SCREENING FOR NEW ANTITRICHOMONAL SUBSTANCES OF MICROBIAL ORIGIN AND ANTITRICHOMONAL ACTIVITY OF TRICHOSTATIN A

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In vitro and *in vivo* screening methods for new antitrichomonal substances were established. Primary screening is based on *in vitro* antitrichomonal activities of culture broths of actinomycetes isolated from soil. With secondary screening, after crude materials obtained from the cultured broths were administered orally to mice, excretion of antitrichomonal activity into urine was examined. Tertiary screening was done by examining therapeutic activity for experimental trichomoniasis in mice with *Trichomonas foetus*.

Using the screening systems, a new antibiotic (setamycin)-producing strain was picked out among about six thousands soil isolates, and the therapeutic efficacy of KM-3851, which was identified as trichostatin A, was found. It was active against *T. foetus* both *in vitro* and *in vivo*.

Many kinds of parasitic diseases remain to be conquered. There are relatively few antiparasitic agents compared with the many antibiotics available for the treatment of bacterial infections.

Trichomoniasis, one of parasitic diseases, is sexually transmitted. Systemic agents are desirable for chemotherapy of the sexually transmitted disease. In 1959, metronidazole was marketed as the first systemic antitrichomonal agent. It was selected for *in vitro* and *in vivo* antitrichomonal activity among about 150 nitroheterocyclic compounds synthesized on the basis of the structure of azomycin which was the first nitroheterocyclic antibiotic possessing antiprotozoal activity.[1,2] Recently, however, *in vivo* resistance to metronidazole and its carcinogenicity and mutagenicity have been reported.[3]

So, we tried to develop a screening method for new antitrichomonal agents from fermented broths of microorganisms to find superior antitrichomonal drugs.

In this paper we present the new *in vitro* and *in vivo* screening methods with evidence for its applicability and the results of the practical screening using the methods.

Materials and Methods

Organisms and Media

A strain of *Trichomonas foetus* provided from Merck Sharp & Dohme Research Laboratories, U.S.A. was maintained at 37°C in DIAMOND's medium[4] supplemented with 10% heat-inactivated calf serum, 100 U/ml of benzylpenicillin and 1.0 mg/ml of streptomycin sulfate, using 48-hour transfers, and used as a test organism.

Trichosel Broth (BBL) supplemented with 5% heat-inactivated calf serum, 100 U/ml of benzylpenicillin and 1.0 mg/ml of streptomycin sulfate, was used for *in vitro* assay of antitrichomonal activity.

ASAMI's medium,[5] SYS medium[6] and Vf bouillon medium[7] which were supplemented with 5% heat-inactivated calf serum, 100 U/ml of benzylpenicillin and 1.0 mg/ml of streptomycin sulfate were also used for growth test.

Mice (ICR) of female and 19~25 g in weight were obtained from commercial sources. Water and feed were provided *ad libitum*.

Primary Screening by *In Vitro* Assay for Antitrichomonal Activity

In the primary screening, the inhibitory activities of cultured broths of soil actinomycetes against *T. foetus* were assayed. An aliquot (200 μl) of a cultured broth of an actinomycete, 100 μl of heat-inactivated calf serum containing 2,000 U/ml of benzylpenicillin and 20 mg/ml of streptomycin sulfate were added to 1.6 ml of Trichosel Broth, and then 100 μl of a diluted cell suspension (2×10^5 cells/ml) prepared from an actively multiplying *T. foetus* culture was delivered into the above medium. The number of the trichomonads to be inoculated was determined with hemocytometer count. After incubation for 48 hours at 37°C, the number of *T. foetus* cells was counted with a hemocytometer. The cultured broths showing reduction of the number over 50% were selected for the secondary screening.

Secondary Screening by Urine Excretion Test

Water extract of dried culture filtrate combined with MeOH extract of mycelium was used as a sample preparation for the test. Each 0.5 ml of a sample solution possessing *in vitro* activity equivalent to that of 200~1,000 μg/ml of metronidazole was administered orally to mice, and then urine was collected for 4 hours. The antitrichomonal activity of the urine was determined by the above *in vitro* assay method with *T. foetus*.

Tertiary Screening by *In Vivo* Assay for Antitrichomonal Activity

A culture of *T. foetus* grown in the Trichosel Broth was inoculated intraperitoneally into mice and reisolated from the mice. The procedures were repeated several times prior to *in vivo* experiment to increase its virulence and infectivity.

A mouse was inoculated intraperitoneally with 1 ml of a 48-hour culture containing about two million *T. foetus* immediately after the oral administration of the test compound as described by CUCKLER *et al.*[8] The drugs were suspended in 0.5% Methocel (methylcellulose) or distilled water. Oral treatment was given once daily for 4 successive days. The experiments were terminated at 7 days after the mice were inoculated with trichomonads. The criteria of antitrichomonal efficacy were based upon the presence of living trichomonads in peritoneal fluid. The living trichomonads were detected microscopically in a 48-hour culture of a washing fluid with sterilized physiological saline solution from infected abdominal cavity in the treated mice.

Chemicals

Metronidazole was obtained from Merck Sharp & Dohme Research Laboratories, U.S.A., trichostatin A was prepared from the fermented broth of the strain KM-3851 in our laboratory as described by TSUJI *et al.*[9]

Results and Discussion

Growth of *T. foetus* in Various Media

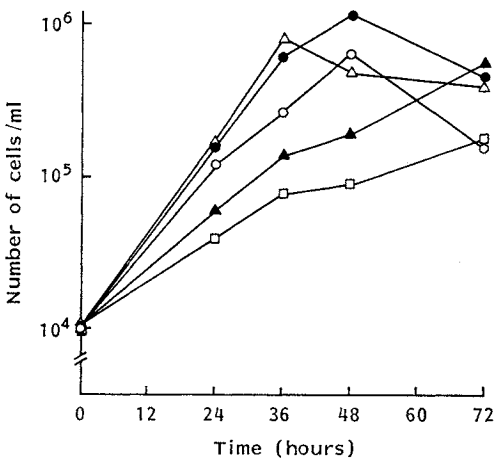
Fig. 1 shows the growth of *T. foetus* in various media tested. Among them DIAMOND's medium, ASAMI's medium and Trichosel Broth supported good growth of the organism. Number of the cells reached maximum after incubation for 48 hours with DIAMOND's medium and Trichosel Broth, and after 36 hours with ASAMI's medium. Trichosel Broth among them was used as *in vitro* assay medium because it was commercially available. With SYS and Vf bouillon media, the growth rate was relatively slow.

Urine Excretion Test with Metronidazole

To investigate the absorption and excretion of metronidazole as model antitrichomonal agent in mice, urine was collected for 4 hours after metronidazole was administered orally, and then the

Fig. 1. Growth of *Trichomonas foetus* in various media.

●: DIAMOND's medium, ○: Trichosel Broth, △: ASAMI's medium, ▲: SYS medium, □: Vf bouillon medium.



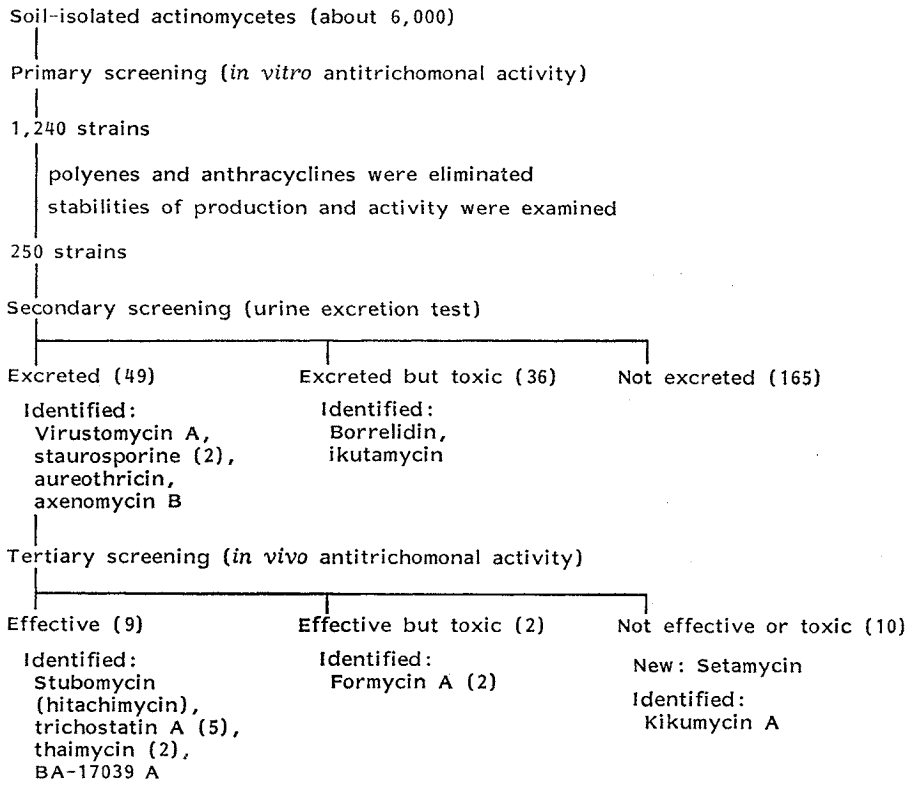
Media was inoculated with 1×10^4 cells/ml of the organism and incubated at 37°C without shaking.

antitrichomonal activity of the urine was determined. As shown in Table 1, the urine preparations from mice to which 50~200 μg of metronidazole was administered showed a significant antitrichomonal activity against *T. foetus*. Considering these results, in the urine excretion test

Table 1. The antitrichomonal activities of urine preparations from mice to which metronidazole was administered.

The amount of metronidazole administered to mice (μg/mouse)	Dilution rate of urine	Antitrichomonal activity (inhibition %)
50	1	79
	1/2	35
100	1	87
	1/2	79
200	1	99
	1/2	83
	1/4	53

Scheme 1. Results of screening for antitrichomonal antibiotics.



A number in the parenthesis indicates a number of strains.

of cultured broths of soil isolates showing *in vitro* antitrichomonal activity, the amount of cultured broth showing the activity equivalent to 100~500 μg of metronidazole was administered orally to mice.

Results of this Screening Program

Broth filtrates of about 6,000 strains of actinomycete soil isolates were submitted to this screening program. As shown in Scheme 1, 1,240 strains among them passed the primary screening and 49 strains passed the secondary screening. Finally, only 9 strains passed the tertiary screening. However, the active products from all passed strains were identified with known antibiotics. Although a new antibiotic setamycin was discovered in this screening work.

Setamycin (KM-6054) is active *in vitro* against trichomonads, some fungi and Gram-positive bacteria. However, the antibiotic was toxic in mice when it was orally administered at the dose over 10 mg/kg, and was not effective to *T. foetus* in mice when it was administered at the dose below 10 mg/kg. The taxonomy, isolation and properties have been reported by ŌMURA *et al.*[10,11] The producing organism, strain KM-6054, was found to be a new genus of the order *Actinomycetales*, and named *Kitasatosporia setae* gen. nov. sp. nov.[12] Recently, the structure was elucidated as shown in Fig. 2.[13]

The antitrichomonal activities of trichostatin A, BA-17039A, stubomycin (hitachimycin), formycin A, kikumycin A, virustomycin A, staurosporine and aureothricin (Fig. 3) among twelve antibiotics identified in this screening work (Scheme 1) have never been known while those of the other four antibiotics (borrelidin,[14,15] axenomycin B,[16,17] thaimycin[18] and ikutamycin[19]) have been reported. Hitachimycin is a 19-membered lactam antibiotic[20] isolated from a culture broth of an actinomycete strain in this screening program.[21] After that hitachimycin was found to be identical with the antitumor antibiotic stubomycin.[22] It is active against Gram-positive bacteria, some fungi, mycoplasmas, and HeLa cells besides trichomonads.[21,22]

Virustomycin A was found as an antivirus antibiotic in our laboratory. It is active against trichomonads and various RNA and DNA viruses and weakely active against some fungi as reported previously.[23,24] The structure and mode of action against *T. foetus* have been reported previously

Fig. 2. Structures of setamycin and virustomycin A.

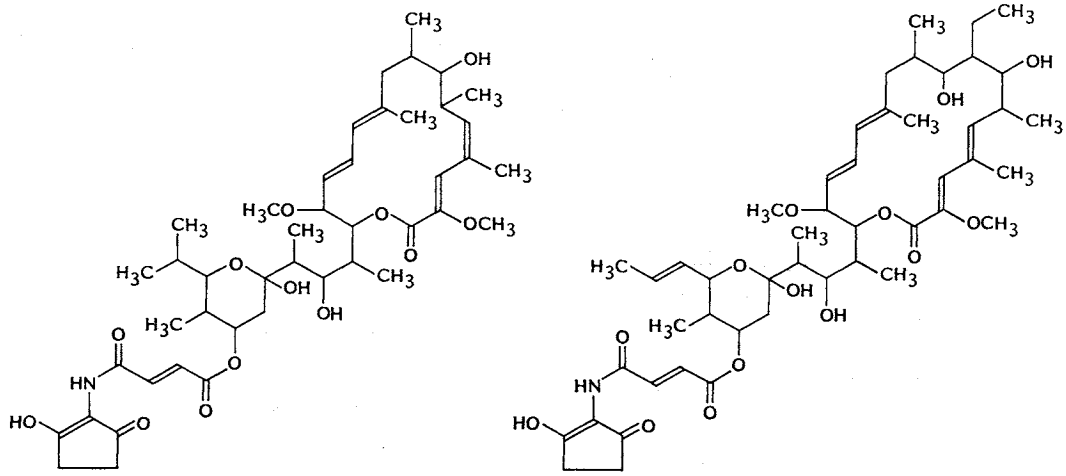
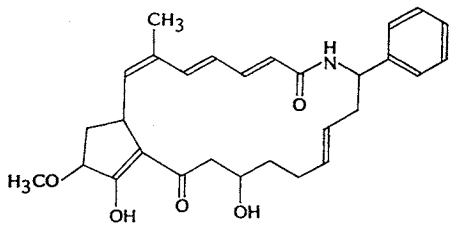
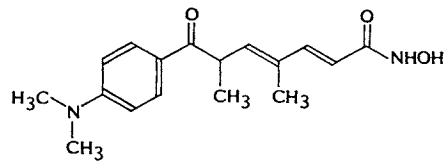


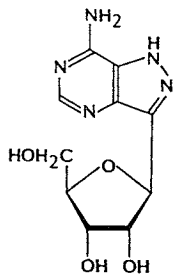
Fig. 3. Structures of stubomycin (hitachimycin), trichostatin A, formycin A, kikumycin A, staurosporine and aureothricin.



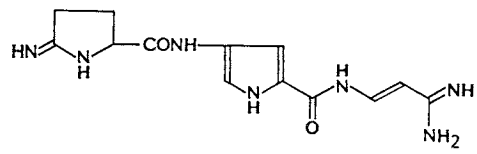
Stubomycin (hitachimycin)



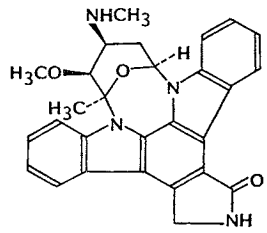
Trichostatin A



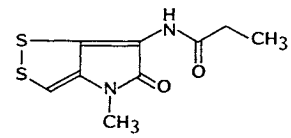
Formycin A



Kikumycin A



Staurosporine



Aureothricin

by the authors (Fig. 2).[24,25]

Trichostatin A is a natural hydroxamic acid active against trichophytons and some other fungi.[9] BA-17039-A is active against HeLa cell, S-180 and adenocarcinoma 755.[26] It is a peptide antibiotic although the structure has not been elucidated. Formycin A is a *C*-nucleoside antibiotic, active against Gram-positive and Gram-negative bacteria and tumor.[27,28] Kikumycin A is a pyrrol-amide antibiotic, active against Gram-positive and Gram-negative bacteria.[29,30] Staurosporine is a microbial alkaloid possessing potent hypotensive, antiedema, antitumor and

Table 2. Antitrichomonal activities of metronidazole and the antibiotics whose antitrichomonal activities were found in this screening work.

Compound	MIC (μg/ml)
Metronidazole	1
Setamycin	1.25
Stubomycin (Hitachimycin)	2
Virustomycin A	1.6
Trichostatin A	0.05
BA-17039-A	1.25
Formycin A	12.5
Kikumycin A	25
Staurosporine	2.5
Aureothricin	1

Table 3. Therapeutic efficacy of trichostatin A and metronidazole on experimental trichomoniasis in mice.

Drug	Daily dose (mg/kg)	Objective symptom[a]	Suppressive symptom[b]	Non-infection[c]	Cured (%)
Trichostatin A	100	2/6	0	4/6	67
	75	2/6	0	4/6	67
	50	4/6	0	2/6	33
	25	4/6	1/6	1/6	17
	12.5	5/6	0	1/6	17
Metronidazole	100	0	0	6/6	100
	75	0	0	6/6	100
	50	2/6	0	4/6	67
	25	2/6	0	4/6	67
	12.5	3/6	1/6	2/6	33
Blank test		4/6	1/6	1/6	17

The method is described in the text. Number of mice; infected/used or cured/used.

[a] Formation of much peritoneal fluid was observed.

[b] Much peritoneal fluid was not formed, but living trichomonads were detected microscopically in a 48-hour culture of washing fluid from infected abdominal cavity with sterilized physiological saline solution.

[c] No living trichomonads were detected.

some antifungal activities. The taxonomy, isolation, properties and structures have been reported by ŌMURA *et al.*[31,32] Aureothricin is a pyrrothine antibiotic, active against Gram-positive and Gram-negative bacteria and some fungi.[33,34]

Table 2 shows the antitrichomonal activities of those antibiotics against *T. foetus*. Trichostatin A among them exhibited the strongest activity. The activity was stronger than that of metronidazole. So, we tried further *in vivo* experiment as described below.

In Vivo Antitrichomonal Activity of Trichostatin A

The therapeutic efficacy of trichostatin A on experimental trichomoniasis in mice was examined compared with that of metronidazole. As shown in Table 3, trichostatin A exhibited moderate effect against peritoneal infections of *T. foetus* in mice when it was administered orally. These results suggest that trichostatin A might be useful as a topical trichomonacide although the activity was somewhat weak compared with that of metronidazole. Further investigations are necessary to evaluate the activities of trichostatin A against other protozoas.

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