Inhibition by Gomisin A, a Lignan Compound, of Hepatocarcinogenesis by 3'-Methyl-4-dimethylaminoazobenzene in Rats

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The effects of gomisin A, a lignan compound of Schizandra fruits, on hepatocarcinogenesis induced by 3'-methyl-4-dimethylaminoazobenzene (3'-MeDAB) in rats were investigated. Gomisin A inhibited both increases of the number and size of glutathione S-transferase placental form (GST-P)-positive foci, a marker enzyme of preneoplasm, and the population of diploid nuclei, as a proliferative state of hepatocytes, in the liver from rats simultaneously treated with 3'-MeDAB. Gomisin A increased GST activity in the liver, by raising the level of GST 1 and 2 isozymes. 3'-MeDAB increased GST activity and GST-P expression. This high level of GST-P induced by 3'-MeDAB was suppressed by additional treatment with gomisin A. In an experiment on simultaneous treatment, gomisin A increased the biliary excretion of 3'-MeDAB-related aminoazo dyes and decreased the content in the liver of rats fed with 0.06% 3'-MeDAB containing diet. In an experiment on pretreatment with 3'-MeDAB, even though no aminoazo dye was detectable in the liver or bile 2-weeks after cessation of 3'-MeDAB-feeding, gomisin A showed a tendency to reduce the preneoplastic changes of increases in GST-P positive foci and diploid nuclei in the liver. These results suggest that gomisin A inhibits the hepatocarcinogenesis induced by 3'-MeDAB by enhancing the excretion of the carcinogen from the liver and by reversing the normal cytokinesis.

Keywords gomisin A; chemoprevention; glutathione S-transferase; ploidy analysis; hepatocarcinogenesis; 3'-methyl-4-dimethylaminoazobenzene

Gomisin A, a lignan compound of Schizandra fruits,¹⁾ has been shown to improve liver injuries induced by hepatotoxic chemicals.²⁻⁶⁾ We previously reported that gomisin A decreased altered clear cell and basophilic foci, in the liver in the promotion stage of hepatocarcinogenesis by 3'-methyl-4-dimethylaminoazobenzene (3'-MeDAB).⁷⁾ Therefore, this lignan compound seems to be a candidate for therapeutic (chemopreventive) drug for not only liver injuries but also hepatocarcinogenesis.

In this study, we investigated the mechanism of gomisin

A for inhibition of hepatocarcinogenesis induced by 3'-MeDAB in rats.

MATERIALS AND METHODS

Materials Gomisin A was isolated from Schizandra fruits¹⁾ in Tsumura Central Research Laboratories, Ibaragi. 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

Fig. 1. Experimental Protocol of Treatment with 3'-MeDAB and/or Gomisin A

and 0.06% 3'-MeDAB were prepared at Nihon Haigoshiryo Co., Aichi. An avidin-biotin-peroxidase (ABC) kit and propidium iodide were purchased from Vector Laboratories Inc., Burlingame, CA and Calbiochem Co., La Jolla, CA, respectively. Glutathione and 1-chloro-2,4-dinitrobenzene were obtained from Wako Pure Chemicals, Tokyo. β -Glucuronidase (Type H-1) was purchased from Sigma Chemical Co., St. Louis, MO. Rabbit antibodies to rat glutathione S-transferase (GST) isozymes, 1-2, 3-4, and placental form (P),⁸⁾ were kindly provided by Drs. Sato and Satoh, Hirosaki University School of Medicine, Hirosaki, Japan. Rat ascites hepatoma AH66 was supplied by the Department of Experimental Therapeutics, Cancer Research Institute, Kanazawa University, Kanazawa, Japan.

Experimental Protocols Male Donryu rats (6-weeks old, Nihon Clea) were divided into groups of ten. Rats were treated with 3'-MeDAB and/or gomisin A according to the protocol⁹⁾ shown in Fig. 1 and killed at designated periods.

Here, because the consumption of gomisin A given *ad libitum* in the 0.03%-containing diet in the 3'-MeDAB pretreatment experiment corresponded to about 30 mg/kg per day, the compound was suspended in 0.5% carboxymethyl cellulose and administered perorally (*p.o.*) at 30 mg/kg once a day for 5 weeks in the simultaneous treatment with 3'-MeDAB.

Immunohistochemistry Three sections, obtained from the right anterior, left anterior, and left median lobes of rat liver, were fixed in 10% phosphate-buffered formalin solution, embedded in paraffin, and stained for GST-P by the ABC method. As a negative control for the specificity of anti-GST antibody binding, preimmune rabbit serum was used instead of antiserum. The number and size of GST-P positive foci ($>200\ \mu\text{m}$ diameter) were measured using a microcomputer imaging device system (Imaging Research Inc., Tokyo).

Assay of GST Activity Total GST activity in the 105000 *g* supernatants of the liver homogenate was measured in 100 mM potassium-phosphate buffer (pH 6.5) containing 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene, according to the method of Habig *et al.*¹⁰⁾

Immunoblot Analysis The liver of each group was *in situ* perfused with physiological saline solution, removed, homogenized, and centrifuged at 105000 *g* for 45 min. The resulting supernatant was used for the Western blotting. A sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 12.5% gel, and the gel was treated sequentially with normal goat serum, rabbit anti-rat GST isozymes (1:1000), biotin labeled goat anti-rabbit IgG and ABC. The peroxidase binding sites were demonstrated by the diaminobenzidine method. AH66, which is a rat ascites hepatoma cell line induced by 3'-MeDAB,¹¹⁾ was used as a GST-P positive control.

Preparation of Nuclei and Flow Cytometry The liver was excised, minced in Locke's solution containing 0.8% sodium citrate at 0°C and homogenized with a Dounce homogenizer. Parenchymal hepatocytes were collected by several centrifugations and suspended in a divalent cation free phosphate-buffered saline (PBS(-)) containing 0.1 M citric acid and 0.1% Triton-X 100 to make hepatocyte

nuclei.¹²⁾ Hepatocyte nuclei were reacted with 50 $\mu\text{g}/\text{ml}$ of propidium iodide in PBS(-) solution.

Red fluorescence from the individual 30000 nuclei, illuminated by an Argon ion laser (488 nm), was measured and analyzed with an EPICS 753 flow cytometer equipped with an MDADS II data analyzer (Coulter Electronic Inc., Hialeah, FL).

Extraction and Determination of Aminoazo Dye 3'-MeDAB-related aminoazo dyes were extracted according to the method reported by Styles *et al.*¹³⁾ and measured by a spectrophotometric method based on that of Miller and Miller.¹⁴⁾ Briefly, the liver from rats 24 h after the finish of treatment was homogenized and heated on a water bath at 100°C for 3 min, and free aminoazo dyes were extracted in a Soxhlet apparatus with methanol. Protein-binding aminoazo dye was extracted with ether from the residual protein after digestion with ethanol and 4.4 M potassium hydroxide. The bile was collected for 24 h after cessation of the treatment and alkalized with 5% ammonium solution; aminoazo dyes were then extracted with ether. A 2 ml portion of the bile was acidified with 0.1 M sodium acetate buffer (pH 3.5) and incubated with 44 units of β -glucuronidase containing 1.5 units of sulfatase, at 37°C for 3 h; after alkalization, deconjugated aminoazo dyes were extracted with ether. For spectrophotometry, the extracts were dissolved in 7 M hydrochloride, and their absorbance at 520 nm was determined using a Hitachi 100-0102 spectrophotometer. For high performance liquid chromatography (HPLC), a Shimadzu LC-5A chromatographic system consisting of an LC-5A liquid pump with a Shiseido Capcellpak C18 (4.6 mm i.d. $\times$ 250 mm), a Yanaco M-315 UV spectrometric detector, and a Shimadzu C-R6A data processor were used. The extracts were dissolved in methanol and injected in a volume of 40 μl , the elutions with a mobile phase (75% methanol in 0.01 M sodium acetate buffer, pH 3.5) and a flow rate of 1.2 ml/min were done at 40°C in a Shimadzu CTO-6A column oven, and the effluent was detected at a wavelength of 424 nm.

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

RESULTS

Histopathological Findings of GST-P Positive Foci

When rats were fed 0.06% 3'-MeDAB-containing diet, GST-P positive foci appeared in the liver (Fig. 2). In the simultaneous treatment with 3'-MeDAB and gomisin A, both the number and size of GST-P positive foci were significantly decreased by gomisin A (30 mg/kg, *p.o.* once a day) over a 5 week period. In the 3'-MeDAB-pretreated groups, GST-P positive foci were retained 12 weeks after cessation and tended to be reduced by feeding of 0.03% gomisin A-containing diet for 10 weeks.

Changes in GST Isozymes Table I shows total GST activity in the liver from rats treated with 3'-MeDAB and/or gomisin A for 5 weeks. GST activity in the liver from untreated rats barely changed during maturation (6-weeks to 11-weeks of age). 3'-MeDAB and gomisin A significantly increased GST activity, but the simultaneous treatment with both agents never changed the increased activity by each alone. Then, we analyzed the GST

Fig. 2. Number (A) and Size (B) of GST-P Positive Foci in the Liver from Rats Treated with 3'-MeDAB and/or Gomisin A

Immunohistochemical determination was made of GST-P positive foci in the liver from rats. Rats were given 0.06% 3'-MeDAB-containing diet for 5 weeks (3'-MeDAB) or simultaneously treated with 0.06% 3'-MeDAB-containing diet and 30 mg/kg gomisin A for 5 weeks (3'-MeDAB + gomisin A), or were pretreated with 0.06% 3'-MeDAB-containing diet for 3 weeks followed by a basal diet for 12 weeks (3'-MeDAB-normal) or by a basal diet for 2 weeks and 0.03% gomisin A-containing diet for 10 weeks (3'-MeDAB-gomisin A). Data are the means $\pm$ S.E. of the number and total area of foci per scanning area from ten rats. ^{a)} Significantly different from the 3'-MeDAB group at $p < 0.05$.

TABLE I. Total GST Activity in the Liver from Rats Treated with 3'-MeDAB and/or Gomisin A for 5 Weeks

Group	GST activity (μ mol/min/mg protein)
Normal	1.38 $\pm$ 0.13
Gomisin A alone	2.16 $\pm$ 0.16 ^{a)}
3'-MeDAB alone	2.29 $\pm$ 0.13 ^{a)}
3'-MeDAB + gomisin A	2.30 $\pm$ 0.09 ^{a)}

Rats were simultaneously given 3'-MeDAB and gomisin A, as indicated in Fig. 1. Data are the means $\pm$ S.E. from five rats. ^{a)} Significantly different from the normal group at $p < 0.05$.

isozymes by immunoblotting using respective antibodies. As shown in Fig. 3, GST-1, -2, -3, and -4 isozymes except for GST-P were detected in the normal liver. 3'-MeDAB clearly increased the expression of GST-P but tended to decrease the level of GST-2 isozyme of the Alpha class, which was seen just above the band of GST-1 isozyme on SDS-PAGE. Gomisin A increased the expression of GST isozymes 1 and 2 (Alfa class) and inhibited the GST-P expression increased by 3'-MeDAB. GSTs 3 and 4 of the Mu class, which have a similar molecular size on the SDS-PAGE, were not influenced by 3'-MeDAB or gomisin A. The high GST-P level induced by treatment with 3'-MeDAB for 3 weeks was maintained even after feeding of a basal diet for 12 weeks, and was decreased by post-treatment with gomisin A for 10 weeks (Fig. 4).

Analysis of Ploidy Classes in Hepatocyte Nuclei Figure 5 shows typical histograms of hepatocyte nuclei from rats at the start of the experiments (6-weeks old) and those at 11-weeks old which had received a basal diet, a 0.06% 3'-MeDAB-containing diet, or 30 mg/kg gomisin A once a day for 5 weeks. The percentage of diploid nuclei (38% of nuclei) from 6-week old rats decreased with aging by 12% while in rats fed 3'-MeDAB it was significantly and

Fig. 3. Western Blot Analysis of GST Isozymes Separated by SDS-PAGE in the Liver from Rats Simultaneously Treated with 3'-MeDAB and/or Gomisin A

Rats were given a basal diet (normal), 30 mg/kg gomisin A (gomisin A), 0.06% 3'-MeDAB-containing diet (3'-MeDAB), or a simultaneous treatment (3'-MeDAB + gomisin A) for 5 weeks. AH66 cells were used as a GST-P positive control. Molecular weight standards (M.W.) are indicated on the left.

rapidly increased up to 5 weeks, thereafter reaching a plateau (about 70%) (Fig. 6), as previously reported.^{13,15} Gomisin A alone did not change the normal progression of polyploidization, but the simultaneous administration of this compound tended to inhibit the increase of diploid nuclei when given with 3'-MeDAB feeding (Fig. 6). Moreover, gomisin A appeared to accelerate the recovery from the ploidy proportion altered by pretreatment with 3'-MeDAB to the normal ploidy pattern (Table II).

3'-MeDAB-Related Aminoazo Dyes in the Liver and Bile

In rats fed 0.06% 3'-MeDAB for 5 weeks, the

Fig. 4. Western Blot Analysis of GST-P Isozyme Separated by SDS-PAGE in the Liver from Rats Pretreated with 3'-MeDAB

Rats were given a basal diet for 15 weeks (normal) or a basal diet for 5 weeks followed by 0.03% gomisin A-containing diet for 10 weeks (normal-gomisin A), or pretreated with 0.06% 3'-MeDAB-containing diet for 3 weeks followed by a basal diet for 12 weeks (3'-MeDAB-normal) or by a basal diet for 2 weeks and 0.03% gomisin A-containing diet for 10 weeks (3'-MeDAB-gomisin A). AH66 cells were used as a GST-P positive control. Molecular weight standards (M. W.) are indicated on the left.

simultaneous administration of gomisin A (30 mg/kg, *p.o.*) significantly lowered the levels of protein-bound and -unbound aminoazo dyes in the liver and increased the amounts of free aminoazo dyes in the bile (Fig. 7). By HPLC analysis, at least one major peak with a retention time (5.5 min) different from 3'-MeDAB (retention time: 17 min) was observed in the bile from rats treated with 3'-MeDAB, and not only was the peak enlarged but many other peaks appeared after the additional treatment with gomisin A (Figs. 8A, B). When the bile was treated with β -glucuronidase and sulfatase, many metabolites except for unchanged 3'-MeDAB were detected (Fig. 8C). However, in rats fed 3'-MeDAB for 3 weeks and a basal diet for 2 weeks, no 3'-MeDAB or its related compounds were detected in the liver or bile by HPLC analysis (Figs. 8D, E).

DISCUSSION

It has been reported that GST-P positive and diploid hepatocytes increase in number and form foci in the preneoplastic stages in the rat liver.^{8,16} The change from polyploid to diploid was also found among phenotypically altered hepatocytes at the early stages of liver carcinogenesis.^{13,17} The present study indicates that gomisin A inhibits these preneoplastic changes in the liver from rats either simultaneously treated or pretreated with 3'-MeDAB.

Both 3'-MeDAB and gomisin A increased the total GST activity in the liver after treatment for 5 weeks. Rat hepatocytes are known to have several GST isozymes.¹⁸ 3'-MeDAB increased the expression of GST-P, but gomisin

Fig. 5. DNA Histograms of Hepatocyte Nuclei

A: Start of experiment. Rats (6 weeks old, start of experiments) were given a basal diet (B), 0.06% 3'-MeDAB-containing diet (C), or 30 mg/kg gomisin A (D) each for 5 weeks. Each histogram is from 30000 nuclei.

Fig. 6. Changes in Percentage of Diploid Nuclei in Hepatocytes from Rats Simultaneously Treated with 3'-MeDAB and/or Gomisin A

Rats were given a basal diet (□), 30 mg/kg gomisin A (○), 0.06% 3'-MeDAB-containing diet (●), and 3'-MeDAB and gomisin A (■) for the indicated periods up to 8 weeks. Data are the means $\pm$ S.E. from five rats. *a, b*) Significantly different from the normal (basal diet) group at $p < 0.005$ and 0.001 , respectively.

A inhibited the GST-P expression and enhanced the Alfa class GST isozymes (1 and 2), without influencing the level of the Mu class isozymes (3 and 4). The increased GST activities by 3'-MeDAB and gomisin A may be caused by the induction of GST-P and the Alfa class GST isozymes, respectively. However, the combined treatment with these agents did not further increase the activity by each agent alone. This may be because gomisin A inhibits any increase in the level of GST-P by 3'-MeDAB. Consequently, the effect of gomisin A on the GST-P expression is coincident with the immunohistochemical observations of GST-P positive foci in the liver.

Styles and co-workers^{19,20}) reported that genotoxic carcinogens such as 3'-MeDAB induce cytokinesis in some binucleated hepatocytes and cause proliferation of diploid cells, and these newly formed diploid cells appear to be incapable of undergoing binucleation or polyploidization. Styles *et al.*¹³) also reported that when 3'-MeDAB was continuously given to rats, the extent of covalent binding of the agent to hepatic protein increased dose-dependently and reached maximum after 5 weeks. In the simultaneous administration with 3'-MeDAB for 5 weeks, gomisin A lowered the contents of protein-bound and -unbound

TABLE II. Effect of Gomisin A on Ploidy Classes in Hepatocytes from Rats Pretreated with 3'-MeDAB

Group	At start of gomisin A			After feeding of gomisin A for 10 weeks		
	Diploid (%)	Tetraploid (%)	Octaploid (%)	Diploid (%)	Tetraploid (%)	Octaploid (%)
Normal	14.7 $\pm$ 1.9	78.7 $\pm$ 2.1	5.5 $\pm$ 0.8	12.0 $\pm$ 1.6	82.0 $\pm$ 1.4	6.0 $\pm$ 0.8
Normal-gomisin A	9.4 $\pm$ 1.0	84.1 $\pm$ 0.8	7.4 $\pm$ 0.5			
3'-MeDAB-normal	55.2 $\pm$ 3.6 ^{a)}	40.5 $\pm$ 3.2 ^{a)}	5.3 $\pm$ 0.4	40.9 $\pm$ 5.0 ^{a)}	53.3 $\pm$ 4.5 ^{a)}	5.5 $\pm$ 0.6
3'-MeDAB-gomisin A	23.7 $\pm$ 5.3 ^{c)}	68.4 $\pm$ 5.1 ^{b,c)}	7.9 $\pm$ 0.9			

Rats were treated with 0.06% 3'-MeDAB-containing diet for 3 weeks and a basal diet for 2 weeks, thereafter the treatment with 0.03% gomisin A-containing diet was begun, according to the experimental protocol for the 3'-MeDAB-pretreatment indicated in Fig. 1. Data are the means $\pm$ S.E. from five rats. *a, b*) Significantly different from the normal group at $p < 0.005$ and 0.05 , respectively. *c*) Significantly different from the 3'-MeDAB-normal group at $p < 0.05$.

Fig. 7. Amounts of Aminoazo Dyes in the Liver and Bile

(A) Protein-bound aminoazo dye in the liver, (B) protein-unbound aminoazo dye in the liver, (C) aminoazo dye in bile. Rats were treated with 0.06% 3'-MeDAB-containing diet (3'-MeDAB) or a combination with 30 mg/kg gomisin A (3'-MeDAB + gomisin A) for 5 weeks. Amounts of aminoazo dyes were colorimetrically determined and expressed the change in the absorbance at 520 nm. Data are the means $\pm$ S.E. from five rats. *a*) Significantly different from the 3'-MeDAB group at $p < 0.05$.

Fig. 8. HPLC Chromatograms of 3'-MeDAB-Related Substances in the Bile and Liver

(A) Bile extract from rats treated with 3'-MeDAB for 5 weeks, (B) bile extract and (C) extract of β -glucuronidase-treated bile from rats simultaneously treated with 3'-MeDAB and gomisins A for 5 weeks, (D) extract of bound components in the liver and (E) extract of β -glucuronidase-treated bile from rats fed 3'-MeDAB for 3 weeks followed by a basal diet for 2 weeks, and (F) an authentic 3'-MeDAB (200 pmol).

3'-MeDAB-related aminoazo dyes in the liver and increased excretion of aminoazo dyes in the bile. 3'-MeDAB is generally conjugated with glucuronate and sulfate in the liver, and a part of the sulfate conjugate binds to the cellular components protein, DNA, and RNA and shows genotoxicity, but most of these conjugates is excreted into the bile.²¹⁻²³) It is also shown that aminoazo dyes are conjugated with glutathione in the liver and excreted into the bile.²⁴) Gomisins A has been reported to induce several microsomal enzymes in the liver,⁵) and in this study it increased total GST activity during its administration, based on the induction of GST-1 and -2 isozymes. Additionally, this lignan compound has activities to increase biliary output and hepatic blood flow.^{4,6}) Therefore, the inhibition by simultaneous treatment with gomisins A of increases in GST-P foci and diploid nuclei by 3'-MeDAB seems to be due to the enhanced clearance of the carcinogen from the liver.

On the other hand, when the gomisins A feeding was started after the pretreatment with 3'-MeDAB for 3 weeks and 2 weeks cessation, at the time no 3'-MeDAB or its metabolites were detectable in the liver and bile, the preneoplastic changes in the liver GST-P positive foci and diploid hepatocytes were also reduced. Accordingly, we propose the possibility that gomisins A causes a switch

back from transformed cells or initiated cells and suppresses the promotion process in hepatocarcinogenesis.

In conclusion, gomisins A, a lignan compound of Schizandra fruits, may inhibit hepatocarcinogenesis by 3'-MeDAB by enhancing the excretion of the carcinogen and reversal of altered hepatocellular polyploidization.

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