

Modification of Mutagenic Activities of Pro-Mutagens by Glyco-Ursodeoxycholic Acid in the Ames Assay

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Biochemical Laboratories, Niigata College of Medical Technology, Niigata 950-2076, ¹Department of Hygiene and Preventive Medicine, Niigata University School of Medicine, Niigata 951-8510, and ²Niigata Cancer Center Hospital, Niigata 951-8566

SHIBUYA, N., NAKAMURA, K., OGOSHI, K., OHTA, T., HORI, Y., KODAMA, K. and YAMAMOTO, M. *Modification of Mutagenic Activities of Pro-Mutagens by Glyco-Ursodeoxycholic Acid in the Ames Assay.* Tohoku J. Exp. Med., 1999, 189 (1), 1-9 — Mutagenicity, co-mutagenicity and anti-mutagenicity of glyco-ursodeoxycholic acid (GUDCA) were examined by the Ames assay using *Salmonella typhimurium* strain TA98 with S9. As pro-mutagens, 2-aminoanthracene (2AA), Benzo[*a*]pyrene (BaP), 3-amino-1-dimethyl-5*H*-pyrido[4, 3-*b*]indole (Trp-P-2), 2-amino-3-methylimidazo[4, 5-*f*]quinoline (IQ) and 2-amino-3, 4-dimethylimidazo[4, 5-*f*]quinoline (MeIQ) were used. In addition to these pro-mutagens, blue-chitin extracts of human gallbladder bile (BCE) collected from the cholecystectomized patients with cholelithiasis were used in order to investigate the role of GUDCA on mutagen(s) actually existing in human bile. It was found that GUDCA did not show mutagenicity in this test system. Concerning the modification of mutagenic activities of pro-mutagens, GUDCA showed the different directions. GUDCA acted as co-mutagen, since it enhanced the mutagenic activities of 2AA and BaP. But, acted as anti-mutagen, since it suppressed the activities of Trp-P-2, IQ and MeIQ, all of which were classified as heterocyclic amines. GUDCA also suppressed the mutagen(s) in human bile. Because of the use of blue-chitin absorbed method for testing bile mutagenicity, the chemicals involved were considered to be heterocyclic amines and other polycyclic compounds. In these we suspect the bile mutagens are heterocyclic amines. Further examination should be directed towards the investigation into the mechanism of anti-mutagenic effects of GUDCA on mutagen(s) actually existing in human bile. — gallbladder cancer; glyco-ursodeoxycholic acid; Ames assay; anti-mutagenicity © 1999 Tohoku University Medical Press

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We have been conducting epidemiological studies on biliary tract cancer (BTC), since the mortality rate for BTC in Niigata prefecture was the highest in Japan. Over the last decade we made a large number of publications and they are recently summarized elsewhere (Yamamoto 1999). Throughout our studies we attempted to employ various kinds of scientific tools in order to reach the final goal of the discovery of the determinants of BTC namely gallbladder cancer (GBC). Among various studies that we have conducted, we came to notice the importance of co-mutagenic effects of bile acids on the occurrence of GBC.

Although many investigators confirmed the co-mutagenic and tumor-promoting effects of bile acids (Narisawa et al. 1974; Reddy et al. 1977; Silverman and Andrews 1977; Kobori et al. 1984; Castleden et al. 1989; Sugezawa and Kaibara 1991; Yamada et al. 1993), they used free bile acids, such as deoxycholic acid (DCA) and lithocholic acid (LCA) for their experiments. But, in human bile, these secondary bile acids do not exist as a free form, but as glycine or taurine conjugates. It is, therefore, a priority to examine the mutagenicity or co-mutagenicity by using these conjugates. Nothing, however, had been known on this matter until our trial (Shibuya et al. 1997).

When we examined mutagenicity and co-mutagenicity of glyco- and tauro-deoxycholic acids (GDCA and TDCA, respectively), it was found that they were not mutagenic in the *Salmonella typhimurium* TA98 and TA100 strains with and without S9mix. However, it was found that they enhanced the mutagenic activities of the pro-mutagens, 2-aminoanthracene (2AA) and benzo[*a*]pyrene (BaP) in the TA98 and TA100 strains with S9mix. Based on these findings, we concluded that GDCA and TDCA may enhance the activity of some mutagens existing in human bile (Shibuya et al. 1997).

During the course of GDCA and TDCA detection in human bile, however, we found that the concentrations of these conjugates in human bile were not different between the patients with gallstone (GS) and GBC. Subsequently, we came to focus on the conjugates of ursodeoxycholic acid (UDCA); glyco-ursodeoxycholic acid (GUDCA) and tauro-ursodeoxycholic acid (TUDCA). Eventually, we found that the higher concentration of GUDCA in bile from the patients with GBC as compared with those with GS. But, TUDCA concentration in bile was not different between patients with GS and GBC (Ogoshi et al. 1997). In Mexico, cases with GBC had a higher concentration of GUDCA than controls with cholelithiasis or choredocholelithiasis in the absence of cancer and controls with normal biliary tracts (Strom et al. 1996). We therefore decided to start examining the mutagenic activity of GUDCA in the Ames assay.

When we made a literature review in regard to the mutagenicity of UDCA related compounds, only limited information was available, that is, there was no mutagenicity of UDCA (Silverman and Andrews 1977), but contradictory findings on co-mutagenicity of UDCA. Silverman and Andrews (1977) reported the lack of co-mutagenicity, but Mori et al. (1996) reported the positive data when

N-nitroso compounds were used as pro-mutagens. Moreover, Earnest (1994) and Seraji (1997) demonstrated that supplemental dietary UDCA inhibited azoxymethane-induced colonic carcinogenesis of rats. Nothing, however, has been disclosed about the effects of its conjugates.

We report here that GUDCA modified the mutagenic activities of pro-mutagens towards the two different directions; co-mutagenic and anti-mutagenic. In the case of human bile, it demonstrated an inhibitory effect on mutagenic substance(s) existing in bile.

MATERIALS AND METHODS

Chemicals used in the present study

GUDCA was purchased from Nacalai Tesque Inc. (Kyoto). The purity of this chemical was more than 99% by high-performance liquid chromatography. As pro-mutagens, 2AA, BaP, 3-amino-1-dimethyl-5*H*-pyrido[4, 3-*b*]indole (Trp-P-2), 2-amino-3-methylimidazo[4, 5-*f*]quinoline (IQ) and 2-amino-3, 4-dimethylimidazo[4, 5-*f*]quinoline (MeIQ) were from Wako Pure Chemical Industries Ltd. (Osaka). Blue-chitin for bile adsorption was purchased from Funakoshi Ltd. (Tokyo).

Ames assay

For the analysis of mutagenic, co-mutagenic and anti-mutagenic activities, the Ames assay (Ames et al. 1975) using the plate incorporation method was employed. Tester strain was *Salmonella typhimurium* TA98 with S9 mix, since this test system was proved to be effective in the previous experiment (Shibuya et al. 1997). The S9 was purchased from Litton Bionetics, Inc. (Kensington, MD, USA). This S9 was prepared from the liver of Sprague-Dawley rat treated with Aroclor 1254.

Test chemicals were dissolved in dimethylsulfoxide (DMSO) for the Ames assay. In each assay duplicated plates were prepared and their results (mean of the duplicated plates) were judged as mutagenic when the two diagnostic criteria were fulfilled; more than 2-fold increase in the revertant colonies in comparison with those of the control (DMSO) plate. The other was the presence of dose-response relationship. Co-mutagenicity or anti-mutagenicity was determined when the 2-fold increase or decrease in revertant colonies in comparison with those of the pro-mutagen treated plate, respectively. Needless to say, the presence of dose-response relationship was also one of the criteria.

The maximum dose of the test compound, GUDCA was 40 μ mol per plate, because the application of 80 μ mol induced bactericidal effects at an initial experiment using 2AA. The five dose levels of GUDCA were established in the present study; 0 (DMSO), 5, 10, 20 and 40 μ mol/plate.

For testing the effects of GUDCA on pro-mutagens, the mixture of 50 μ l solution of GUDCA and the same volume of either of BaP, 2AA, Trp-P-2, IQ or

MeIQ was examined by the Ames assay. The dose of these pro-mutagens were 0.1 μg for 2AA, 0.5 μg for BaP, 1.25 ng for Trp-P-2, 700 pg for IQ and 150 pg for MeIQ.

Experiment using human gallbladder bile

Bile samples were collected from patients who had undergone cholecystectomy surgery for cholelithiasis. Bile was obtained from the resected gallbladder and stored at -20°C until analysis. Just prior to the Ames assay, samples were sterilized by passing bile through a 0.45-mm Milipore filter.

Ten ml of sterilized sample was diluted 50-fold with water and the diluent was pumped through a column of blue-chitin prepared by washing with 20 ml of water, 30 ml of eluent (methanol-ammonia solution [50 : 1, v/v]) and again 30 ml of water. The column was then washed with water (20 ml) and blue-chitin absorbed substance eluted with the above eluent (200 ml). The eluate was evaporated to dryness under low pressure. This blue-chitin extract from human bile (BCE) was dissolved with DMSO. Mutagenicity of the samples was tested by the Ames assay by using a preincubation method (Yahagi et al. 1977). The six dose levels were established such as 0, 15.6, 31.3, 62.5, 125 and 250 μl bile-equivalent groups. The dose of GUDCA was established as 10 $\mu\text{mol}/\text{plate}$, since the use of 20 $\mu\text{mol}/\text{plate}$ in combination with bile extracts induced bactericidal effects.

RESULTS

Mutagenicity of GUDCA

It was found that GUDCA, did not show any mutagenic action in this test system (open circle of Fig. 1B).

Effects of GUDCA on pro-mutagens

When the mixture of GUDCA and pro-mutagen was tested, we obtained peculiar results; GUDCA enhanced mutagenic activities of some of the pro-mutagens, but suppressed the others. The revertant colonies induced by 2AA (0.1 $\mu\text{g}/\text{plate}$) increased in a dose-related manner when 0, 5, 10, 20 and 40 μmol of GUDCA was added to the plate (Fig. 1A). The numbers of revertant colonies in the corresponding groups were 56, 132, 218, 353 and 1056. The treatment with 80 μmol of GUDCA, however, induced lethal effects on *Salmonella typhimurium* (data not shown). When we employed BaP (0.5 $\mu\text{g}/\text{plate}$) as a pro-mutagen, we found that there was a dose-related increase in the numbers of revertant colonies up to the group of 40 μmol of GUDCA treatment (closed circle of Fig. 1B). The number of colonies in the control group (BaP only) was 142 and that of 40 μmol of GUDCA group was 290, showing the significant increase.

In contrast to the effects on 2AA and BaP, GUDCA showed anti-mutagenic effects when Trp-P-2, IQ and MeIQ were employed as pro-mutagens. With regard

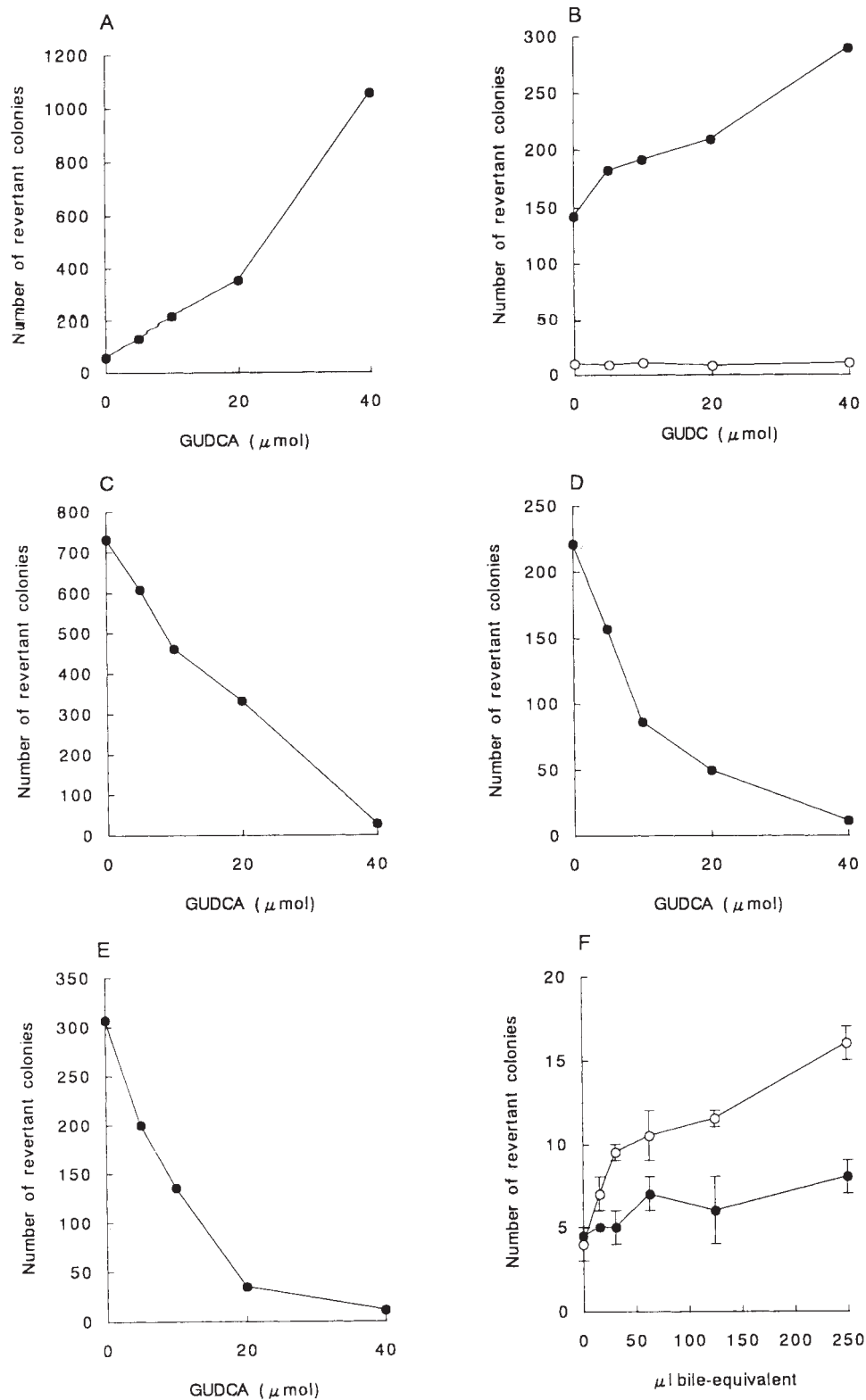


Fig. 1. Effects of GUDCA on mutagenicity of pro-mutagens (2AA, BaP, Trp-P-2, IQ, MeIQ) and blue-chitin extract from human bile (BCE) in the presence of S9mix. Tester strain was *Salmonella typhimurium* TA98. Pro-mutagens: A, 2AA (0.1 μg); B, BaP (0.5 μg, ●) and GUDCA only (○); C, Trp-P-2 (1.25 ng); D, IQ (700 pg); E, MeIQ (150 pg); F, BCE with 10 μmol of GUDCA (○, means ± s.d.) or DMSO (●, means ± s.d.).

to toxic effect on bacterial growth, we applied tests for viability of bacteria using nutrient agar plates (Ames et al. 1975) and observed no toxicity on the doses of GUDCA with pro-mutagens (Trp-P-2, IQ, MeIQ). As shown in Fig. 1 (C, D and E), the revertant colonies decreased gradually when the amount of GUDCA increased. There was a linear relationship up until the dose of 40 μmol of GUDCA. In the case of IQ and MeIQ, the dose-effect relationship showed the similar pattern. The revertant colonies in the group of 10 μmol of GUDCA was 86 and decreased rapidly from 221 colonies in the control group (IQ only), while the numbers of colonies decreased gradually after 10 μmol of GUDCA treatment. In the case of MeIQ, the numbers of revertant colonies were 307 in the control group, and decreased lineally down to 35 in the group of 20 μmol group, then reached the plateau in the 40 μmol group.

Effects of GUDCA on blue-chitin extract from human bile

It is needless to say that the revertant colonies increased with increasing the dose of the tested bile sample added (open circle of Fig. 1F), only because we used a mutagenic-positive sample by the Ames assay. In the case of BCE, the numbers of colonies increased from 5 to 16 in the control and 250 μl bile-equivalent dose groups, respectively. When BCE was preincubated with 10 μmol of GUDCA, the numbers of revertant colonies were found to be lower than those in the corresponding dose group of BCE (closed circle of Fig. 1F). The doses of BCE with GUDCA were non-toxic for bacteria; this fact was confirmed by the bacteria-viability test mentioned above. Based on these findings, it is suggested that GUDCA acted as an anti-mutagenic role on unknown mutagen(s) existing in human bile.

DISCUSSION

We started this experiment, being suggested by the findings that the concentration of GUDCA was higher in gallbladder bile from the patients with GBC. It was a matter of our primitive concern whether GUDCA increased prior to the occurrence of GBC or simply the result of ill-condition and how it acts on DNA of epithelial cells of the gallbladder.

UDCA is classified as the tertiary bile acid and it is formed in the liver by epimerization of the secondary cholic acid, LCA (Sherlock and Dooley 1997; Greenberger and Isselbacher 1998). It is reported that amount of UDCA, though found in trace amount in normal condition, increased with the condition of cirrhosis or cholestasis. In addition, glycine conjugate was found in excess in bile as compared with the taurine conjugate (usually the ratio of these conjugates are about 3 : 1 in normal condition), when the patients had cirrhosis or cholestasis. In the case of GBC, many patients have cholestasis and this pathological condition of cholestasis may increase the concentration of GUDCA in bile.

In order to find out the answer of the second question how it acts on DNA of

the epithelial cells of the gallbladder, we examined the mutagenicity of GUDCA in the Ames assay. Then, we found no mutagenicity of GUDCA. This finding is in agreement with those by Silverman and Andrews (1977), who, however, used UDCA instead of GUDCA. To our knowledge, no information concerning the conjugate of UDCA, namely GUDCA is available.

With regards to the co- or anti-mutagenicity, however, we happened to find out that GUDCA acted towards the two different directions against pro-mutagens. Of five chemicals employed, GUDCA enhanced the mutagenic activities of 2AA and BaP, whereas it suppressed those of Trp-P-2, IQ and MeIQ. In our previous study (Shibuya et al. 1997), we used only 2AA and BaP, and the dosages of these chemicals were proved to be effective for testing, but we used Trp-P-2, IQ and MeIQ for the first time in combination with GUDCA. There are two possible explanations; one is that the action of GUDCA may differ according to the kinds of pro-mutagens employed, the other is that the experiment was not undertaken under the optimal condition in combination of pro-mutagens with GUDCA. From the point of view of chemical characteristics, 2AA and BaP are classified as aromatic amine and polycyclic aromatic, respectively and the latter 3 chemicals belong to heterocyclic amines. It should be particularly noted that GUDCA acted toward the same direction, so-called anti-mutagenicity when heterocyclic amines were used as pro-mutagens. Before getting a final answer, we should conduct additional experiments in order to exclude the second possibility.

When we tested a combination of GUDCA and BCE, an anti-mutagenic action was observed against mutagen(s) absorbed in blue-chitin. Up to the present, no one knows what kind of chemicals involved in human bile, although heterocyclic amines are highly suspected, since the blue-chitin extraction method was employed and these chemicals were proved mutagenic in the tester strain of TA98 with S9mix. Of five pro-mutagens used in the present experiment, Trp-P-2 was actually detected in human bile (Manabe and Wada 1990). Except for this information, nothing has been known in regard to the mutagens which belong to heterocyclic amines in human bile. Final solution may be obtained when ultimate mutagen(s) are identified in human bile.

Further examination will be necessary to elucidate the patho-physiological role of GUDCA which was produced in increased amounts in patients with GBC. Especially it should be directed towards the investigation into the mechanism of anti-mutagenic effects of GUDCA on mutagen(s) actually existing in human bile.

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