Effects of Gomisin A on the Promotor Action and Serum Bile Acid Concentration in Hepatocarcinogenesis Induced by 3'-Methyl-4-dimethylamino-azobenzene

Ken-ichi MIYAMOTO,*^a Kazuyoshi HIRAMATSU,^a Yumiko OHTAKI,^{a,b} Masanao KANITANI,^b Masaaki NOMURA,^a and Masaki ABURADA^b

Faculty of Pharmaceutical Sciences, Hokuriku University,^a Ho-3 Kanagawa-machi, Kanazawa 920-11, Japan and Tsumura & Co.,^b 12-7, Nibancho, Chiyoda-ku, Tokyo 102, Japan. Received May 12, 1995; accepted June 20, 1995

The effects of gomisin A, a lignan component of *Schizandra* fruits, on the promotion stage of hepatocarcinogenesis initiated by 3'-methyl-4-dimethylamino-azobenzene (3'-MeDAB) in male Donryu rats were investigated. When different types of tumor promoters, phenobarbital (PB) and deoxycholic acid (DCA), were administered for 5 weeks after initiation by 3'-MeDAB, preneoplastic alterations in the liver, determined by glutathione S-transferase placental form (GST-P), were markedly increased. Gomisin A significantly inhibited the increase in number and size of GST-P positive foci, regardless of the promotor. This lignan inhibited the increase in serum bile acid concentration by administration of DCA, but hardly influenced the serum bile acids in the PB-combined group. These results suggest that the inhibitory effect of gomisin A on the promotive action of DCA is based on improving bile acid metabolism, but regarding the action of PB, the effect could not be elucidated from the metabolism of bile acids.

Key words gomisin A; phenobarbital; deoxycholic acid; glutathione S-transferase placental form; serum bile acid; 3'-methyl-4-dimethylamino-azobenzene

We have reported that gomisin A, a lignan component of *Schizandra* fruits,¹⁾ suppressed the appearance of altered foci in the liver, such as clear cell foci, basophilic cell foci,²⁾ and glutathione S-transferase placental form (GST-P) positive foci, both with simultaneous treatment and after short-term treatment with a carcinogen, 3'-methyl-4-dimethylamino-azobenzene (3'-MeDAB), in rats.^{3–5)} Therefore, gomisin A is thought to inhibit the promotion stage of hepatocarcinogenesis. Phenobarbital (PB) is a well-known promotor of chemical carcinogenesis in the liver.⁶⁾ Among bile acids, a secondary bile acid, deoxycholic acid (DCA), has been reported to initiate and/or promote rat hepatocarcinogenesis.^{7–9)} This paper deals with the effects of gomisin A on the promotion by PB and DCA in hepatocarcinogenesis initiated by 3'-MeDAB and on the serum concentration and composition of bile acids.

MATERIALS AND METHODS

Materials Gomisin A was isolated from *Schizandra* fruits¹⁾ in Tsumura Central Research Laboratories, Ibaraki. 3'-MeDAB was purchased from Tokyo Kasei, Inc., Tokyo. The basal diet (CE-2) was obtained from Nihon Clea Co., Tokyo, and diets containing 0.03% gomisin A and 0.06% 3'-MeDAB were prepared at Nihon Haigoshiryo Co., Aichi. Deoxycholic acid (DCA) and 5 α -pregnane-3 α ,20 α -diol were from Sigma Chemical Co., St. Louis, MO, U.S.A. Phenobarbital was obtained from Hoei Yakukoh Co., Osaka. An avidin-biotin-peroxidase (ABC) kit was purchased from Vector Laboratories, Inc., Burlingame, CA, U.S.A. Rabbit antibody to rat glutathione S-transferase placental form (GST-P)¹⁰⁾ was kindly provided by Drs. Sato and Satoh, Hirosaki University School of Medicine, Hirosaki.

Experimental Protocol Male Donryu rats (6-weeks old, Nippon SLC, Hamamatsu) were divided into groups

of eight. Rats were fed 0.06% 3'-MeDAB in the diet for 3 weeks, ordinary feed for 2 weeks, and then given 0.05% PB in water, 100 mg/kg DCA (suspended in 0.5% carboxymethyl cellulose) orally once a day, and/or 0.03% gomisin A in the diet for 5 weeks.

Immunohistochemistry GST-P positive foci in the liver preparations were stained using the ABC method with anti-GST-P antibody, as previously reported.³⁾ The number and size of GST-P positive foci (>200 μ m diameter) were measured using a microcomputer imaging device system (Imaging Research, Inc., Tokyo).

Bile Acid Analysis At the finish of the experiments, rats were fasted for 24 h, serum was collected, and bile acids were assayed by the modified method of Okuyama.^{11,12)} Bile acids were separated from each other using a high-performance liquid chromatography with a column of immobilized 3 α -hydroxysteroid dehydrogenase (Sekisui Chemical Co., Osaka) and assayed by fluorescence with an internal standard of 5 α -pregnane-3 α , 20 α -diol.

Statistical analysis was done using the Student's *t*-test and Welch's test.

RESULTS AND DISCUSSION

During the experiments, gomisin A (0.03% in the diet) had little influence on the diet and water consumption or body and liver weight of rats pretreated with 3'-MeDAB. PB (0.05% in water) suppressed the increase in body weight and the water consumption, but increased the liver weight. DCA (100 mg/kg) tended to suppress both the body weight and the diet consumption.

As shown in Table 1, two tumor promoters, PB and DCA, significantly increased the number and size of GST-P positive foci in the liver, compared with pretreatment with 3'-MeDAB alone. Gomisin A significantly suppressed the increase in number and size of foci in the liver, not only in those untreated with promoters but also

* To whom correspondence should be addressed.

in those treated with each promotor. This lignan also significantly decreased the size of GST-P positive foci treated with DCA and PB. These results indicate that gomisins A inhibits the promotion stage of hepatocytes initiated by 3'-MeDAB, regardless of treatment with tumor promoters. It has been reported that gomisins A increases microsomal enzymes in the liver,¹³⁾ similarly to PB, and also accelerates biliary output and hepatic blood flow.^{14,15)} However, because at two weeks after the cessation of 3'-MeDAB the carcinogen and its metabolites were not detectable in the liver,⁴⁾ it is difficult to consider whether gomisins A influences the action and metabolism of the carcinogen. It is possible that gomisins A affects the metabolism and excretion of these promoters when simultaneously administered.

Our previous studies²⁻⁵⁾ and this study indicate that gomisins A inhibits the hepatocarcinogenesis initiated by 3'-MeDAB, regardless of the presence of tumor promoters. This lignan component may therefore affect some endogenous factors of carcinogenesis. Bile acids have been shown to enhance experimental carcinogenesis in the liver and colon and are proposed as potential endogenous tumor promoters.^{7,16-18)} Therefore, the serum bile acid concentration was measured at the finish of experiment, and the results are shown in Table 2. Lithocholic acid and its conjugates were not detected by this assay system using 3 α -dehydroxylation, because the major part of the bile acid is present as conjugated forms with sulfate and glucuronate at the 3-position.^{19,20)} The ratio of taurine conjugates vs. glycine conjugates was generally higher in the treated rats

than in the age-matched normal rats, although there was no statistical significance. Even at 7 weeks after the pretreatment with 3'-MeDAB, the total bile acid concentration and the composition rate of DCA in serum were higher and the rate of ursodeoxycholic acid (UDCA) was lower than in the normal rats. Gomisins A improved the bile acid composition, accompanied by a marked decrease of DCA. After DCA feeding, the total bile acid concentration in serum, particularly DCA itself and its 7 α -hydroxylated product cholic acid (CA), was increased, but was significantly decreased by co-administration with gomisins A. DCA has not only potent tumor promotor action but initiator action due to its genotoxicity.^{8,21)} Hydrophobic or cytotoxic bile acids, such as DCA, are good substrates of GSTs, and gomisins A induces microsomal enzymes, including alpha class GST isoenzyme,^{4,13)} and increases bile flow.^{14,15)} Gomisins A may increase the detoxification of DCA and decrease the DCA concentration by facilitation of 7 α -hydroxylation of the bile acid in the liver or inhibition of the dehydroxylation of CA in the intestine. Consequently, gomisins A seems to inhibit the promotion mediated by endogenous and exogenous bile acids.

On the other hand, PB decreased the serum bile acid concentration but increased the composition rate of both β -muricholic acid (MCA) and chenodeoxycholic acid (CDCA) (Table 2). These results agree with previous evidence that PB caused an increase in MCAs, hyodeoxycholic acid, and CDCA in the liver after a chronic feeding.²²⁾ Delzenne *et al.*²²⁾ described that although the modification of liver bile acids is important in modulating hepatocarcinogenesis, the quantitative perturbations of bile acid homeostasis induced by PB are not implicated in the positive modulation of hepatocarcinogenesis, because the increased bile acids are comparatively low in toxicity and do not substantially contribute to tumor promotion. Therefore, the positive effect of PB on hepatocarcinogenesis may be based on other actions, such as mitogenic activity on hepatocytes.²³⁾ There is a report that the promotor action of PB may be related to an increase in transforming growth factor- β_1 expression and an inhibition of epidermal growth factor action in proliferating hepatocytes,²⁴⁾ but the effects of gomisins A on these growth factors have not been determined. Gomisins A significantly inhibited the promotor action

Table 1. Number and Size of GST-P Positive Foci in the Liver

Group	Number/cm ²	Area (mm ² /cm ²)
3'-MeDAB control	0.67 ± 0.06	0.11 ± 0.04
– gomisins A	0.34 ± 0.10 ^{a)}	0.07 ± 0.03
– DCA	1.67 ± 0.17 ^{b)}	0.43 ± 0.08 ^{b)}
– DCA + gomisins A	0.94 ± 0.32 ^{c)}	0.19 ± 0.07 ^{c)}
– PB	6.41 ± 0.59 ^{b)}	1.74 ± 0.19 ^{b)}
– PB + gomisins A	3.63 ± 0.77 ^{a,c)}	1.02 ± 0.22 ^{a,c)}

Eight male Donryu rats in a group were fed 0.06% 3'-MeDAB-containing diet for 3 weeks, ordinary feed for 2 weeks, and then given a 0.03% gomisins A-containing diet and/or 100 mg/kg DCA or 0.05% PB water for 5 weeks. Data are the mean ± S.E. a) Significantly different from the 3'-MeDAB control at $p < 0.05$ and 0.01, respectively. c) Significantly different from the respective gomisins A-untreated group at $p < 0.05$.

Table 2. Serum Bile Acid Composition

Group	Total (nmol/ml)	Composition (%)						T/G ratio
		α -MCA	β -MCA	UDCA	CA	CDCA	DCA	
Normal control	2.69 ± 0.02	8.9 ± 0.2	21.9 ± 0.3	53.0 ± 0.4	6.0 ± 0.2	7.7 ± 0.4	2.5 ± 0.1	2.6 ± 0.1
3'-MeDAB control	4.84 ± 0.28 ^{a)}	10.1 ± 1.8	29.7 ± 3.4	28.4 ± 4.6 ^{a)}	15.2 ± 3.3	10.6 ± 3.3	6.0 ± 0.6 ^{a)}	10.8 ± 1.8
– gomisins A	6.43 ± 0.65 ^{a)}	6.7 ± 0.8	17.4 ± 1.6 ^{b)}	55.8 ± 2.0 ^{b)}	11.4 ± 1.6	8.0 ± 1.0	0.6 ± 0.2 ^{a,b)}	6.9 ± 0.5
– DCA	13.64 ± 1.76 ^{a,b)}	3.4 ± 0.6 ^{a,b)}	8.2 ± 1.3 ^{a,b)}	17.8 ± 3.8 ^{a)}	29.0 ± 3.1 ^{a)}	6.7 ± 0.8	34.9 ± 15.6 ^{a,b)}	4.1 ± 1.0
– DCA + gomisins A	5.87 ± 0.66 ^{a,c)}	3.0 ± 0.2 ^{a,b)}	7.7 ± 0.4 ^{a,b)}	21.2 ± 2.8 ^{a)}	33.6 ± 1.5 ^{a,b)}	4.1 ± 1.6	30.4 ± 3.2 ^{a,b)}	6.5 ± 1.9
– PB	2.82 ± 0.50 ^{b)}	11.6 ± 1.0	35.0 ± 2.0 ^{a)}	18.8 ± 1.9 ^{a)}	12.6 ± 0.4 ^{a)}	19.8 ± 0.7 ^{a)}	2.0 ± 0.9 ^{b)}	12.8 ± 2.3
– PB + gomisins A	4.14 ± 1.54	13.5 ± 1.9	31.2 ± 2.0 ^{a)}	16.4 ± 2.0 ^{a)}	17.0 ± 2.1 ^{a)}	18.8 ± 1.6 ^{a)}	3.1 ± 1.4	8.9 ± 1.7

At the finish of the experiments, rats were fasted for 24 h, serum was collected, and bile acids (α - and β -MCA, α - and β -muricholic acid; UDCA, ursodeoxycholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid) were analyzed. T/G ratio, taurine conjugates/glycine conjugates. Data are the mean ± S.E. of eight rats. a) Significantly different from the age-matched normal rats at $p < 0.01$. b) Significantly different from the 3'-MeDAB control at $p < 0.01$. c) Significantly different from the 3'-MeDAB-DCA group at $p < 0.01$.

of PB on hepatocarcinogenesis (Table 1), while barely affecting the PB action on bile acid (Table 2). The negative promotion effect of gomisin A seems to be due to the enhanced metabolism and excretion of PB from the liver by its pharmacologic actions.

In conclusion, gomisin A inhibited the promotion of hepatocarcinogenesis initiated by 3'-MeDAB, regardless of the promotor used. This lignan component also inhibited the increase in serum bile acids caused by administration of a promotor bile acid, DCA, and particularly, the improvement of bile acid composition seems to be important to the negative promotor action. The inhibition by gomisin A of PB action could not be elucidated from bile acid metabolism.

REFERENCES

- 1) Ikeya Y., Taguchi H., Yoshioka I., Kobayashi H., *Chem. Pharm. Bull.*, **27**, 1383 (1979).
- 2) Miyamoto K., Wakusawa S., Nomura M., Sanae F., Sakai R., Sudo K., Ohtaki Y., Takeda S., Fujii Y., *Jpn. J. Pharmacol.*, **57**, 71 (1991).
- 3) Nomura M., Nakachiyama M., Hida T., Ohtaki Y., Sudo K., Aizawa T., Aburada M., Miyamoto K.-I., *Cancer Lett.*, **76**, 11 (1994).
- 4) Ohtaki Y., Nomura M., Hida T., Miyamoto K., Kanitani M., Aizawa T., Aburada M., *Biol. Pharm. Bull.*, **17**, 808 (1994).
- 5) Nomura M., Ohtaki Y., Hida T., Aizawa T., Wakita H., Miyamoto K., *Anticancer Res.*, **14**, 1967 (1994).
- 6) Peraino C., Fry R. J. M., Stafeldt E., *Cancer Res.*, **31**, 1506 (1971).
- 7) Tsuda H., Masui T., Imaida K., Fukushima S., Ito N., *Jpn. J. Cancer Res., (Gann)*, **75**, 871 (1984).
- 8) Tsuda H., Asamoto M., Kagawa M., Uwagawa S., Inoue K., Inui M., Ito N., *Carcinogenesis*, **9**, 1103 (1988).
- 9) Armstrong D., Cameron R. D., *Cancer Lett.*, **57**, 153 (1991).
- 10) Sato K., Kitahara A., Satoh K., Ishikawa T., Tatematsu M., Ito N., *Jpn. J. Cancer Res. (Gann)*, **75**, 199 (1984).
- 11) Okuyama S., Kokubun N., Higashidate S., Uemura D., Hirata Y., *Chem. Lett.*, **1979**, 1443.
- 12) Sakakura H., Suzuki M., Kimura N., Takeda H., Nagata S., Maeda M., *J. Chromatogr.*, **621**, 123 (1993).
- 13) Takeda S., Maemura S., Sudo K., Kase Y., Arai I., Ohkura Y., Funo S., Fujii Y., Aburada M., Hosoya E., *Nippon Yakurigaku Zasshi*, **87**, 169 (1986).
- 14) Maeda S., Takeda S., Miyamoto Y., Aburada M., Harada M., *Jpn. J. Pharmacol.*, **38**, 347 (1985).
- 15) Takeda S., Kase Y., Arai I., Hasegawa M., Sekiguchi Y., Funo S., Aburada M., Hosoya E., Mizoguchi Y., Morisawa S., *Nippon Yakurigaku Zasshi*, **88**, 321 (1986).
- 16) Reddy B. S., Narasawa T., Weisburger J. H., Wynder E. L., *J. Natl. Cancer Inst.*, **56**, 441 (1976).
- 17) Cameron R. G., Imaida K., Tsuda H., Ito N., *Cancer Res.*, **42**, 2426 (1982).
- 18) Blair P. C., Popp J. A., Bryant-Varela B. J., Thompson M. B., *Carcinogenesis*, **12**, 59 (1991).
- 19) Uchida K., Endo T., *Taisha*, **17**, 119 (1980).
- 20) Murata N., Beppu T., Idezuki Y., *Seitai No Kagaku*, **36**, 132 (1985).
- 21) Kitazawa S., Denda A., Tsutsumi M., Tsujiuchi T., Hasegawa K., Tamura K., Maruyama H., Konishi Y., *Carcinogenesis*, **11**, 1323 (1989).
- 22) Delzenne N. M., Taper H. S., Roberfroid M., *Arch. Toxicol.*, **68**, 394 (1994).
- 23) Japundzic M., Knezevic B., Djordjevic-Camba V., Japundzic I., *Exp. Cell Res.*, **48**, 163 (1967).
- 24) Jirtle R. L., Meyer S. A., *Digest. Dis. Sci.*, **36**, 659 (1991).