Grapefruit juice intake does not enhance but rather protects against aflatoxin B\textsubscript{1}-induced liver DNA damage through a reduction in hepatic CYP3A activity

Masaaki Miyata\textsuperscript{1}, Hiroki Takano, Lian Q. Guo, Kiyoshi Nagata and Yasushi Yamazoe

Division of Drug Metabolism and Molecular Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Aramaki, Aoba-ku, Sendai 980-8578, Japan
\textsuperscript{1}To whom correspondence should be addressed
Email: miyata@mail.pharm.tohoku.ac.jp

Influence of grapefruit juice intake on aflatoxin B\textsubscript{1} (AFB\textsubscript{1})-induced liver DNA damage was examined using a Comet assay in F344 rats given 5 mg/kg AFB\textsubscript{1} by gavage. Rats allowed free access to grapefruit juice for 5 days prior to AFB\textsubscript{1} administration resulted in clearly reduced DNA damage in liver, to 65\% of the level in rats that did not receive grapefruit juice. Furthermore, rats treated with grapefruit juice extract (100 mg/kg per os) for 5 days prior to AFB\textsubscript{1} treatment also reduced the DNA damage to 74\% of the level in rats that did not receive grapefruit juice. No significant differences in the portal blood and liver concentrations of AFB\textsubscript{1} were observed between grapefruit juice intake rats and the controls. In an Ames assay with AFB\textsubscript{1} using Salmonella typhimurium TA98, lower numbers of revertant colonies were detected with hepatic microsomes prepared from rats administered grapefruit juice, compared with those from control rats. Microsomal testosterone 6\textsuperscript{b}-hydroxylation was also lower with rats given grapefruit juice than with control rats. Immunoblot analyses showed a significant decrease in hepatic CYP3A content, but not CYP1A and CYP2C content, in microsomes of grapefruit juice-treated rats than in non-treated rats. No significant differences in hepatic glutathione S-transferase (GST) activity and glutathione content was observed in the two groups. GSTA5 protein was not detected in hepatic cytosol of the two groups. In microsomal systems, grapefruit juice extract inhibited AFB\textsubscript{1}-induced mutagenesis in the presence of a microsomal activation system from livers of humans as well as rats. These results suggest that grapefruit juice intake suppresses AFB\textsubscript{1}-induced liver DNA damage through inactivation of the metabolic activation potency for AFB\textsubscript{1} in rat liver.

Introduction

Aflatoxin B\textsubscript{1} (AFB\textsubscript{1}) is one of the most common mycotoxins found in human foodstuffs such as corn, peanuts and cotton seeds. This mycotoxin is a potent hepatocarcinogen in experimental animals and also probably in humans (1–3). Because of detection in a variety of human foodstuffs, AFB\textsubscript{1} is believed to be a carcinogen of human health risk. This chemical is biotransformed by cytochrome P450 (CYP) to the reactive intermediate aflatoxin B\textsubscript{1} 8,9-epoxide (AFBO) to exert its carcinogenic effect (4–6). AFBO is capable of reacting with DNA, resulting in the production of DNA damage. There is considerable evidence suggesting that CYP3A2 and CYP2C11, but not CYP1A2, are involved in the production of AFBO in rats (7–9). CYP1A1, CYP2B and CYP3A also metabolize AFB\textsubscript{1} to other metabolites, aflatoxin M\textsubscript{1}, aflatoxin P\textsubscript{1} and aflatoxin Q\textsubscript{1}. The production of these metabolites is considered to represent detoxification reactions (10,11). Thus, CYP3A forms are involved in detoxification and activation of AFB\textsubscript{1}. Another major detoxification pathway is glutathione conjugation of AFBO by glutathione S-transferase (GST) (12). The rat GSTA5 subunit (also called Yc2) plays a major role in protection against AFB\textsubscript{1}-induced DNA damage (13,14). Pretreatment of rats with GST inducers such as ethoxyquin, oltipraz and butylyhydroxytoluene resulted in protection against AFB\textsubscript{1}-induced DNA damage and carcino genesis (15). Thus, AFBO production and its glutathione conjugating activity are considered to be major determinants of susceptibility to AFB\textsubscript{1}-induced hepatocarcinogenesis.

Numerous epidemiological studies suggest a role of dietary habit in human cancer incidence. Foods sometimes contain carcinogens as well as anticarcinogenic compounds. It has been shown that various fruits, vegetables and teas reduce chemical tumorigenesis in experimental animals. Anticarcinogenic activity of components in these foods has been widely investigated (16). Grapefruit juice is known to alter the pharmacokinetics and efficacies of various drugs catalyzed by CYP3A, including dihydropyridine calcium channel blockers and cyclosporin A (17–19). Co-administration of grapefruit juice with these drugs results in a substantial increase in their oral bioavailability. Microsomal metabolism of these drugs was inhibited by components of grapefruit juice (20–22). The effects of grapefruit juice are mainly attributed to the inhibition of CYP3A activities in the gastrointestinal tract rather than in the liver in human (23,24). Inhibition of P-glycoprotein activity, which controls excretion of these drugs into the intestinal lumen, is also at least in part involved in the increase in bioavailability with grapefruit juice (25). Thus, we examined whether grapefruit juice intake enhances AFB\textsubscript{1}-induced liver DNA damage concomitant with an increase in hepatic and portal blood concentrations of AFB\textsubscript{1} in rats.

Unexpectedly, we have shown that grapefruit juice intake suppresses AFB\textsubscript{1}-induced liver DNA damage in rats. Furthermore, the suppressive effect is likely to be attributable to a decrease in the capacity for metabolic activation of AFB\textsubscript{1} through hepatic CYP3A activity, rather than an effect on transport systems or metabolism in the gastrointestinal tract.
Metabolites were detected at 240 nm.

Grapefruit juice extract (100 mg/kg in saline) once a day for 5 days by gavage as described previously (26).

Comet assay
Rats were allowed free access to grapefruit juice for 5 days or were given grapefruit juice extract (100 mg/kg in saline) once a day for 5 days by gavage prior to oral administration of AFB1 (5 mg/kg). Rats were given GFJE 3 h following the final treatment with grapefruit juice extract. Rats were killed 3 h after AFB1 treatment to remove the livers. Comet assays were conducted as described previously (27,28). Liver nuclei were prepared and placed in chilled lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, 1% sarkosyl, 10% DMSO and 1% Triton X-100, pH 10) and kept at 0°C for >60 min, then in chilled alkaline solution (300 mM NaOH and 1 mM EDTA, pH 13) at 0°C for 10 min. Electrophoresis was performed at 0°C for 15 min at 40 V and ~50 mA. The slides were neutralized and stained with 50 μl of a 20 μg/ml ethidium bromide solution. Fifty nuclei were analyzed per mouse at 200× magnification by fluorescence microscopy. The differences between the length of the whole comet and the diameter of the head were calculated.

Inhibition of metabolic activation with grapefruit juice extract
Grapefruit juice extract was dissolved in methanol. The grapefruit juice extract was preincubated at 37°C for 10 min in a reaction mixture containing microsomes, cofactors and bacteria prior to addition of AFB1. The reaction was started by addition of AFB1. All reactions were carried out at 37°C for 10 min.

Liver and serum AFB1 concentration
Blood samples were taken from portal veins of rats treated with AFB1 (5 mg/kg) or vehicle-treated groups, no significant difference was found (P<0.05, GFJ, grapefruit juice; GFJE, grapefruit juice extract).

GST activity
GST activities were determined as described previously (32). The reaction mixture for 1-chloro-2,4-dinitrobenzene (CDNB) GST activity (100 μl) contained 2 μg cytosolic protein, 1 mM reduced glutathione (GSH) and 1 mM CDNB in 100 mM potassium phosphate buffer (pH 7.5). The reaction mixture for 1,2-dichloro-4-nitrobenzene (DCNB) GST activity (100 μl) contained 2 μg cytosol, 1 mM GSH and 1 mM DCNB in 100 mM potassium phosphate buffer (pH 7.5). The reaction was performed at 25°C for 2.5 min. GST activities were measured at 340 and 345 nm for CDNB and DCNB, respectively.

Liver GSH content
Liver was homogenized in a 4-fold volume of cold 1 M perchloric acid solution with 2 mM EDTA. After the denatured protein was removed by centrifugation, the supernatant was assayed for GSH according to the method of Griffith (33). The reaction mixture (1 ml) contained 5 mM EDTA, 0.6 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 0.2 mM NADPH, 0.2 ml supernatant and 0.5 U GSH reductase in 100 mM potassium phosphate buffer (pH 7.5). Incubation was initiated by the addition of GSH reductase and the rate of formation of 2-nitro-5-thiobenzoic acid was measured at 412 nm.

Inmunoblot analysis
Microsomal proteins were separated by SDS-PAGE and transferred to a nitrocellulose sheet. The sheets were immunostained with polyclonal antibodies against GSH transferase, GST A5 and J.D.Hayes (Dundee University, Dundee, UK). Grapefruit juice (Tropicana) distributed by Kirin Beverages Inc. (Tokyo, Japan) was used. Polyclonal antibodies against rat GSTA5 were kindly provided by Dr J.D.Hayes (Dundee University, Dundee, UK). Grapefruit juice extract was prepared by extraction of grapefruit juice with ethyl acetate as described previously (26).
AFB1-induced migration of tailed nuclei was unexpectedly reduced in grapefruit juice-pretreated rats to just 65% of the tail observed in liver nuclei from control rats that were not pretreated with grapefruit juice. To determine whether lipid-soluble components of grapefruit juice such as furanocoumarin derivatives suppress AFB1-induced liver DNA damage in rats, the influence of pretreatment with an ethyl acetate extract of grapefruit juice was examined. One milligram of grapefruit juice extract is derived from 1 ml of grapefruit juice. Grapefruit juice extract (100 mg/kg per os) was administered to rats once a day for 5 days before AFB1 treatment. AFB1-induced migration of tailed nuclei was reduced in grapefruit juice extract-pretreated rats to 74% of the vehicle-pretreated rats (Figure 1B).

Absorption of AFB1
Grapefruit juice intake is known to inhibit metabolism and transport of drugs in the intestinal tract. Therefore, the portal blood concentration of AFB1 was measured to determine whether grapefruit juice intake affects absorption of AFB1 from the intestine. However, no significant difference in AFB1 concentration between rats with and without grapefruit juice intake was observed until 45 min after 5 mg/kg AFB1 administration (Figure 2A). Thus, hepatic AFB1 content at 45 min after AFB1 administration was measured to compare the level of influx and efflux of AFB1 across the liver between rats of the two groups. No significant difference was observed in hepatic AFB1 content between the two groups (Figure 2B).

Metabolic activation of AFB1 in liver microsomes
The capacity for metabolic activation of AFB1 in rat liver microsomes was evaluated by the Ames assay. At 0.5 μg/plate AFB1, the number of revertant colonies increased, depending on the amount of microsomal protein (from 25 to 100 μg/plate) (Figure 3). Numbers of revertant colonies were significantly decreased with liver microsomes prepared from grapefruit juice intake rats compared with those from control rats. In rats, two forms of cytochrome P450, CYP2C11 and CYP3A2, are known to metabolically activate AFB1. To evaluate the influence of grapefruit juice intake on CYP3A2 and CYP2C11 activities in liver microsomes, testosterone 6β-hydroxylase and 16α-hydroxylase activities were measured as indicators of CYP3A2 and CYP2C11, respectively.

Microsomal testosterone 6β-hydroxylation by liver microsomes in grapefruit juice intake rats was decreased to 50% of the control liver level (Figure 4). Grapefruit juice intake tended to decrease microsomal testosterone 16α-hydroxylase activity in rat livers, but there was no statistically significant difference in activity between the grapefruit juice intake rats and the control rats (n = 5).

Thus, to assess whether the decrease in metabolic activation potency is attributable to the change in content of CYP3A forms, immunoblot analyses of CYP3A forms were performed using antibodies raised against CYP3A4. As shown in Figure 5, the hepatic CYP3A level in grapefruit juice intake rats was reduced to 50% of control livers. On the other hand, no significant differences in CYP1A, CYP2C and mEH levels were found between rats with and without grapefruit juice intake (Figure 6A).

Detoxification of AFB1 reactive intermediates in liver
GST and mEH mediate inactivation of the AFB1 reactive intermediate AFBO prior to its reaction with DNA. Typical substrates for GST (CDNB and DCNB) were thus used to evaluate GST activity in liver cytosol (32). These GST activities were not significantly different between control and grapefruit juice intake groups (Figure 7A and B).
Furthermore, no differences in GSH content (Figure 7C) in liver and mEH protein level (Figure 6A) in liver microsomes between the two groups were observed. GSTA5 was identified as the major AFBO-metabolizing enzyme (14). Thus, the expression level of GSTA5 protein was measured by western blot analysis. GSTA5 protein was not detected in liver cytosol of either the control or grapefruit juice intake male rats (Figure 6B).

### Inhibition of metabolic activation of AFB1 with grapefruit juice extract

Metabolic activation of AFB1 was assessed with the Ames assay to evaluate whether grapefruit juice components inhibit the metabolic activation of AFB1. A clear increase in revertant colonies was detected in the presence of an activation system using rat microsomes (50 μg protein/reaction) (Figure 8A). A 50% decrease in AFB1-induced mutagenicity was observed in the presence of grapefruit juice extract (25 μg/reaction) in the preincubation mixture. On the other hand, no clear increase in revertant numbers was observed in the Ames test with 50 μg human liver microsomes (0.5 μg AFB1/plate) (Figure 8B). Addition of more than 20 times higher amounts of AFB1 resulted in the production of revertants. Addition of grapefruit juice extract inhibited AFB1-induced mutagenesis in the presence of an activation system using human microsomes.

### Discussion

The present study has employed the Comet assay to demonstrate that grapefruit juice intake protects rats against AFB1-induced liver DNA damage. This assay also revealed that pretreatment of rats with grapefruit juice extracts containing furanocoumarin derivatives protected against the genotoxic effects of the mycotoxin. These results suggest that lipid-soluble compounds in grapefruit juice that can be extracted with ethyl acetate are the major components protecting against DNA damage caused by AFB1. A significant

![Fig. 5. Quantitation of CYP3A forms in liver microsomes. Microsomal proteins (5 μg) were subjected to SDS–PAGE on 8% gels and electrically transferred to nitrocellulose membranes for immunostaining with anti-CYP3A4 antibody. CYP3A1/2 levels were quantitated as described in Materials and methods. Data are shown as means ± SD (n=5). *Significant difference from control group at P<0.05.](image1)

![Fig. 6. Immunoblot analyses of drug metabolizing enzymes. (A) Microsomal proteins (5 μg) were subjected to SDS–PAGE on 8% gels. The proteins were immunostained with anti-rat CYP3A2, anti-rat CYP1A1, anti-rat CYP2C11 and anti-rat mEH antibodies. Lanes 1–3, liver microsomes of rats without grapefruit juice intake; lanes 4–6, liver microsomes of grapefruit juice intake rats. (B) Cytosolic proteins (10 μg) were subjected to SDS–PAGE on 12% gels. The proteins were immunostained with anti-rat GSTA5 antibody. Lane 1, female rat liver microsomes; lanes 2–4, liver microsomes of male rats without grapefruit juice intake; lanes 5–7, liver microsomes of grapefruit juice intake male rats; lane 8, female mouse microsomes.](image2)

![Fig. 7. GST activities and GSH content in rat liver. GST activity in rat liver cytosol was measured using (A) CDNB and (B) DCNB as substrates. (C) GSH content in rat livers. Liver cytosols were prepared from rats allowed free access to grapefruit juice for 5 days. Data are shown as means ± SD (n=5).](image3)
content was observed in livers of grapefruit juice intake rats by the GSTA5 subunit. The change results in protection against inhibition of hepatic CYP3A activity and a decrease in microsomal CYP3A content. This result supports the idea that grapefruit juice components inhibit hepatic CYP3A4 activity (37,38). Grapefruit juice components containing these furanocoumarin dimers inhibit not only CYP3A4, but also CYP1A2, CYP2C9 and CYP2C19 (39). Several reports suggest that CYP3A4 and CYP1A2 are the major forms producing AFBO in humans (40–42). Furthermore, our data showed that grapefruit juice intake can affect hepatic CYP3A activity in rats. Although grapefruit juice is known to selectively inhibit intestinal CYP3A activity, Kivistö et al. suggested that in the human a large intake of grapefruit juice suppresses the activity of CYP3A in both the liver and the intestine (43). These facts suggest the possibility that AFB1-induced DNA damage is also suppressed by grapefruit juice intake in humans through a reduction in hepatic CYP3A activity.

In an Ames assay of AFB1 genotoxicity, a 20-fold higher concentration of the mycotoxin was required to generate bacterial revertants in the presence of human liver microsomes than was required in the presence of rat liver microsomes (Figure 8); it should be noted that equivalent levels of testosterone 6β-hydroxylation were detected in both rat and human liver microsomes. Thus, the metabolic activity of AFB1 in human liver microsomes is lower than that in rat liver microsomes.

In the present study we have demonstrated that grapefruit juice intake protects against AFB1-induced liver DNA damage in rats and a decrease in hepatic CYP3A activity is at least in part involved in this protection.
References


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