Effects of Gomisin A on Hepatocarcinogenesis by 3'-Methyl-4-dimethylaminoazobenzene in Rats

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ABSTRACT—We examined the effects of gomisin A on tumor promotion in the liver after a short-term feeding of 3'-methyl-4-dimethylaminoazobenzene (3'-MeDAB) to rats, compared with the effects of phenobarbital. Male Donryu rats were fed ad libitum a diet containing 0.06% 3'-MeDAB and 0.03% or 0.01% gomisin A or water containing 0.05% phenobarbital. Gomisin A and phenobarbital did not cause any proliferative and neoplastic lesions by themselves in 40 weeks of feeding. Altered foci in the liver increased with a peak at 12 weeks after the rats were fed 3'-MeDAB. Gomisin A decreased the number of hepatic altered foci such as the clear cell and basophilic cell type foci in the early stages. Phenobarbital enhanced neoplastic alterations so that the number and size of the foci were much larger in the phenobarbital-combined group than in the 3'-MeDAB-control group. Thus, phenobarbital acted as a promoter of cells initiated by 3'-MeDAB; on the other hand, gomisin A showed a weak suppressive effect on tumor promotion.

We have reported that gomisin A, a lignan component isolated from *Schizandra* fruits (1), inhibits liver injuries induced by several hepatotoxic chemicals (2–5) and enhances regeneration of the liver after a partial hepatectomy (6–8). These effects of gomisin A are based on its ability to promote the recovery of liver functions, protect hepatocytes, inhibit cytotoxic cells in the liver, and increase hepatic blood flow, etc. On the other hand, phenobarbital, which induces liver metabolizing enzymes and accelerates liver cell proliferation, enhances hepatocarcinogenesis when given after a

short-term treatment with hepatocarcinogens (9–11).

Because of the above-described properties of gomisin A, we determined its effects on hepatocarcinogenesis. In this study, we histopathologically examined the effects of gomisin A on the progression of carcinogenesis in the liver after a short-term treatment with 3'-MeDAB, which selectively causes parenchymal cell carcinoma in the liver of rats (12).

MATERIALS AND METHODS

Materials

Gomisin A (Fig. 1) was isolated from *Schizandra* fruits (1). The basal diet (CE-2)

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Fig. 1. Structure of Gomisin A.

was obtained from Japan Clea Co., Tokyo. Diets containing 0.01% and 0.03% gomisin A and 0.06% 3'-MeDAB were prepared at Nihon Haigoushiryo Co., Aichi. Phenobarbital (Hoei Yakukoh Co.) was dissolved in water at a concentration of 0.05%.

Rats and feeding protocol

Male Donryu rats (6-weeks-old, Japan Clea) were divided into 8 groups, and 5 rats were housed in a cage. Animals were fed and given water containing drugs ad libitum according to the following protocol (11) (Table 1) and killed for histopathological study at 0, 5, 10, 20, and 40 weeks after starting the feeding

gomisin A and phenobarbital.

Histopathological observation

The liver was excised and fixed in 10% phosphate-buffered formalin solution. Three sections, obtained from the right anterior, left anterior, and left median lobes, were stained with hematoxylin and eosin (H.E.). The number, areas and size of hepatic altered foci were measured in H.E.-stained sections using an image processor (Digitizer GT-4100, Photoron Co., Ltd.) with an Image Measuring System (Finetec Co., Ltd.).

Statistical analyses were done by Student's *t*-test and the Welch test.

RESULTS

During the experiments, gomisin A, even at a high dosage (0.03% of the diet), hardly influenced the diet and water consumption and the body and liver weights of rats pretreated or not pretreated with 3'-MeDAB. Phenobarbital suppressed the increase of body weight and water consumption, perhaps due to its bitter taste, but increased the liver weight, so the ratio of liver weight vs. body weight of rats given phenobarbital was significantly higher than that of the control and gomisin A groups (Table 2 and Fig. 2). No behavioral change was apparent in rats fed gomisin A or phenobarbital.

Table 1. Feeding protocol of 0.06% 3'-MeDAB-containing diet (3'-MeDAB) and gomisin A or phenobarbital (PB)

Group	Feeding period		
	3 weeks	2 weeks	40 weeks
1	Basal diet	Basal diet	Basal diet
2	Basal diet	Basal diet	0.03% Gomisin A-containing diet
3	Basal diet	Basal diet	0.01% Gomisin A-containing diet
4	Basal diet	Basal diet	0.05% PB-containing water
5	3'-MeDAB	Basal diet	Basal diet
6	3'-MeDAB	Basal diet	0.03% Gomisin A-containing diet
7	3'-MeDAB	Basal diet	0.01% Gomisin A-containing diet
8	3'-MeDAB	Basal diet	0.05% PB-containing water

Table 2. Body and liver weight and relative liver weight of rats after feeding gomisin A (G) or phenobarbital (PB) for 40 weeks

Group	Treatment	Body weight (g)	Liver weight (g)	Liver/body (%)
1	Basal-Basal	585.3 $\pm$ 46.7	19.6 $\pm$ 1.8	3.3 $\pm$ 0.2
2	Basal-0.03% G	586.2 $\pm$ 57.0	20.5 $\pm$ 2.2	3.5 $\pm$ 0.2
3	Basal-0.01% G	586.8 $\pm$ 51.7	20.0 $\pm$ 1.9	3.4 $\pm$ 0.4
4	Basal-0.05% PB	570.0 $\pm$ 32.5	24.9 $\pm$ 2.1*	4.4 $\pm$ 0.2*
5	3'-MeDAB-Basal	591.0 $\pm$ 71.2	20.2 $\pm$ 2.9	3.5 $\pm$ 0.6
6	3'-MeDAB-0.03% G	585.3 $\pm$ 36.1	21.8 $\pm$ 2.3	3.7 $\pm$ 0.3
7	3'-MeDAB-0.01% G	594.8 $\pm$ 66.3	22.7 $\pm$ 3.3	3.8 $\pm$ 0.5
8	3'-MeDAB-0.05% PB	550.0 $\pm$ 49.3	27.7 $\pm$ 3.6**	5.1 $\pm$ 0.8**

Values are the mean $\pm$ S.D. *,** Significant difference with $P < 0.01$ from groups 1 and 5, respectively.

Fig. 2. Body and liver weight changes in rats during feeding of 3'-MeDAB and gomisin A or phenobarbital. Upper: body weight. Lower: liver weight. ○: 0.06% 3'-MeDAB-containing diet alone, ▲: combined with 0.01% gomisin A-containing diet, ●: combined with 0.03% gomisin A-containing diet, △: combined with 0.05% phenobarbital-containing water.

In rats fed gomisin A or phenobarbital alone, no neoplastic change was histopathologically observed until 40 weeks. The number of histopathological lesions observed in the liver preparations from 3'-MeDAB-pretreated rats is shown in Table 3. Hepatic altered foci were observed in almost all the rats fed the 3'-MeDAB-containing diet. The total number of altered foci in the 3'-MeDAB-control group rapidly increased, reached a peak at 10 weeks (12 weeks after the stopping the 3'-MeDAB diet), and thereafter decreased and maintained a level of 4 to 6 foci/cm²/rat (Fig. 3). Gomisin A inhibited the appearance of the altered foci in the early period of the feeding: at a high dosage of gomisin A (0.03%), small foci (below 700 μ m diameter) were clearly fewer than in the control group during the experiment and large foci (over 701 μ m diameter) were

very few; and at a low dosage of gomisin A (0.01%), the number of altered foci was less than in the control group until 10 weeks and then gradually increased. In the phenobarbital-combined group, the number of large foci (over 701 μ m diameter) was significantly higher than that in the control group during the experiment and a certain number of hyperplastic nodules and hepatomas was also observed.

As shown in Table 4, we classified the foci of hepatocellular alterations as previously indicated (13). Most commonly observed hyperplastic foci in the 3'-MeDAB-treated groups were of the clear cell type, and gomisin A decreased the number of such types of foci in the early stage. A high incidence of basophilic cell foci was temporarily observed at 5 weeks in the control and 0.01% gomisin A groups. In

Table 3. Histopathological lesions in the liver during feeding of gomisin A (G) or phenobarbital (PB) after treatment with 3'-MeDAB

Group	Period of feeding (week)	No. of rats with lesions /No. of rats examined	Number of foci with the following diameters (No./cm ² /rat)		
			below 700 μ m	over 701 μ m	
5 Basal diet	0	10/10	5.89 $\pm$ 3.61	0.02 $\pm$ 0.04	(0)
	5	9/10	6.35 $\pm$ 3.92	0.48 $\pm$ 0.69	(0)
	10	10/10	7.24 $\pm$ 3.68	0.37 $\pm$ 0.36	(0)
	20	9/10	3.39 $\pm$ 2.20	0.30 $\pm$ 0.43	(0.09 $\pm$ 0.20)
	40	10/10	5.47 $\pm$ 3.43	0.34 $\pm$ 0.59	(0)
6 0.03% G	5	10/10	3.44 $\pm$ 2.54	0.05 $\pm$ 0.14	(0)
	10	10/10	3.09 $\pm$ 1.89 ^{*2}	0.11 $\pm$ 0.24	(0)
	20	10/10	3.09 $\pm$ 1.85	0.31 $\pm$ 0.69	(0)
	40	9/10	3.84 $\pm$ 2.44	0.35 $\pm$ 0.33	(0.05 $\pm$ 0.15)
7 0.01% G	5	9/10	3.06 $\pm$ 2.13 ^{*1}	0.34 $\pm$ 0.39	(0)
	10	10/10	4.71 $\pm$ 2.71	0.17 $\pm$ 0.28	(0)
	20	10/10	5.27 $\pm$ 3.33	0.39 $\pm$ 0.32	(0)
	40	10/10	5.69 $\pm$ 4.11	0.64 $\pm$ 0.83	(0.18 $\pm$ 0.31)
8 0.05% PB	5	10/10	5.21 $\pm$ 3.15	1.46 $\pm$ 1.09 ^{*1}	(0.09 $\pm$ 0.19)
	10	10/10	10.56 $\pm$ 6.43	1.18 $\pm$ 1.32	(0.11 $\pm$ 0.34)
	20	10/10	4.77 $\pm$ 2.93	1.63 $\pm$ 1.25 ^{*2}	(0.64 $\pm$ 0.66 ^{*1})
	40	10/10	5.56 $\pm$ 2.58	1.29 $\pm$ 1.10 ^{*1}	(0.71 $\pm$ 0.62)

Data are the mean $\pm$ S.D. Data in parentheses indicate the number of hyperplastic nodules and hepatomas.

^{*1,*2} significant difference from the corresponding period of group 5, with $P < 0.05$ and $P < 0.01$.

Fig. 3. Change in number of altered foci in the liver. Data indicate the mean of the number of foci during feeding of the basal diet (○), 0.01% gomisin A-containing diet (▲), 0.03% gomisin A-containing diet (●) and 0.05% phenobarbital-containing water (△) after treatment with 0.06% 3'-MeDAB for 3 weeks and suspension for 2 weeks.

the 0.03% gomisin A group, basophilic cell foci were few for 20 weeks and increased only at 40 weeks. The other types were very few in the control and gomisin A groups. Phenobarbital increased every type of neoplastic lesion, and a particular change was a very high incidence of eosinophilic cell foci.

DISCUSSION

The two-step theory has been proposed for carcinogenesis by physical, chemical, and viral carcinogens (14). Phenobarbital enhanced the hepatic tumor incidence when it was fed to rats after their exposure to a carcinogen, while the simultaneous feeding of phenobarbital and carcinogen reduced carcinogenesis (9). Thus, phenobarbital acts as a promoter of progress

in initiated cells. Kitagawa and Sugano (11) have reported that subsequent feeding of 3'-MeDAB (3 weeks) and phenobarbital (36 weeks) caused a considerable number of neoplastic changes in the livers of rats.

It has been reported that gomisin A accelerates the liver regeneration and increases the cell division after a partial hepatectomy (7, 8) as mentioned for phenobarbital (15). In this study, we then examined the effects of gomisin A on the tumor progression in the liver after a short-term feeding of 3'-MeDAB to rats, in comparison to the effects of phenobarbital. In groups fed gomisin A or phenobarbital alone, no hepatic alteration was observed during this experiment. Altered foci in the liver increased after the feeding of 3'-MeDAB and reached a peak 12 weeks after treatment with the carcinogen. Phenobarbital enhanced the alterations so that the number and size of the foci were much larger in the phenobarbital group than in the control group. In contrast, gomisin A decreased the number in early stages. In the morphological classification and typing of hepatocellular alterations, the clear cell type of alteration accounted for most of the foci, and basophilic cell foci appeared with a high incidence in the early stage after 3'-MeDAB feeding. The numbers of clear cell and basophilic cell foci were decreased by subsequent feeding of gomisin A. In the phenobarbital-combined group, a large number of the eosinophilic cell type and a gradual increase of the mixed cell type were characteristic changes. These results suggest that gomisin A inhibits or delays the promotion step of cells initiated by a carcinogen, while phenobarbital strongly promotes hepatocarcinogenesis.

Gomisin A, like phenobarbital, enhances hepatic regeneration and increases the mitotic index after a partial hepatectomy (7, 8, 15), but the former suppressed and the latter promoted hepatocarcinogenesis. It may be concluded that the effects of gomisin A on the liver after hepatectomy are events different from the carcinogenic actions. Ito et al. (16, 17) indicated that some hyperplasia increased in the early period after feeding of carcinogens

Table 4. Number of altered foci found in the liver during feeding gomisin A or phenobarbital after treatment with 3'-MeDAB

Group	Period of feeding (week)	Type of foci (Number/cm ² /rat)				
		Clr.	Vac.	Eos.	Bas.	Mix.
5 Basal diet	5	4.07 ± 2.97	0.39 ± 0.38	0.14 ± 0.22	2.13 ± 1.58	0.10 ± 0.21
	10	6.66 ± 3.48	0.13 ± 0.42	0.13 ± 0.42	0.54 ± 0.63	0.14 ± 0.31
	20	2.93 ± 1.81	0.25 ± 0.63	0.04 ± 0.12	0.39 ± 0.51	0.08 ± 0.18
	40	4.78 ± 3.12	0.37 ± 0.65	0.39 ± 0.73	0.27 ± 0.29	0
6 0.03% G	5	2.84 ± 2.47	0.09 ± 0.29	0.11 ± 0.23	0.40 ± 0.37* ²	0.05 ± 0.16
	10	2.28 ± 1.52* ³	0.05 ± 0.17	0.10 ± 0.21	0.50 ± 0.72	0.28 ± 0.41
	20	2.89 ± 1.92	0.09 ± 0.18	0.06 ± 0.17	0.37 ± 0.50	0
	40	2.90 ± 2.18	0.15 ± 0.31	0.10 ± 0.19	0.97 ± 0.90	0.08 ± 0.17
7 0.01% G	5	1.02 ± 0.69* ¹	0.30 ± 0.42	0.24 ± 0.43	1.72 ± 1.25	0.12 ± 0.25
	10	4.30 ± 2.60	0.06 ± 0.19	0.12 ± 0.26	0.29 ± 0.49	0.11 ± 0.24
	20	5.06 ± 3.11	0.22 ± 0.31	0	0.37 ± 0.58	0
	40	4.93 ± 3.98	0.24 ± 0.25	0.48 ± 0.51	0.64 ± 0.75	0.05 ± 0.15
8 0.05% PB	5	2.13 ± 3.47	0.09 ± 0.19* ¹	3.04 ± 1.49* ⁴	1.04 ± 0.84	0.37 ± 0.35
	10	8.39 ± 7.05	0.13 ± 0.21	2.20 ± 1.66* ³	0.65 ± 1.03	0.37 ± 0.33
	20	2.03 ± 1.88	0.48 ± 0.61	2.29 ± 2.08* ²	0.79 ± 0.78	0.81 ± 0.74* ¹
	40	3.14 ± 2.02	0.34 ± 0.36	1.81 ± 0.88* ⁴	0.65 ± 0.79	0.92 ± 0.90

Data are the mean ± S.D. Abbreviations: G, gomisin A; PB, phenobarbital; Clr., clear cell type; Vac., Vacuolar cell type; Eos., eosinophilic cell type; Bas., basophilic cell type; Mix., mixed cell type. *¹, *², *³, *⁴ Significant difference from the corresponding period of group 5, with P < 0.05, P < 0.01, P < 0.005, and P < 0.001, respectively.

contain reversible lesions and a few of the lesions are then committed to neoplastic transformation. Gomisin A suppressed the appearance of the lesions in the early stages after 3'-MeDAB. It is possible that gomisin A decreases the reversible fractions by improving of hepatic functions (6–8). Other molecular mechanisms of inhibition by gomisin A of tumor promotion should be considered and investigations on these mechanisms are in progress in our laboratory.

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REFERENCES

- 1 Ikeya, Y., Taguchi, H., Yoshioka, I. and Kobayashi, H.: The constituents of *Schizandra chinensis* Baill. I. Isolation and structure determination of five lignans, gomisin A, B, C, F and G, and the absolute structure of schizandrin. *Chem. Pharm. Bull. (Tokyo)* **27**, 1383–1394 (1979)
- 2 Macda, S., Sudo, K., Miyamoto, Y., Takeda, S., Shinbo, M., Aburada, M., Ikeya, Y., Taguchi, H. and Harada, M.: Pharmacological studies on schizandra fruits. II. Effects of constituents of schizandra fruits on drug induced hepatic damage in rats. *Yakugaku Zasshi* **102**, 579–588 (1982) (Abs. in English)
- 3 Takeda, S., Maemura, S., Sudo, K., Kase, Y., Arai, I., Ohkura, Y., Funo, S., Fujii, Y., Aburada, M. and Hosoya, E.: Effects of gomisin A, a lignan component of *Schizandra* fruits, on experimental liver injuries and liver microsomal

- drug-metabolizing enzymes. *Folia Pharmacol. Japon.* **87**, 169–187 (1986) (Abs. in English)
- 4 Hikino, H., Kiso, Y., Taguchi, H. and Ikeya, Y.: Antihepatotoxic actions of lignoids from *Schizandra chinensis* fruits. *Planta Med.* **50**, 213–218 (1984)
- 5 Kiso, Y., Tohkin, M., Hikino, H., Ikeya, Y. and Taguchi, H.: Mechanism of antihepatotoxic activity of Wuweizisu C and Gomisins A. *Planta Med.* **51**, 331–334 (1985)
- 6 Maeda, S., Takeda, S., Miyamoto, Y., Aburada, M. and Harada, M.: Effect of gomisins A on liver functions in hepatotoxic chemicals-treated rats. *Japan. J. Pharmacol.* **38**, 347–353 (1985)
- 7 Takeda, S., Kase, Y., Arai, I., Hasegawa, M., Sekiguchi, Y., Funo, S., Aburada, M., Hosoya, E., Mizoguchi, Y. and Morisawa, S.: Effects of TJN-101 ((+)-(6s,7s,R-biar)-5,6,7,8-tetrahydro-1,2,3,12-tetramethoxy-6,7-dimethyl-10,11-methylenedioxy-6-dibenzo[a,c]cyclooctenol) on liver regeneration after partial hepatectomy, and on regional hepatic blood flow and fine structure of the liver in normal rats. *Folia Pharmacol. Japon.* **88**, 321–330 (1986) (Abs. in English)
- 8 Takeda, S., Kase, Y., Arai, I., Ohkura, Y., Hasegawa, M., Sekiguchi, Y., Tatsugi, A., Funo, S., Aburada, M. and Hosoya, E.: Effects of TJN-101, a lignan compound isolated from *Schizandra* fruits, on liver fibrosis and on liver regeneration after partial hepatectomy in rats with chronic liver injury induced by CCl₄. *Folia Pharmacol. Japon.* **90**, 51–65 (1987) (Abs. in English)
- 9 Peraino, C., Fry, R.J.M. and Staffeldt, E.: Reduction and enhancement by phenobarbital of hepatocarcinogenesis induced in the rat by 2-acetylaminofluorene. *Cancer Res.* **31**, 1506–1512 (1971)
- 10 Kitagawa, T. and Sugano, H.: Enhancement of azo-dye hepatocarcinogenesis with dietary phenobarbital in rats. *Japan. J. Cancer Res. (Gann)* **68**, 255–256 (1977)
- 11 Kitagawa, T. and Sugano, H.: Enhancing effect of phenobarbital on the development of enzyme-altered islands and hepatocellular carcinomas initiated by 3'-methyl-4-(dimethylamino)azobenzene or diethylnitrosamine. *Japan. J. Cancer Res. (Gann)* **69**, 679–687 (1978)
- 12 Giese, J.E., Clayton, C.C., Miller, E.C. and Baumann, C.A.: The effect of certain diets on hepatic tumor formation due to m'-methyl-p-dimethylaminoazobenzene and o'-methyl-p-dimethylaminoazobenzene. *Cancer Res.* **6**, 678–684 (1946)
- 13 Squire, R.A. and Levitt, M.H.: Report of a workshop on classification of specific hepatocellular lesions in rats. *Cancer Res.* **35**, 3214–3223 (1975)
- 14 Farber, E.: The sequential analysis of liver cancer induction. *Biochim. Biophys. Acta* **605**, 149–166 (1980)
- 15 Japundzic, M., Knezevic, B., Djordjevic-Camba, V. and Japundzic, I.: The influence of phenobarbital-Na on the mitotic activity of parenchymal liver cells during rat liver regeneration. *Exp. Cell Res.* **48**, 163–167 (1967)
- 16 Ito, N., Hananouchi, Sugihara, S., Shirai, T., Tsuda, H., Fukushima, S. and Nagasaki, H.: Reversibility and irreversibility of liver tumors in mice induced by the α isomer of 1,2,3,4,5,6-hexachlorocyclohexane. *Cancer Res.* **36**, 2227–2234 (1976)
- 17 Ito, N.: Development of carcinomas and pre-neoplastic changes in rat liver. *Gan to Kagakuryoho* **7**, Supp. **I**, 55–63 (1980) (Abs. in English)