Effects of Citrus Limonoids on Glutathione S-Transferase Activity in Mice

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Citrus limonoids were studied as inducers of the detoxifying enzyme system glutathione S-transferase in mice. Among eight limonoids studied, nomilin was shown to be the most potent inducer in the liver and in the small intestinal mucosa. In the forestomach, the inducing activity was low but the enzyme activity was significantly elevated when compared with that of the control. In the lung and colon no appreciable enzyme activity was induced by nomilin. Three other limonoids, obacunone, isoobacunoic acid, and ichangin, were shown to be enzyme inducers in the liver and some other tissues studied. Limonin, on the other hand, showed only marginal activity as an enzyme inducer in the small intestinal mucosa and was inactive in all other tissues studied. Limonol showed small inducing activity in the forestomach while deoxylimonin was not active in any of the tissues examined. Since anticarcinogens such as nomilin and kahweol are potent inducers of detoxifying enzymes, citrus limonoids such as obacunone, isoobacunoic acid, and others may be effective inhibitors of chemically induced carcinogenesis.

Limonoids are a group of chemically related triterpene derivatives found in the Rutaceae and Meliaceae families. Limonin and nomilin, bitter members of the group, occur widely in citrus (Hasegawa et al., 1980). Bitterness due to limonin in a variety of citrus juices is a major problem in the citrus industry and has significant negative economic impact (Maier et al., 1977).

Citrus limonoids contain a furan moiety attached to the D-ring lactone at the 3-position. Previous studies on furan-containing natural products have suggested that the furan moiety is responsible for the induction of the detoxifying enzyme system glutathione S-transferase (GST) (EC 2.5.1.18). Kahweol and cafestol, two furan-containing diterpenes isolated from green coffee beans, are inducers of increased GST activity in various tissues in mice (Lam et al., 1982). Studies on the inducing capacity of derivatives obtained by the modification of the functional groups on the dieterpene molecules have led to the conclusion that the furan moiety is the critical site for enzyme-inducing activity (Lam et al., 1987).

The GST enzymes are one of the major enzyme systems responsible for the detoxification of xenobiotics (Chasseaud, 1979; Jakoby and Habig, 1980). They catalyze the adduct formation of glutathione with electrophiles, including reactive carcinogenic species, to water-soluble substances that are readily excreted. Enhancement of the activity of GST suggests an increase in the ability of the organism to detoxify carcinogens. Thus, any substance that can elicit increased activity of this detoxifying enzyme system may be potential anticarcinogens that can inhibit chemically induced cancer formation.

The GST enzyme inducers cafestol and kahweol have been found to inhibit mammary tumor formation in Sprague–Dawley rats (Wattenberg and Lam, 1984). Other GST inducers such as phenolic antioxidants, benzyl isothiocyanates, and coumarins have been found to inhibit chemically induced tumorigenesis in laboratory animals (Lam et al., 1979; Wattenberg et al., 1979, 1980; Sparnins and Wattenberg, 1981). To test further the hypothesis that natural products possessing a furan moiety are also inducers of the GST enzyme system and thus are potential chemopreventive agents, we have investigated the inducing activity of several citrus limonoids. This study reports the enzyme-inducing effects of eight citrus limonoids in various tissues of ICR/Ha mice.

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MATERIALS AND METHODS

Chemicals. All citrus limonoids were isolated from grapefruit seeds. Seed meals were washed thoroughly with hexanes, and the limonoids were extracted with acetone. The most abundant limonids, limonin and nomilin, were obtained by fractional crystallization successively from methylene chloride and acetone. The other limonoids were isolated from the mother liquor by silica gel column chromatography separation. The column was eluted with increasing concentrations of ethyl acetate in hexane. The structures of the limonoids were confirmed by NMR spectral analysis.

Pure 3-*tert*-butyl-4-hydroxyanisole (BHA; >99.6% by HPLC) was obtained by fractional crystallization from commercial butylated hydroxyanisole (Sigma Chemical Co., St. Louis, MO). Glutathione (GSH) was purchased from Sigma. 1-Chloro-2,4-dinitrobenzene (CDNB) was obtained from Aldrich Chemical Co., Milwaukee, WI.

Animals. Female ICR/Ha mice were obtained from the Harlan Sprague–Dawley Laboratory (Indianapolis, IN). Mice, 8 weeks old, were fed a semipurified diet (ICN Nutritional Biochemicals, Cleveland, OH) for 1 week before they were divided into experimental and control groups with three mice per group. The experimental groups were given a total of three doses of the test compounds suspended in cottonseed oil by oral intubation once every 2 days. The control group was given cottonseed oil alone. Twenty-four hours after the last intubation, the mice were killed by cervical dislocation and the lung, forestomach, the proximal one-third of the small intestinal mucosa, colon, and the liver were removed for enzyme preparation. The tissues were homogenized in 1.15% KCl solution by means of a Brinkman homogenizer. The cytosol after 100000*g* centrifugation for 1 h was obtained and frozen at −80 °C until use. Each sample represents one tissue from each individual animal.

GSH S-Transferase Assay. The activity of cytosolic GSH S-transferase was assayed according to the method of Habig et al. using CDNB as the substrate (Habig et al., 1974). The reaction was monitored at 340 nm in a Hewlett-Packard HP8450A or a Beckman DU65 spectrophotometer. Assays were performed at 30 °C in 0.1 M phosphate buffer, pH 6.5, in the presence of 5 mM GSH and 1 mM CDNB. Complete assay mixture without enzyme was used as the control.

RESULTS

Eight citrus limonoids (Figure 1) were tested as inducers of the detoxifying enzyme GST. In general, limonoids with both the A- and A′-rings intact, such as limonin, limonol, and deoxylimonin, were not active as enzyme inducers

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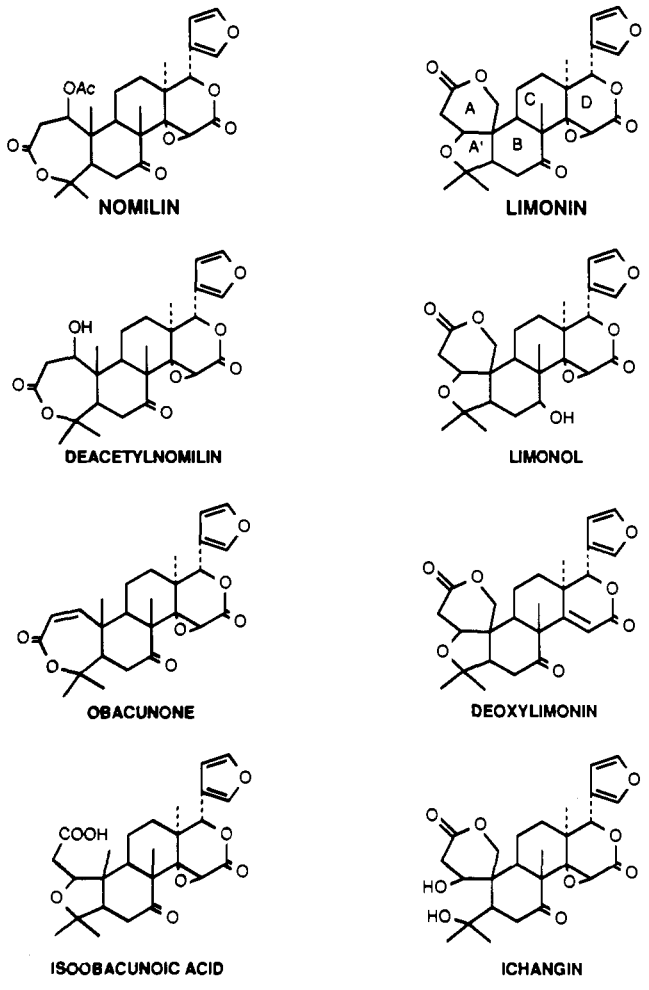

Figure 1. Structures of citrus limonoids used in this study.

(Table I). The highest inducing activity was found in the cytosol of the small intestinal mucosa of nomilin-treated mice. At a dose of 20 mg/intubation, nomilin was able to elicit 4.51 times the enzyme activity of the control. With the 5-mg dose, the GST activity was 3 times higher than that of the control. The inducing activity at the lowest dose was comparable to that obtained with 7.5 mg of BHA. Since the molecular weight of nomilin is 3 times as high as that of BHA, it is estimated that the limonoid is at least 3 times more active on a molar basis than the antioxidant as an enzyme inducer in the small intestinal mucosa of mice. In this same tissue, three other limonoids, obacunone, isoobacunoic acid, and ichangin, also showed significant potential as enzyme inducers.

Limonin, on the other hand, induced increased GST activity at a modest 50% higher than that of the control at the 20-mg dose in the small intestinal mucosa. At the lowest dose of 5 mg it was not significantly different from the control.

In the liver, the effects of the limonoids as enzyme inducers are less than those observed in the mucosa. At all three doses employed, the increased GST activity induced by nomilin was less than that obtained by BHA. Nevertheless, at 20-mg dose, the enzyme activity was 3.79 times higher than that of the control. At 10-mg dose, most nomilin-related compounds, obacunone, ichangin, and isoobacunoic acid, were active enzyme inducers, with the last limonoid being the most potent. In this tissue, limonin, limonol, and deoxylimonin were inactive at the dosages tested.

In the cytosols of the forestomach, the increased activity by nomilin and derivatives was less than 50%. Limonin

Table I. Effects of Citrus Limonoids and Butylated Hydroxyanisole on the Activity of Glutathione *S*-Transferase in the Tissues of Female ICR/Ha Mice

		small intestinal mucosa		liver		forestomach		lung		colon	
compound	dose,[b] mg	GST act.,[c] μmol/min per mg protein	ratio TEST/CON	GST act.,[c] μmol/min per mg protein	ratio TEST/CON	GST act.,[c] μmol/min per mg protein	ratio TEST/CON	GST act.,[c] μmol/min per mg protein	ratio TEST/CON	GST act.,[c] μmol/min per mg protein	ratio TEST/CON
control		0.33 ± 0.04		1.15 ± 0.19		0.66 ± 0.08		0.36 ± 0.05			
BHA[a]	7.5	1.08 ± 0.03[d]	3.25	5.24 ± 0.60[d]	4.55	0.84 ± 0.18	1.29	0.45 ± 0.07	1.24		
limonin	5	0.45 ± 0.08[e]	1.33	1.29 ± 0.35	1.12	0.69 ± 0.13	1.05	0.35 ± 0.11	0.98		
limonin	10	0.45 ± 0.09	1.36	1.24 ± 0.23	1.08	0.60 ± 0.12	0.92	0.33 ± 0.04	0.91		
limonin	20	0.49 ± 0.06[f]	1.46	1.42 ± 0.17	1.23	0.68 ± 0.20	1.03	0.34 ± 0.03	0.93		
nomilin	5	1.00 ± 0.03[d]	3.00	2.86 ± 0.60[d]	2.48	0.70 ± 0.16	1.07	0.34 ± 0.07	0.96		
nomilin	10	1.39 ± 0.15[d]	4.17	3.96 ± 0.81[d]	3.44	0.76 ± 0.15	1.16	0.39 ± 0.04	1.07		
nomilin	20	1.50 ± 0.35[d]	4.51	4.36 ± 1.02[d]	3.79	0.94 ± 0.39	1.43	0.32 ± 0.04	0.87		
control		0.75 ± 0.02		2.21 ± 0.21		0.80 ± 0.04		0.50 ± 0.10		0.65 ± 0.03	
nomilin	10	2.09 ± 0.32[d]	2.77	6.05 ± 1.08[d]	2.74	1.04 ± 0.01[d]	1.3	0.51 ± 0.05	1.02	0.77 ± 0.13	1.18
deacetylnomilin	10	0.80 ± 0.10	1.06	3.12 ± 0.62	1.41	0.99 ± 0.20	1.24	0.63 ± 0.05	1.27	0.79 ± 0.05[e]	1.22
obacunone	10	1.24 ± 0.05[d]	1.65	5.55 ± 0.76[d]	2.51	1.01 ± 0.02[d]	1.26	0.50 ± 0.06	1.00	0.75 ± 0.05[e]	1.15
isoobacunoic acid	10	1.26 ± 0.06[d]	1.67	7.77 ± 0.39[d]	3.52	1.17 ± 0.07[d]	1.47	0.52 ± 0.08	1.03	0.73 ± 0.09	1.12
limonol	10	0.89 ± 0.10	1.18	2.65 ± 0.45	1.20	1.01 ± 0.12[e]	1.26	0.48 ± 0.08	0.96	0.66 ± 0.05	1.01
deoxylimonin	10	0.74 ± 0.03	0.98	2.31 ± 0.21	1.05	0.84 ± 0.08	1.06	0.54 ± 0.07	1.08	0.69 ± 0.06	1.05
ichangin	10	1.10 ± 0.20[e]	1.45	4.04 ± 0.69[e]	1.83	1.05 ± 0.02[d]	1.32	0.54 ± 0.10	1.09	0.79 ± 0.07[e]	1.20

[a] 3-*tert*-Butyl-4-hydroxyanisole (Sigma), recrystallized from hexanes–acetone. [b] Limonoids given as fine suspension in 0.3 mL of cottonseed oil; BHA readily soluble in cottonseed oil; control given cottonseed oil only. [c] GST activity determined according to the method of Habig et al. (1974) using CDNB as the substrate. [d] *P* values obtained by the two-tailed Student's *t*-test ($n = 3$); $P < 0.005$. [e] $P < 0.05$. [f] $P < 0.01$.

and deoxylimonin were not active while limonol showed a 26% increase in GST activity. In the cytosols of the lung, no significant increase of GST activity was observed as a result of limonoid treatment. In the colon, slight elevation of enzyme activity was obtained from animals treated with three limonoids, deacetylnomilin, obacunone, and ichangin.

DISCUSSION

In all citrus limonoids, a furan moiety is attached to the D-ring lactone (Dreyer, 1968). Unlike the fused furan structure in cafestol and kahweol (Djerassi et al., 1958), the triterpene nucleus of limonoids is located at the 3-position of the furan function (Dreyer, 1968; Arnott et al., 1960). Therefore, the difference in the arrangement of the furan moiety does not appear to be an important factor in the induction of increased GST activity. At a low dose of 5 mg/intubation, nomilin was able to increase the enzyme activity 3 times above the control level in the small intestinal mucosa. The presence of a furan group, on the other hand, does not ensure high activity as an enzyme inducer. Limonin, which has a structure differing from that of nomilin only on the A-ring, is not an effective compound in this study. Thus, the A- and A′-ring moieties also appear to play a role in the induction of GST activity. The importance of the A- and A′-rings is further illustrated by the activity of ichangin, which has a structure that is similar to that of limonin. Unlike limonin, where the A- and A′-rings are fused, ichangin has an opened A′-ring. This latter structure appears to differ from that of limonin sufficiently to give the compound an overall inducing activity.

The ability of a substance to induce increased activity of the detoxifying enzymes GST has been correlated with its inhibitory action against carcinogens. Green coffee beans and the isolated diterpene esters are potent inducers of GST activity and are effective anticarcinogens against DMBA-induced mammary tumors (Lam et al., 1982; Wattenberg and Lam, 1984). Other natural products and phenolic antioxidants that induce GST activity are also inhibitors that protect laboratory animals from carcinogen-induced tumors (Lam et al., 1979; Wattenberg et al., 1979, 1980; Sparnins and Wattenberg, 1981). The antioxidant BHA used as a positive control in this study is an effective inhibitor against a variety of carcinogens in different animal models (Wattenberg and Lam, 1983). The observation that nomilin is 3 times more active as an enzyme inducer in mouse intestinal mucosa than BHA suggests that this and other limonoids may be effective anticarcinogens. In vivo tumor protection experiments confirmed that nomilin is an inhibitor of benzo[*a*]pyrene-induced neoplasia in the forestomach of mice (Lam and Hasegawa, 1989).

In general, commercial citrus juices contain limonoids at levels below the bitterness threshold of 6 ppm. However, they contain very high concentrations of limonoid glucosides (Hasegawa et al., 1988). Orange juices contain the highest amounts, averaging 320 ppm total limonoid glucosides. Grapefruit juices average 195 ppm. Lemon juices contain the lowest levels, averaging 90 ppm. Recently, at the Pasadena Laboratory, a number of species of bacteria were found to hydrolyze the glucosides of limonoids. Therefore, these glucosides may be hydrolyzed in the intestinal flora to liberate the limonoid aglycones. For this reason, citrus juices may be excellent sources of GST inducing limonoids.

Registry No. Glutathion *S*-transferase, 50812-37-8; nomilin, 1063-77-0; obacunone, 751-03-1; isoobacunoic acid, 751-28-0; ichangin, 10171-61-6; limonin, 1180-71-8; limonol, 989-61-7; deoxylimonin, 989-23-1; deacetylnomilin, 3264-90-2.

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