ANTITRYPANOSOMAL EFFECTS OF TRADITIONAL CHINESE HERBAL MEDICINES ON BLOODSTREAM FORMS OF *TRYPANOSOMA BRUCEI RHODESIENSE IN VITRO*

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Abstract. The antitrypanosomal activity of traditional Chinese herbal medicines and these crude drug ingredients were determined using axenic cultured bloodstream forms of *Trypanosoma b. rhodesiense* which is one of the two causative agents of African sleeping sickness in man. The drugs tested were 8 traditional Chinese herbal medicines and these 14 crude drug ingredients. Of these traditional Chinese medicines examined, san'o-shasin-to and oren-gedoku-to showed most potent antitrypanosomal effect. The minimal effective concentration (MEC) which killed all bloodstream form populations within 24 hours of both drug exposure was 125 μg/ml. The 50% effective concentration (EC_{50}) of san'o-shashin-to and oren-gedoku-to was 63 and 74 μg/ml, respectively. In the crude drug ingredients tested, *Scutellaria baicalensis G.* and *Coptis japonica M.* which are the main components of san'o-shasin-to and oren-gedoku-to, showed the most powerful antitrypanosomal activity. The MEC and EC_{50} value of these crude drug ingredients were 30 and 60 μg/ml, and 20 and 36 μg/ml.

INTRODUCTION

Trypanosoma brucei rhodesiense is one of the two important pathogenic protozoan parasites which cause African sleeping sickness in man, and is transmitted by the tsetse fly. These parasitic diseases occur in a large area of Africa between latitudes 14°N and 29°S, following the geographic distribution of the tsetse fly. Over 50 million people in Africa suffer from infection with pathogenic trypanosomes. Until recently, effective drugs for treatment against human trypanosome infection have been pentamidine, suramin, and melarsoprol. All these drugs have adverse side-effects, for example, melarsoprol which is an effective drug for late stage of African sleeping sickness, causes reactive encephalopathy in 5-10% of patients treated, with a fatal outcome in 1-5% (Kuzoe, 1993; Wang, 1995). Resistance to pentamidine of *Trypanosoma b. gambiense* and to melarsoprol of both *T. b. gambiense* and *T. b. rhodesiense* occurs (Kuzoe, 1993; Wang, 1995). The recently developed DFMO is effective against *T. b. gambiense* infection but not for disease caused by *T. b. rhodesiense* (Kuzoe, 1993; Wang, 1995; Bales *et al,* 1989; Bacchi *et al,* 1990; Iten *et al,* 1995). New drugs, effective against African try-panosome infection are eagerly awaited.

Many present medicines are derived directly or indirectly from medicinal plants. Since the use of Chinese herbal medicines is widespread in Japan (Natori, 1980; Hidaka *et al,* 1992), an ethnopharmacological approach may prove to be a rich source of drug discovery. In the recent investigation, Freiburghaus *et al* (1996) screened antitrypanosomal activity of African plants used in traditional medicine in Uganda against *T. b. rhodesiense in vitro* and revealed the potent efficacy of 8 lipophilic extracts of 5 plants as chemotherapeutic agents for the treatment of African sleeping sickness. For this reason, we determined the antitrypanosomal effects of traditional Chinese herbal medicines and these crude drug ingredients using axenically cultured bloodstream forms of *T. b. rhodesiense in vitro* for developing the new drugs for African sleeping sickness.

MATERIALS AND METHODS

Preparation of traditional Chinese herbal medicines

The traditional Chinese herbal medicines used in this experiment were provided by Tsumura Co

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Ltd (Tokyo, Japan). The crude drug ingredients of 8 traditional Chinese herbal medicines are shown in Table 1. Each mixture of crude drug was boiled in 600 ml of distilled water and extracted for 1 hour. The decoction was passed through 4 sheets of gauze and lyophilized. The yield of each traditional Chinese medicine was as follows: sho-saiko-to, 6.94g; dai-saiko-to, 8.02g; san'o-shashin-to, 2.63g; orengedoku-to, 2.34g; daio-botanpi-to, 3.91g; mokuboi-to, 2.35g; toki-shakuyaku-san, 5.51g; juzendaiho-to, 8.05g.

Preparation of water extract of crude drug ingredients

Ten grams of each ingredient of traditional Chinese medicine was extracted with 500 ml of distilled water at room temperature for 1 hour. Each decoction was passed through 4 sheets of gauze and lyophilized.

Trypanosomes

The pleomorphic clone of *Trypanosoma brucei rhodesiense* (ILRAD 1501), kindly provided by Dr T Fukuma, Department of Parasitology, Kurume University, Kurume, Japan, was used to initiate cultivation. A culture adapted bloodstream forms in axenic condition was used for *in vitro* drug screening test.

Culture medium

Dulbecco's modified minimum essential medium (D-MEM, pH 7.0, GIBCO BRL, Life Technologies, Inc, Rockville, MD, USA) supplemented with 0.3% sodium bicarbonate was used for the cultivation of bloodstream forms of *T. b. rhodesiense* as described by Yabu *et al* (1990). The medium was sterilized by Millipore filter (0.22 μm). Immediately before use, the culture medium was supplemented with 10 μM 2,9-dimethyl-4,7-diphenyl-1,10-phenon- throline disulfonic acid (bathocuproine sulfonate, BCS; Dojin Chemical Co, Kumamoto, Japan), 100 μM cysteine (Wako Pure Chemical Co, Osaka, Japan), 0.2 IU/ml insulin (GIBCO), 1 mM sodium pyruvate (Wako) and 20 % heat-inactivated bovine calf serum (GIBCO).

Axenic culture of trypanosomes

Bloodstream forms removed from infected mouse were diluted with D-MEM supplemented with 10 μM BCS, 100 μM cysteine, 0.2 IU/ml insulin, 1 mM sodium pyruvate and 20 % FBS to a density of 1 x 10^4 trypanosomes/ml. Then 5 ml of this trypanosome suspension was added to 25 cm^2 tissue culture flasks (Costar Co, Cat. No 3055, Massachusetts, USA) and incubated at 37°C in an atmosphere of 5 % CO_2 and 95 % air. After incubation for 24 hours, 1 ml of fresh medium was added to the flasks without removing the medium, thereafter 1 ml of culture medium was changed every day. Culture-adapted long slender bloodstream forms were transferred into 25 cm^2 tissue culture flasks (Costar) containing 5 ml of D-MEM supplemented with 10 μM BCS, 100 μM cysteine and 20% FBS, and maintained therein.

***In vitro* assays**

Drug sensitivity of *in vitro* cultured bloodstream forms of *T. b. rhodesiense* was determined according to the slightly modified method previously described by Minagawa *et al* (1997). Drugs were dissolved in complete culture medium to the concentration of 10 mg/ml and passed through 0.22 μm filter for sterilization. The drug solution was serially diluted from 2 mg to 15 μg/ml in culture medium in 96-well tissue culture plate (Costar, Cat No. 3596) as follows: The first well was placed 100 μl of drug solution (2 mg/ml) and diluted serially to the other wells containing 50 μl culture medium. Then, same volume of trypanosome suspension (2 x 10^5/ml) was added to each well and cultured at 37°C in an atmosphere of 5 % CO_2 and 95 % air for 24 hours. Control culture (without drug) was incubated under the same condition. Suramin (Antrypol, Imperial Chemical Pharmaceutical Ltd, Manchester, England) was also dissolved and diluted with culture medium and used for commercially available drug control. After incubation for 24 hours, living trypanosomes were counted by hemacytometer. Living trypanosomes after drug exposure was expressed as percentage of control cultures. The percentage values of living trypanosomes were plotted against the corresponding drug concentration on a semi-logarithmic scale. EC_{50} values (defined as the concentration which inhibited 50% growth of trypanosomes) were quantified by linear interpolation according to the modified method of Huber and Koella (1993). The minimum effective concentration (MEC) was also defined as the lowest concentration of drug in which no trypanosomes with normal morphology or mortality.

Table 1

Crude drug ingredients of traditional Chinese herbal medicines.

Plant	Family	Part	Composition (g)							
			[a]A	B	C	D	E	F	G	H
Bupleuram falcatum L.	Umbelliferae	Root	7.0	6.0	-	-	-	-	-	-
Pinellia ternata B.	Araceae	Tuber	5.0	4.0	-	-	-	-	-	-
Scutellaria baicalensis G.	Labiatae	Root	4.0	3.0	3.0	3.0	-	-	-	-
Zizyphus vulgaris L.	Rhamnaceae	Fruit	4.0	3.0	-	-	-	-	-	-
Panax ginseng C.A. Meyer	Araliaceae	Root	4.0	-	-	-	-	3.0	-	3.0
Glycyrrhiza glabra L.	Leguminosae	Root	3.0	-	-	-	-	-	-	1.5
Zingiber officinale R.	Zingiberaceae	Rhizome	1.0	1.0	-	-	-	-	-	-
Paeonia lactiflora P.	Paeoniaceae	Root	-	3.0	-	-	-	-	4.0	3.0
Citrus aurantium L.	Rutaceae	Fruit	-	2.0	-	-	-	-	-	-
Rheum palmatum L.	Polygonaceae	Rhizome	-	1.0	3.0	-	2.0	-	-	-
Coptis japonica M.	Ranunculaceae	Rhizome	-	-	3.0	2.0	-	-	-	-
Phellodeudron amurense R.	Rutaceae	Cortex	-	-	-	1.5	-	-	-	-
Gardenia jasminoides E.	Rubiaceae	Fruit	-	-	-	2.0	-	-	-	-
Prunus persia B.	Rosaceae	Fruit	-	-	-	-	4.0	-	-	-
Paeonia suffruticosa A.	Paeoniaceae	Cortex	-	-	-	-	4.0	-	-	-
Benincasa hispida T.	Cucurbitaceae	Seed	-	-	-	-	6.0	-	-	-
Cinnamomum cassia B.	Lauraceae	Cortex	-	-	-	-	-	3.0	-	3.0
Sinomenium acutum R.	Menispermaceae	Wood	-	-	-	-	-	4.0	-	-
Atractylodes lancea D.C.	Compositae	Rhizome	-	-	-	-	-	-	4.0	3.0
Alisma orientale J.	Alismataceae	Rhizome	-	-	-	-	-	-	4.0	-
Poria cocos W.	Polyporaceae	Sclerotin	-	-	-	-	-	-	4.0	3.0
Cnidium officinale M.	Umbelliferae	Rhizome	-	-	-	-	-	-	3.0	3.0
Angelica acutiloba K.	Umbelliferae	Root	-	-	-	-	-	-	3.0	3.0
Rehmannia glutinosa L.	Scrophulariaceae	Root	-	-	-	-	-	-	-	3.0
Astragalus membranaceus B.	Leguminosae	Root	-	-	-	-	-	-	-	3.0

a: A, Sho-saiko-to; B, Dai-saiko-to; C, San'o-shasin-to; D, Oren-gedoku-to; E, Daio-botanpi-to; F, Moku-bio-to; G, Toki-shakuyaku-san; H, Juzen-daiho-to

Cytotoxicity assays

Cytotoxicity of traditional Chinese herbal medicines tested was determined according to the slightly modified method of Freiburghaus *et al* (1996). Mouse lymphoma L-1210 cells were seeded in 96-well tissue culture plates (Costar) at a density of 1 x 10^5 cells/ml in 50 µl D-MEM per well supplemented with 20% FBS. A twofold serial dilution ranging from 2,000 to 2 µg/ml of Chinese herbal medicine in 50 µl culture medium was added. Then culture plate was incubated as described for *in vitro* assay. Control (without drug) was incubated under same condition. After incubation for 24 hours the maximum tolerated concentration (MTC) was determined microscopically. The MTC was defined as the highest concentration of Chinese herbal medicine which did not affect growth of L-1210 cells. Selectivity indices (SI) were then calculated by dividing MTC for L-1210 cells by MEC for *T. b. rhodesiense* (Kaminsky *et al,* 1996; Freiburghaus *et al,* 1996).

RESULTS AND DISCUSSION

Animal infective bloodstream forms of *T. b. rhodesiense* (ILRAD 1501) can be maintained in 25 mM HEPES-buffered D-MEM supplemented with 10 µM BCS, 100 µM cysteine and 20% fetal bovine serum for more than 2 years *in vitro* (Yabu *et al,*1990). In this culture condition, trypanosome populations increased in number to 7 to 8 x 10^6 trypanosomes/ml, by day 4 after initiation of the culture at 1 x 10^4/ml. Fig 1 shows a growth curve for this clone of bloodstream forms when culture was initiated at 1 x 10^5/ml, the initial density for drug assays. Before determination of antitrypanosomal activity of traditional Chinese herbal medicines, infectivity of bloodstream forms maintained in culture for more than 2 years was examined. All mice inoculated with bloodstream forms (1 x 10^2 trypanosomes) of *T. b. rhodesiense* which had been cultured for over 2 years became parasitemia on day 4 and, thereafter, successive waves of parasitemia were seen in infected mice.

Antitrypanosomal activity of traditional Chinese herbal medicines, sho-saiko-to, dai-saiko-to, san'o-shashin-to, oren-gedoku-to, daio-botanpi-to, moku-boi-to, toki-shakuyaku-san and juzen-daiho-to were determined using animal infective cultured bloodstream forms of *T. b. rhodesiense in vitro*.

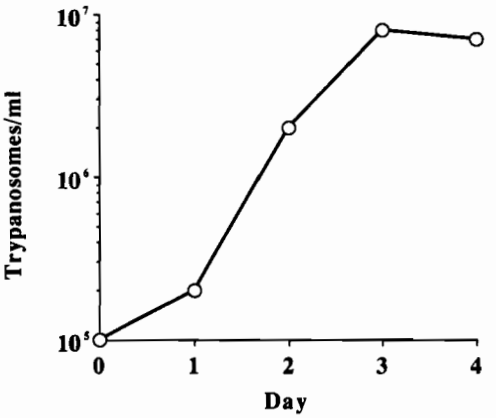

Fig 1–Growth of bloodstream forms of *T. b. rhodesiense* ILRAD 1501 in 96 well tissue culture plates in D-MEM supplemented with 10 µM BCS, 100 µM cysteine and 20% fetal bovine serum at 37°C.

The ingredients of these traditional Chinese medicines tested are shown in Table 1. The effects of these drugs against cultured bloodstream forms was demonstrated as the minimal effective concentration (MEC) which died all bloodstream forms within 24 hours of drug exposure (Minagawa *et al,* 1997). Among the various traditional Chinese herbal medicines tested, san'o-shashin-to and oren-gedoku-to showed the most potent antitrypanosomal activity (Table 2). The MEC value of both drugs was 125 µg/ml. The EC_{50} value of these drugs were 63 and 74 µg/ml. Dai-saiko-to and daio-botanpi-to also showed antitrypanosomal effects. However, these drugs were less effective than san'o-shasin-to and oren-gedoku-to. The MEC value of dai-saiko-to and daio-botanpi-to was 250 µg/ml. The antitrypanosomal activity of sho-saiko-to and mokuboi-to was less effective, the MEC value was 500 µg/ml, respectively. Among 14 different types of crude drug ingredients used, *Scutellaria baicalensis G.* and *Coptis japonica M.* showed powerful trypanocidal activity (Table 3). The MEC values of these drug ingredients were 30 and 60 µg/ml, respectively. The EC_{50} value of these ingredients were 20 and 36 µg/ml, respectively. Since *C. japonica M.* and *S. baicalensis G.* are main ingredients of san'o-shasin-to and oren-gedoku-to (Table 1), potent antitrypanosomal effect of san'o-shasin-to and oren-gedoku-to may be reflected in the activity of *Scutellaria baicalensis G.* and *Coptis japonica M.*

Table 2

Effects of traditional Chinese herbal medicines on the growth of bloodstream forms of *T. b. rhodesiense in vitro.*

Drug	EC_{50} value for *T. b. rhodesiense* (μg/ml)	MEC value for *T. b. rhodesiense* (μg/ml)	MTC value for L1210 cells (μg/ml)	SI*
Sho-saiko-to	121	500	250	0.5
Dai-saiko-to	146	250	250	1.0
San'o-shashin-to	63	125	125	1.0
Oren-gedoku-to	74	125	125	1.0
Daio-botanpi-to	167	250	250	1.0
Moku-boi-to	200	500	250	0.5
Toki-shakuyaku-san	317	1,000	500	0.5
Juzen-daiho-to	383	1,000	500	0.5
Suramin	328	500	250	0.5

*SI, MTC for L1210 cells/MEC for *T. b. rhodesiense*

Table 3

Effects of crude drug ingredients of traditional Chinese herbal medicines on the growth of bloodstream forms of *T. b. rhodesiense in vitro.*

Plant	Part	MEC value (μg/ml)	EC_{50} value (μg/ml)
Bupleuram falcatum L.	Root	1,000	460
Pinellia ternata B.	Tuber	> 1,000	ND
Scutellaria baicalensis G.	Root	30	20
Zizyphus vulgaris L.	Fruit	1,000	640
Panax ginseng C. A. Meyer	Root	1,000	600
Glycyrrhiza glabra L.	Root	500	440
Zingiber officinale R.	Rhizome	500	370
Rheum palmatum L.	Rhizome	250	130
Phellodeudron amurense R.	Cortex	500	370
Gardenia jasminoides E.	Fruit	1,000	490
Paeonia suffruticosa A.	Cortex	250	120
Prunus persia B.	Fruit	> 1,000	ND
Coptis japonica M.	Rhizome	60	36
Benincasa hispida T.	Seed	500	380

ND : not determined.

Our investigation of present study confirmed traditional Chinese herbal medicines possess an effective antitrypanosomal activity and suggested an ethnopharmacological approach may prove to be a rich source of drug discovery.

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