TETROCARCINS, NEW ANTITUMOR ANTIBIOTICS

3. ANTITUMOR ACTIVITY OF TETROCARCIN A

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Tetrocarcin A, isolated from a *Micromonospora* culture showed activity against experimental i.p. inoculated tumors such as Ehrlich carcinoma, MH134 hepatoma, B16 melanoma. But it was not active against solid tumors such as sarcoma 180 and Ehrlich carcinoma. It was marginally active against the growth of solid Lewis lung carcinoma without prolonging the life span of the tumor-bearing mice. It was active against P388 leukemia (i.v. - i.v. system). It did not show myelosuppression and nephrotoxicity in mice. DNA and protein synthesis of P388 cells in culture were more significantly suppressed than RNA synthesis by tetrocarcin A.

Tetrocarcin, a group of antibiotics with a novel carbon skelton[1] was isolated from the culture broth of *Micromonospora chalcea* KY11091.[1,2,3] This antibiotic is active against Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*, but is weakly active against Gram-negative bacteria, such as *Escherichia coli* and *Klebsiella pneumoniae*. The preliminary result of antitumor activity of tetrocarcins against P388 leukemia and sarcoma 180 was described in the previous reports.[1,3]

This paper describes the antitumor activity against various murine tumors, bone marrow toxicity in mice and the effect of tetrocarcin A on the macromolecule synthesis of P388 cells in culture.

Materials and Methods

Chemicals

Tetrocarcin A was prepared according to the method of TOMITA *et al.*[3] Mitomycin C (Kyowa Hakko Kogyo) was used as a reference antitumor agent. [^{3}H]Thymidine ([6-^{3}H]thymidine, 22.9 Ci/mmole), [^{3}H]uridine ([5-^{3}H]uridine, 30.0 Ci/mmole) and [^{3}H]leucine (L-[4,5-^{3}H]leucine, 52.0 Ci/mmole) were obtained from the Radiochemical Center Amersham (England).

Animals

Male mice of *dd*Y, CDF_1 hybrid strain (BALB/c, female × DBA/2, male) weighing 18~22 g, male mice of BDF_1 hybrid strain (C57BL/6, female × DBA/2, male) weighing 20~22 g, male mice of C3H/He/J weighing 18~22 g and male mice of C57BL/6 weighing 20~23 g were obtained from Shizuoka Agricultural Co-operative Association for Laboratory Animals (Hamamatsu).

Tumors

Sarcoma 180 and Ehrlich carcinoma were maintained by successive i.p. passage of 1 week interval in *dd*Y male mice, P388 in DBA/2 mice, Lewis lung tumor by s.c. passage of 14 days interval in the axillary region of C57BL/6 male mice, and MH134 hepatoma by i.p. passage of 12 days interval in C3H/He/J mice.

For antitumor test, sarcoma 180 were transplanted s.c. at 5×10^6 cells/mouse. P388 leukemia was implanted i.p. at 1×10^6 and 1×10^5 cells/mouse for i.p. - i.p. system and i.v. - i.v. system, respectively.

Ehrlich carcinoma was transplanted s.c. or i.p. at 1×10^6 cells/mouse. Lewis lung carcinoma was transplanted s.c. at 2^3 mm^3 by trocar. B16 melanoma was transplanted i.p. at 0.1 ml of 20% homogenate/mouse and MH134 was inoculated i.p. at 1×10^6 cells/mouse.

With ascites tumors, antitumor activity was evaluated by the T/C (%) where T and C represent the mean survival of the treated animal and that of control tumor-bearing animals, respectively. Each of the experiment was terminated on the 50th or 60th day after inoculation. For the solid tumors, the antitumor activity was evaluated by the T/C(%), where T and C represent the mean size of tumor of the treated animal and that of control animal respectively. Tumor diameters were measured periodically with callipers and the tumor volume was calculated by the formula represented in NCI protocol for screening[4]: volume (mm^3)$=1/2$ ab^2, where a and b represent the larger and smaller diameter, respectively.

LD_{50} was calculated from the number of survivors at 14 days after a single intravenous or intraperitoneal administration into *dd*Y mice by Behrens-Karber method.

For white blood cell count, 20 μl sample of supra-orbital venous blood was mixed with 9.98 ml of Cellkit-7 (Toa Medical Electro. Co. Ltd.) solution and counted by Microcell Counter (Toa Medical Electro. Co. Ltd.) after lysis of erythrocytes with Saponin-S (Toa Medical Electro. Co. Ltd.). Differential counts of white blood cells were performed on Wright-stained smear of pretreatment blood on day 4 after treatment which was found to be nadir of WBC depression.

For assessing renal toxicity, blood urea nitrogen (BUN) was measured according to the method reported previously.[5]

Culture of P388 Cells

P388 cells were cultured in RPMI 1640 (Grand Island Biological Co., Ltd., U.S.A.: GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 50 μM of mercaptoethanol, 100 U/ml of penicillin G and 100 μg/ml of streptomycin. For testing the growth inhibitory activity, 1×10^4 cells inoculated for 24 hours at 37°C in a humidified atmosphere of 5% CO_2 in air were treated with drugs for 1 or 72 hours and the cells were counted at the end of 96 hours inoculation after the start of the experiments.

Incorporation of the Labelled Precursors into Macromolecule

Logarithmically growing P388 cells (1×10^5/ml) were cultured with tetrocarcin A at 37°C in humidified 5% CO_2 in air. [^{3}H]Thymidine (2 μCi/ml), [^{3}H]uridine (2 μCi/ml) and [^{3}H]leucine (2 μCi/ml) was added at 0, 1, 2, 3 and 4 hour after addition of the drug and cells were cultured for 1 hour. The collected cells on membrane filters GF/F (Whatman Co. Ltd., England) were washed with cold phosphate buffered saline solution (pH 7.0) and were washed with 5% cold trichloroacetic acid. The filters were dried and their radioactivity was determined in vials containing toluene scintillation fluid with 2,5-diphenyloxazole by a liquid scintillation counter (Aloca Lsc-653).

Results

Acute Toxicity of Tetrocarcin A

The LD_{50} of tetrocarcin A by a single intraperitoneal and intravenous dose were 54.2 and 64.0 mg/kg, respectively.

Antitumor Activity

A summary of antitumor activity of tetrocarcin A was shown in Table 1. For the solid tumor of sarcoma 180 and Ehrlich carcinoma, tetrocarcin A did not show a significant inhibitory activity on tumor growth by a single or multiple administration. The i.v. administration of tetrocarcin A gave T/C% of 42 and 33 against the growth of Lewis lung carcinoma on day 8 by a single and multiple doses respectively, but the inhibitory activity of tumor growth was not observed on day 14. The prolongation of the life span of treated mice was not obtained by the administration routes and schedules examined.

Against i.p. implanted Ehrlich carcinoma bearing mice, a single injection of tetrocarcin A gave only 125% of T/C%, but a multiple i.p. administration gave T/C% of 185 at a dose of 5 mg/kg/day for 7 days.

Table 1. Antitumor activity of tetrocarcin A on mouse tumor systems.

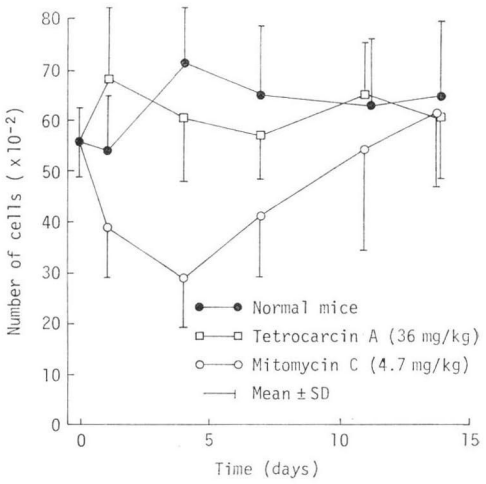
site	Tumor	Drug		Number of animals	T/C%	(50 days survivors)	Optimal dose (mg/kg/day)
		Route	Schedule				
sc	Sarcoma 180	iv	day 1	6	82		40
sc	Sarcoma 180	iv	day 1~5	6	49		40
sc	Ehrlich carcinoma	ip	day 1	5	47		25
sc	Lewis lung carcinoma	iv	day 1	5	42		40
sc	Lewis lung carcinoma	iv	day 1~5	5	33		10
ip	Ehrlich carcinoma	ip	day 1	5	125		50
ip	Ehrlich carcinoma	ip	day 1~7	5	185		5
ip	B16 melanoma	ip	day 1	5	130		40
ip	B16 melanoma	ip	day 1,3,5,7	5	179		15
ip	MH134 hepatoma	ip	day 1	5	156	(2/5)	20
ip	MH134 hepatoma	ip	day 1~4	5	172		2.5
iv	P388 leukemia	iv	day 1	5	130		40
iv	P388 leukemia	iv	day 1~5	5	157		10

A significant activity was exhibited against mice bearing B16 melanoma inoculated intraperitoneally. A maximum T/C% (179%) was obtained at a dose of 15 mg/kg/day × 4 days. Tetrocarcin A gave 156% maximum T/C% on MH134 hepatoma-bearing (i.p.) mice, and 40% of treated mice survived more than 50 days at a dose of 20 mg/kg. Significant activity (over 125% of T/C%) was obtained against P388 bearing mice inoculated intravenously with T/C% of 130 and 157 by a single (40 mg/kg) and multiple (10 mg/kg/day × 5) administration.

Effect on WBC

Fig. 1 shows the white blood cell count after intravenous dose of $2/3 \times LD_{50}$ of tetrocarcin A or mitomycin C. None of the mice treated with tetrocarcin A showed a significant decrease in WBC. But, the WBC count in mice treated with mitomycin C was 46% of saline-control on 4 days after injection. Tetrocarcin A showed a slight increase in the percentage of neutrophil in WBC differential count. However, this increase was not statistically significant, although it might show the irritation by tetrocarcin A.

Fig. 1. Effect of tetrocarcin A on WBC in mice.



Renal Toxicity

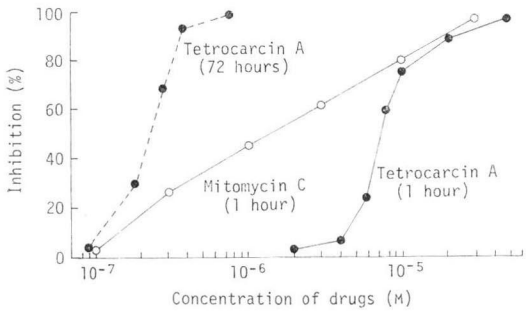
Tetrocarcin A did not affect BUN (BUN value = 20 mg%) by an intravenous dose of $2/3 \times LD_{50}$.

In Vitro Effect

In vitro studies using P388 leukemia cells revealed that tetrocarcin A possessed a potent cytotoxicity at low concentrations. Approximately 50% growth inhibition was observed at a concentration of 6.2 ×

Fig. 2. Effect of tetrocarcin A on the growth of P388 *in vitro*.

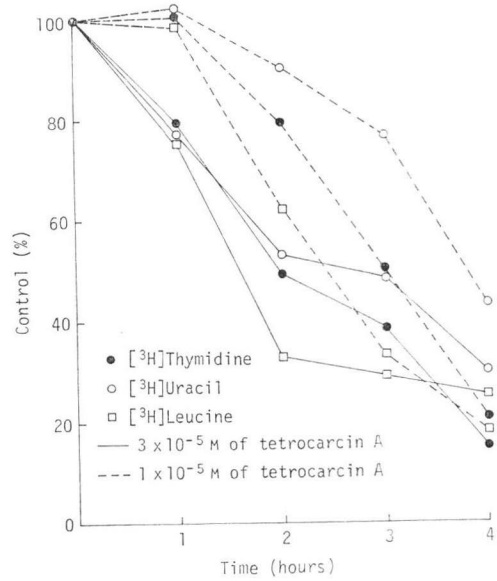
Logarithmically growing P388 leukemia cells were incubated with drugs for 1 or 72 hours. Number of cells were counted at 72 hours after addition of drugs.



10^{-6} M after incubation for 1 hour. When cells were incubated with tetrocarcin A for 72 hours, cell growth was more severely inhibited than 1 hour incubation and 50% growth inhibition was approximately at a concentration of 2.2×10^{-7} M (Fig. 2).

Fig. 3. Effect of tetrocarcin A on macromolecule synthesis.

The labelled precursors were added at the indicated time after addition of tetrocarcin A and the radioactivity was measured after 1 hour incorporation.



Effect on Macromolecule Synthesis

At the concentrations of 1×10^{-5} and 3×10^{-5} M of tetrocarcin A, the incorporation of [^{3}H]leucine and [^{3}H]thymidine into macromolecules were more strongly inhibited than that of [^{3}H]uridine (Fig. 3).

Discussion

Tetrocarcin A gave a significant activity against i.p. implanted B16 melanoma, MH134 hepatoma and Ehrlich carcinoma. But, it was less active on solid tumor of Lewis lung carcinoma and inactive on solid tumor of sarcoma 180, Ehrlich carcinoma and Yoshida sarcoma (unpublished data).

As WBC and WBC differential counts were not affected at a dose of $2/3\times LD_{50}$, the myelosuppressive activity of tetrocarcin A was not significant in mice.

Tetrocarcin A was reported to inhibit RNA synthesis primarily and some effect on protein synthesis without affecting DNA synthesis in *B. subtilis*.[3] However, protein synthesis and DNA synthesis were more severely inhibited than RNA synthesis in P388 leukemia cells *in vitro*. The step inhibited by tetrocarcin A might be different between mammalian cells and microorganisms.

The antitumor spectrum and the antitumor effect of tetrocarcin A was not significant as compared with the antitumor antibiotics used clinically, such as mitomycin C or adriamycin, whose primary site of action was DNA. If the primary site inhibited by tetrocarcin A was confirmed to be not on nucleic acids, the antibiotics might be interesting for further clinical studies.

References

1) Tomita, F.; T. Tamaoki, K. Shirahata, M. Kasai, M. Morimoto, S. Ohkubo, K. Mineura & S. Ishii: Novel antitumor antibiotics, tetrocarcins. J. Antibiotics 33: 668~670, 1980

2) Tomita, F. & T. Tamaoki: Tetrocarcins, novel antitumor antibiotics. 1. Producing organisms, fermentation and antimicrobial activity. J. Antibiotics 33: 940~945, 1980

3) TAMAOKI, T.; M. KASAI, K. SHIRAHATA, S. OKUBO, M. MORIMOTO, K. MINEURA, S. ISHII & F. TOMITA: Tetrocarcins, novel antitumor antibiotics. 2. Isolation, characterization and antitumor activity. J. Antibiotics 33: 946~950, 1980
4) GERAN, R. I.; N. H. GREENGERG, M. M. MACDONALD, A. M. SCHUMACHER & B. J. ABBOTT: Protochols for screening chemical agents and natural products against animal tumors and other biological systems. Cancer Chemother. Rep., Part 3, 3: 1~103, 1972
5) BRATAUTIERE, J.P.; H.T. PHIG & M. BAILLY: Direct enzymatic determintion of urea in plasma and urine with a centrifugal analyzer. Clinic. Chem. 22: 1614~1617, 1976