Jpn. J. Cancer Res. 82, 1234–1238, November 1991

Inhibitory Effect of Sarcophytol A on Development of Spontaneous Hepatomas in Mice

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The inhibitory effect of sarcophytol A, a cembrane-type diterpene isolated from a marine soft coral, *Sarcophyton glaucum*, on development of spontaneous hepatomas was investigated in C3H/HeNCrj mice. A total of 80 mice were divided equally into two groups. The experimental and control groups were given basal diets with and without 0.01% sarcophytol A, respectively. At week 65 of the experiment, mice were examined for hepatomas. The percentages of hepatoma-bearing mice of the subgroup with three or more tumors and the tumor diameters of the group treated with sarcophytol A were smaller than those of the control group. Ridit analysis revealed that these differences were statistically significant. The body weight gain, and the food intake were not significantly different between these two groups. Analysis of blood serum revealed that feeding the diet containing 0.01% sarcophytol A for 65 weeks did not show any adverse effects. These results suggest that sarcophytol A inhibits the development of spontaneous hepatomas without toxicity, and should be considered as a possible cancer chemopreventive agent for hepatomas in humans.

Key words: Sarcophytol A — Cancer chemoprevention — Hepatoma — C3H mouse

Finding non-toxic agents associated with strong inhibitory effects on development of various cancers is a fundamental requirement for cancer chemoprevention in high-risk groups of humans. Human intervention trials with possible cancer chemopreventive agents, such as retinoids, vitamins C and E, piroxicam, and calcium, are now being conducted in the U.S.[1-3] However, further studies to find new inhibitory and non-toxic agents are required.

Sarcophytol A (Fig. 1) is a cembrane-type diterpene, isolated from the marine soft coral *Sarcophyton glaucum*.[4] Topical applications of sarcophytol A significantly inhibited tumor promotion by teleocidin in a two-stage carcinogenesis experiment on mouse skin.[5] Sarcophytol A in the diet significantly inhibited the development of large bowel cancer induced by methylnitrosourea in rats.[6] Sarcophytol A did not show any toxic effect on animals in those experiments. Kinetic studies on sarcophytol A indicated that ^{3}H-labeled sarcophytol A administered orally to mice is excreted through the hepatobiliary system (unpublished observations). These results prompted us to investigate anticarcinogenic effects of sarcophytol A on development of hepatomas. In this paper, we report that sarcophytol A inhibited development of spontaneous hepatomas in mice without any toxicity.

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[5] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; RBP, retinol-binding protein.

MATERIALS AND METHODS

Animals and chemicals A total of 80 male C3H/HeNCrj mice, 8 weeks old, were purchased from Charles River Japan Inc., Atsugi. They were housed 10 mice/plastic cage on wood chip bedding in an air-conditioned room. Sarcophytol A was isolated from *S. glaucum* collected off Ishigaki Island, Okinawa, and purified as reported previously.[4] Diets containing 0.01% (w/w) sarcophytol A or without the compound (basal diet) were prepared once a month at Oriental Yeast Co., Tokyo, and stored at 4°C.

Experimental procedure All the mice were given the basal diet for the first 6 weeks. They were then divided into two groups (40 mice each). The experimental group was given the diet containing 0.01% sarcophytol A from 14 weeks of age, and the control group was given the basal diet. The mice were kept for a further 65 weeks on each diet. The body weight and the amount of diet consumed were measured once a week. At week 65 of the experiment, all mice in each group were killed under ether anesthesia by drawing blood from the abdominal aorta. The serum was analyzed for albumin, total bilirubin, glutamic pyruvic transaminase, γ-glutamyl transpeptidase, and total cholesterol by using an autoanalyzer (Type 736-60, Hitachi Electric Co., Tokyo) and for retinol-binding protein (RBP),[5] which is a rapid-turnover protein used as an index of hepatic parenchymal function,[7] by enzyme immunoassay (Immunoenzyme RBP, Fujirebio Co., Tokyo).

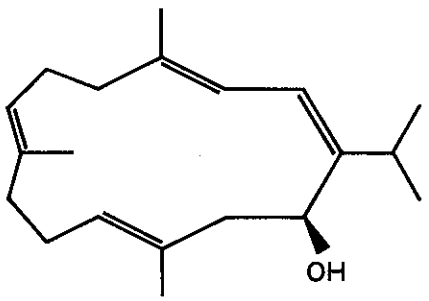

Fig. 1. Chemical structure of sarcophytol A.

Following laparotomy of all mice, detailed macroscopic observation was performed to evaluate the numbers and the diameters of hepatomas. All the tumor tissues were fixed in 10% buffered formalin, embedded in paraffin blocks, and processed by routine histological methods with the use of hematoxylin and eosin stains. Hepatomas were classified into two types, type A, a simple nodular growth of liver parenchymal cells, and type B, areas of papilliform and adenoid growth of tumor cells, as reported by Walker *et al.*[8]

Statistical evaluation of data Differences between experimental results were tested for statistical significance by means of Ridit analysis and Student's *t* test.

RESULTS

General observations There were no significant differences in the body weight gain and amount of food consumed between the experimental and control groups, although the average body weight of the experimental group given the diet containing sarcophytol A was slightly smaller than that of the control group throughout the experiment (Table I). The amount of sarcophytol A consumed was 10.8 mg/kg of body weight/day at week 33 and 12.6 mg/kg of body weight/day at week 65 (Table I).

Development of hepatomas The percentages of hepatoma-bearing mice in the experimental and control groups were 75.0% and 85.0%, at week 65 of the experiment, respectively, and average numbers of hepatomas per mouse were 1.43±1.32 and 1.73±1.18, respectively (Table II). Table III summarizes the distribution of the total numbers of hepatomas per mouse, divided into three subgroups. As Table III clearly indicates, sarcophytol A treatment reduced the percentage of hepatoma-bearing mice of the subgroup with three or more tumors from 30.0% to 15.0%. In the subgroup without hepatomas, 25.0% of the mice were in the experimental group, compared to the mice in the control (15.0%) at week 65 of the experiment. Ridit analysis revealed that the differences in these two parameters were statistically significant between the two subgroups ($P<0.05$). The tumor size was also examined (Table IV). It is apparent that sarcophytol A-treated animals bore smaller tumors than those of the control group, indicating that sarcophytol A treatment inhibited or retarded the growth of tumors ($P<0.001$). Hepatomas were microscopically observed and classified into types A and B. Type A tumor was composed of well differentiated cells with basophilic, eosinophilic, clear or vacuolated cells, and its growth appeared to be by expansion. Type B hepatoma was composed of atypical and basophilic cells with hyper-

Table I. Body Weight, and Weights of Consumed Diet and Sarcophytol A

Groups given the diet[a]	At week 1	At week 33	At week 65
	Body weight (g)		
With sarcophytol A	28.8±2.1[b]	39.5±2.4	36.5±1.7
Without sarcophytol A	28.9±2.1	42.2±2.3	37.5±2.6
	Consumed diet (g/mouse/day)		
With sarcophytol A	4.21±0.18	4.25±0.24	4.56±0.26
Without sarcophytol A	4.05±0.10	4.23±0.11	4.06±0.15
	Consumed sarcophytol A (mg/kg of body weight/day)		
With sarcophytol A	14.6±0.4	10.8±1.1	12.6±1.2

a) In the experimental group, mice were given the standard laboratory diet containing 0.01% sarcophytol A from week 0. The experiment was terminated at week 65. Each group consisted of 40 mice.
b) Mean±SD.

Table II. Incidences and Numbers of Hepatomas

Groups given the diet[a]	No. of mice	Incidence (%)	Average number of hepatomas per mouse
With sarcophytol A	40	30 (75.0)	1.43±1.32[b]
Without sarcophytol A	40	34 (85.0)	1.73±1.18

a) See Table I.
b) Mean±SD.

Table III. Distribution of Total Numbers of Hepatomas per Mouse

Groups given the diet[a]	No. of mice	Total numbers of hepatomas per mouse		
		0	1–2	3–
With sarcophytol A	40	25.0%	60.0%	15.0%
Without sarcophytol A	40	15.0%	55.0%	30.0%

a) See Table I.
P (overall) <0.05.

Table IV. Distribution of Tumor Size

Groups given the diet[a]	Total number of tumors/ No. of mice	Tumor size in diameter (mm)			
		-4	5-9	10-19	20-
With sarcophytol A	57/40	54.4%	21.1%	17.5%	7.0%
Without sarcophytol A	69/40	30.4%	24.6%	31.9%	13.0%

a) See Table I.
P (overall) < 0.001.

Table V. Analysis of Blood Serum

Groups given the diet[a]	Albumin (g/dl)	Total bilirubin (mg/dl)	GPT (IU/liter)	γ-GTP (IU/liter)	Total cholesterol (mg/dl)	RBP (pmol/g)
With sarcophytol A	2.62±0.40[b]	0.33±0.10	45.8±30.1	0.82±1.03	156±46	33.6±8.9
Without sarcophytol A	2.71±0.52	0.38±0.10	51.2±37.1	0.59±0.91	169±62	35.3±10.6

a) See Table I.
b) Mean±SD.
The abbreviations used are: GPT, glutamic pyruvic transaminase; γ-GTP, γ-glutamyl transpeptidase; RBP, retinol-binding protein.

chromatic nuclei arranged in an irregular trabecular pattern. The proportions of A and B in the experimental group were 87.2% and 12.8%, whereas those in the control group were 80.5% and 19.5%. There was no significant difference in the ratio between these two groups. These results indicated that sarcophytol A treatment does not affect the histology of hepatomas.

Analysis of blood serum Serum analysis showed no significant difference between the two groups (Table V). The data indicated that sarcophytol A does not cause any damage to liver functions.

DISCUSSION

The present study has shown that feeding of a diet containing sarcophytol A inhibited the development of spontaneous hepatomas in mice (C3H/HeNCrj). As described in the introduction, sarcophytol A was first reported to be a potent antitumor promoter in mouse skin. Subsequently, sarcophytol A was demonstrated to inhibit methylnitrosourea-induced large bowel carcinogenesis in rats. It is well known that the C3H/HeNCrj mouse is a strain prone to develop spontaneous hepatomas. Hepatocarcinogenesis of this mouse does not need any chemical initiator or tumor promoter. Therefore, it should be established whether sarcophytol A has a potency to inhibit spontaneous liver tumors (developing in high incidence) in C3H mice, besides inhibiting chemical carcinogenesis. Fujiki *et al.* recently reported that sarcophytol A was effective in inhibiting the development of spontaneous mammary tumors in SHN mice (assumed to be induced by mouse mammary tumor virus) and spontaneous leukemia of AKR mice.[9, 10] Thus, sarcophytol A seems to inhibit various kinds of carcinogenic systems. Although the mechanisms of action of sarcophytol A have not been clearly elucidated yet, sarcophytol A suppresses the proliferation of tumor cells and is likely to inhibit the carcinogenesis itself based on the following data. Our present results demonstrated that sarcophytol A reduced the percentage of hepatoma-bearing mice in the subgroup with three or more tumors, as well as reducing the growth of those tumors. These results are compatible with our observation that sarcophytol A inhibited the proliferation of human hepatocellular carcinoma cell line, PLC/PRF/5, *in vitro* (manuscript in preparation).

Recently, Fujiki *et al.*[11] reported that sarcophytol A inhibited tumor promotion by other 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-type tumor promoters, TPA itself and aplysiatoxin, as well as teleocidin. However, sarcophytol A did not inhibit the specific binding of ^{3}H-TPA to a particulate fraction of mouse skin or the activation of protein kinase C *in vitro*,[5] both of which are common biochemical effects of the TPA-type tumor promoters. Therefore, it is assumed that sarcophytol A inhibits the process of tumor promotion at a step after

the binding of TPA-type tumor promoters to the phorbol ester receptors. Some derivatives of sarcophytol A, such as sarcophytol A acetate and compound Y, which is a secocembranoid carboxylic acid derivative obtained by cleaving the cembrane ring of sarcophytol A, did not show strong inhibitory effects on tumor promotion, whereas sarcophytol B, which has two hydroxyl groups in its cembrane ring was as active as sarcophytol A.[5] The results indicated that there are certain structural requirements for inhibitory activity. Verma *et al.* reported that sarcophytol A bound to albumin and several proteins in the liver fraction.[12] Frenkel *et al.* have extensively studied the effects of sarcophytol A and its derivatives on induction of H_2O_2 formation in human polymorphonuclear leukocytes by tumor promoters, whose tumor-promoting activities were assumed to be related to H_2O_2 formation by polymorphonuclear leukocytes. Sarcophytol A suppressed H_2O_2 formation by TPA-activated human polymorphonuclear leukocytes.[13] In addition, we also observed that the culture medium of PLC/PRF/5 cells treated with sarcophytol A showed a lower α-fetoprotein level and an increased albumin level, compared with those of the cells without sarcophytol A treatment (unpublished results). These results suggest that sarcophytol A may induce differentiation through a gene transcriptional switch from α-fetoprotein to albumin.

No clinical symptoms were induced by the diet containing 0.01% sarcophytol A in any of the mice during the 65 weeks of the experiment. All the mice tolerated well the long-term feeding of sarcophytol A at doses of 10 to 15 mg/kg of body weight/day, which were assumed to be pharmacologically effective. Furthermore, sarcophytol A showed no adverse effect on blood serum parameters, and so presumably on liver function. A higher dose of sarcophytol A administered in a similar experimental model is currently under investigation in our laboratory. The non-toxicity of sarcophytol A is promising for its clinical application as a chemopreventive agent.

In general, five main steps are required for development of cancer chemopreventive agents; isolation of an active compound, *in vitro* short-term tests, *in vivo* long-term animal experiments, study of pharmacokinetics and toxicity tests, and application to humans.[14] The third step, *in vivo* long-term animal experiments and toxicity tests in animals, has been completed with sarcophytol A. The next step will be the examination of acute and chronic toxicities in humans. It is generally accepted that one cannot predict the efficacy of compounds in humans directly based on results from animal experiments. Conducting of clinical intervention trials using agents proved to be effective as well as safe in animal models is the only way to resolve this problem, and such a step seems justified at this time.

ACKNOWLEDGMENTS

We are grateful to Dr. T. Tanaka, First Department of Pathology, Gifu University School of Medicine, and Dr. T. Yamada, First Department of Internal Medicine, Gifu University School of Medicine, for advice on histological analysis. We thank Dr. S. Nishiwaki, Cancer Prevention Division, National Cancer Center Research Institute, for valuable discussions. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan.

(Received April 22, 1991/Accepted July 29, 1991)

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