	>30	4.4
KS-505a	10×4	i.p.	0/4	13.0	4.4
Alazopeptin	50×4	i.p.	0/4	10.3	5.8
Pentamidine	1×4	i.p.	4/4	>30	4.4
Pentamidine	0.2×4	i.p.	1/4	>17.3	6.0
T. b. rhodesiense STIB 900 mouse model					
KS-505a	42×4	i.p.	1/4	>28.5	8.0
Pentamidine	20×4	i.p.	2/4	>49.3	8.5
Pentamidine	10×4	i.p.	1/4	>45.8	8.5

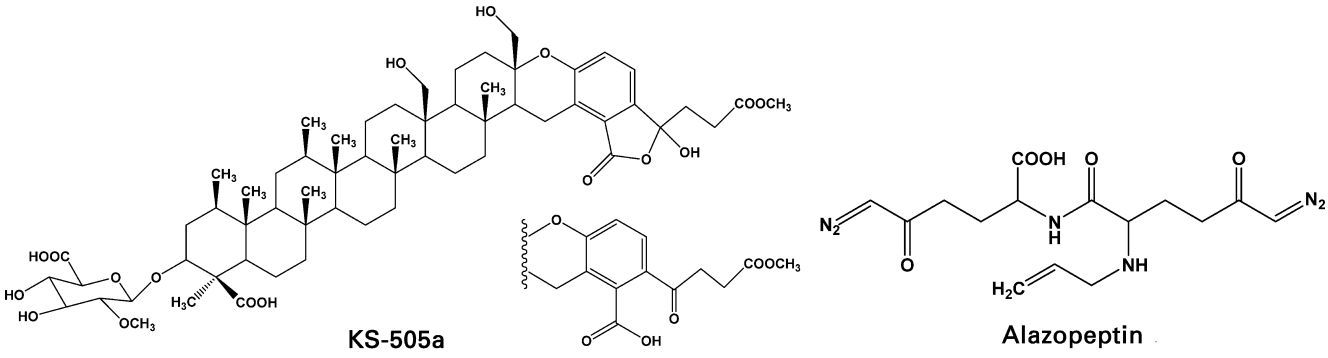

Fig. 1 Structures of KS-505a and alazopeptin.

Therefore, it may be useful to study *T. brucei* PDEs using KS-505a as an inhibitor of mammalian CaM-PDE. The relationship between mammalian PDEs and TbPDEs remains of significant interest.

Alazopeptin is an azaamino acid antibiotic [19], and is reported to have antitumor, antibacterial, and glutamine and purine antagonist activity [20]. Alazopeptin contains two moles of DON and alanine in the structure (Fig. 1). Recently, Hofer *et al.* reported that DON exhibited *in vitro* and moderate *in vivo* antitrypanosomal activities [21]. Its *in vivo* antitrypanosomal activity was static (not curative), furthermore, the mode of action of DON was via inhibition of CTP synthetase of *T. brucei* [21]. Alazopeptin may inhibit *Trypanosma* species in the same manners as DON.

The above results reveal that KS-505a and alazopeptin are promising lead compounds for new types of antitrypanosomal drugs. Further investigations of the antitrypanosomal potential of KS-505a and alazopeptin are in progress.

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