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Protective effects of baicalein and wogonin against benzo[*a*]pyrene- and aflatoxin B_1-induced genotoxicities

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Abstract

To evaluate the protective effects of baicalein and wogonin against benzo[*a*]pyrene- and aflatoxin (AF) B_1-induced toxicities, the effects of these flavonoids on the genotoxicities and oxidation of benzo[*a*]pyrene and AFB_1 were studied in C57BL/6J mice. Baicalein and wogonin reduced benzo[*a*]pyrene and AFB_1 genotoxicities as monitored by the *umu*C gene expression response in *Salmonella typhimurium* TA1535/pSK1002. Baicalein added *in vitro* decreased liver microsomal benzo[*a*]pyrene hydroxylation (AHH) activity with an ic_{50} of 33.9 ± 1.4 μM at 100 μM benzo[*a*]pyrene. Baicalein also inhibited AFQ_1 and AFB_1-epoxide formation from AFB_1 (50 μM) oxidation (AFO) with ic_{50} values of 22.8 ± 1.4 and 5.3 ± 0.8 μM, respectively. However, the *in vitro* inhibitory effects of wogonin on AHH and AFO activities in liver microsomes were less than those of baicalein as inhibition by 500 μM wogonin was only about 51–65%. Treatment of mice with liquid diets containing 5 mM baicalein and wogonin resulted in 22 and 49% decreases in hepatic AHH activities, respectively. Baicalein treatment resulted in 39 and 32% decreases in AFQ_1 and AFB_1-epoxide formation from liver microsomal AFO, respectively. Wogonin treatment resulted in 39 and 47% decreases in AFQ_1 and AFB_1-epoxide formation, respectively. A 1-week pretreatment with wogonin significantly decreased hepatic DNA adduct formation in mice treated with 200 mg/kg of benzo[*a*]pyrene via gastrogavage. These *in vitro* and *in vivo* effects suggested that baicalein and wogonin might have beneficial effects against benzo[*a*]pyrene- and AFB_1-induced hepatic toxicities and that wogonin had a stronger protective effect *in vivo.*

Keywords: Baicalein; Wogonin; Liver; Benzo[*a*]pyrene; Aflatoxin B_1; Cytochrome P450

1. Introduction

Benzo[*a*]pyrene and AFB_1 are important environmental pollutants, and their tumorigenic effects have been studied extensively in experimental animals [1,2]. Humans are exposed to benzo[*a*]pyrene and related polycyclic aromatic hydrocarbons via the inhalation of industrial and automobile emissions, cigarette smoke, and the consumption of charred food. AFB_1, a secondary metabolite produced by *Asparagillus flavus*, is known to be a potent hepatocarcinogen in experimental animals and probably in humans [3]. Benzo[*a*]pyrene and AFB_1 are of concern because of the large amounts released from the combustion process and the widespread contamination of foodstuffs through mold infestation, respectively. Bioactivation is required for the toxic action of both benzo[*a*]pyrene and AFB_1. Microsomal CYP-dependent monooxygenase consists of CYP hemoproteins, NADPH-CYP reductase, and phospholipids. This monooxygenase is a key enzyme in the metabolic activation of benzo[*a*]pyrene and AFB_1 [4,5]. The epoxide metabolites, benzo[*a*]pyrene 7,8-diol-9,10-epoxide and AFB_1 *exo*-epoxide, formed by CYP-catalyzed oxidation, have been known for their ability to form DNA adducts and lead to tumorigenesis [6,7]. Suppression of their CYP-mediated activation may beneficially reduce the risk of DNA adduct formation.

An important way for protective agents to prevent car-

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Abbreviations: AF, aflatoxin; AFB_1, aflatoxin B_1; AHH, benzo[*a*]pyrene hydroxylation; CYP, cytochrome P450; and AFO, aflatoxin B_1 oxidation.

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cinogenesis is by decreasing the incidence of initiation events that occur during tumor development. Metabolic activation followed by DNA adduct formation is one of the main events during this initiation process [8]. A review of several reports suggests that inhibition of metabolic activation might reduce the tumorigenic risk of exposure to chemical carcinogens [9]. Flavonoids are of interest due to their broad biological activities, including enzyme inhibition, and their antioxidative, hepatoprotective, and tumor-suppressing activities [10,11]. A series of reports revealed that natural flavonoids could modulate benzo[*a*]pyrene and AFB_1 metabolism [12–14]. Elangovan *et al.* [15] reported that treatment of mice with a powdered Hindustan Level Pellet diet containing 1% quercetin and luteolin reduced the incidence of fibrosarcoma induced by 3-methylcholanthrene. This reduction was accompanied by greater reduced levels of lipid peroxides and CYP and increased activity of glutathione *S*-transferase than found in the 3-methylcholanthrene-treated group. Baicalein and wogonin, mainly present as glucuronide conjugates, are the major flavonoids in *Scutellariae radix* that have been commonly used in traditional Chinese medicine. The glucuronides of baicalein and wogonin can constitute up to 20 and 3% of the dry weight of *Scutellariae radix,* respectively [14]. Baicalein and wogonin have been known for their contribution to the pharmacological activity of *Scutellariae radix* [16,17]. It has been shown that baicalein and wogonin significantly decrease liver damage induced by acetaminophen, CCl_4, and β-galactosamine in the rat [16]. Lee *et al.* [18] reported that baicalein inhibits tumor promotion caused by 12-*O*-tetradecanoylphorbol-13-acetate in benzo[*a*]pyrene-initiated CD-1 mouse skin. However, co-administration of baicalein with 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) or 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) showed toxicity in *Salmonella typhimurium* TA100, i.e. in the Ames assay [19,20]. Elliger *et al.* [21] reported that, when measured by the Ames test, wogonin was mutagenic in the presence of NADPH and liver S9 from Aroclor 1254-treated rats. To elucidate the protective effects of baicalein and wogonin, their *in vitro* and *in vivo* effects on the genotoxicities and oxidation of benzo[*a*]pyrene and AFB_1 were analyzed in the present study.

2. Materials and methods

2.1. Chemicals and enzymes

Biacalein and wogonin were isolated from the root of *Scutellariae baicalensis* by the National Research Institute of Chinese Medicine. The purity of the flavonoids was ≥97%, as determined by HPLC and NMR analyses. NADH, NADPH, glucose-6-phosphate, RNase A, 2-nitrophenyl-β-d-galactopyranoside, benzo[*a*]pyrene, glutathione, rat glutathione *S*-transferase (a mixture of α and μ class enzymes), AFB_1, and AFQ_1 were purchased from the Sigma Chemical Co. 3-Hydroxybenzo[*a*]pyrene was purchased from the NCI Chemical Carcinogen Reference Standard Repositories, and proteinase K from Boehringer Mannheim. Acetone, *n*-hexane, and chloroform were obtained from Merck Taiwan Ltd.

2.2. Animal treatment and microsomal and cytosolic preparations

Male C57BL/6J mice (5-weeks-old, weighing 13–15 g) were purchased from the National Laboratory Animal Breeding and Research Center. Before experimentation, mice were allowed a 1-week acclimation period at the air conditioned (25 ± 1^{o}) animal quarters and an automatically controlled photoperiod of 12 hr of light daily. Liquid diets with or without baicalein or wogonin (5 mM) were prepared as described previously [14]. Mice ($N = 6$ per group) were fed *ad lib.*, and their daily dietary intake was monitored. Washed microsomes and cytosol were prepared from mouse liver by differential centrifugation 16 hr after the last feeding [14]. Sera were collected from the hearts of ether-anesthesized mice, and serum alanine transaminase activity was determined using a commercial kit from Abbott Laboratories Ltd. The serum concentrations of the aglycones and glucuronide/sulfate conjugates of baicalein and wogonin after dietary treatments were determined by HPLC [22].

2.3. Genotoxicity analysis

The genotoxicities of baicalein and wogonin in the presence or absence of microsomes or cytosols were determined in *S. typhimurium* TA 1535/pSK1002 following the *umu* genotoxicity assay method [23]. Liver microsomes and cytosols were prepared from untreated mice, and 50 pmol microsomal CYP or 3 mg cytosolic protein was used in this analysis. Flavonoids were dissolved in DMSO and added to the bacterial incubation mixture containing 100 μM benzo[*a*]pyrene or 5 μM AFB_1. The same volume of DMSO was added to the control. The final concentration of DMSO in the incubation medium was less than 1%. Reactions were carried out at 37^{o} for 2 hr. Bacterial growth was monitored by measuring the absorbance at 600 nm. Expressed β-galactosidase activity was determined using 2-nitrophenyl-β-d-galactopyranoside as a substrate. Induction of *umu* gene expression is presented as units of β-galactosidase standardized by bacterial growth. For the *umu* genotoxicity test, a compound that induces β-galactosidase expression to a level higher than two times of the control value is classified as genotoxic [24].

2.4. Enzyme assays

CYP content was determined by the spectrometry method of Omura and Sato [25]. NADPH-CYP reductase activity was determined following the method of Phillips and Langdon [26] using cytochrome *c* as a substrate. AHH

activity was assayed by fluorometric determination of the formation of 3-hydroxybenzo[*a*]pyrene [27]. AFO activity was determined as described previously [5]. Metabolites of AFO were separated and analyzed by HPLC using an Econosphere C18 column (4.6 × 250 mm, Alltech). Formation of AFQ_1 was determined using an external standard. Formation of AFB_1-epoxide was determined as the glutathione conjugate. The glutathione conjugate was analyzed by HPLC and quantified using the extinction coefficient of 21.8 $mM^{-1}cm^{-1}$ for the conjugate [28]. Microsomal protein concentration was determined by the method of Lowry *et al.* [29].

2.5. Benzo[a]pyrene–DNA adduct analysis

Mice were fed a liquid diet containing 5 mM baicalein or wogonin for 1 week. On the last day, the animals were treated with 200 mg/kg of benzo[*a*]pyrene through gastrogavage. Control mice received the liquid diet for 1 week before the administration of benzo[*a*]pyrene. Livers were removed 16 hr after the administration of benzo[*a*]pyrene, and DNA was isolated by phenol/chloroform extraction and ethanol precipitation following the method of Gupta [30]. The quality of DNA was analyzed by the absorbance ratio of A_{260}/A_{280} (≥1.8). Benzo[*a*]pyrene–DNA adduct formation was analyzed by ^{32}P-postlabeling using DNA modified by benzo[*a*]pyrene 7,8-diol-9,10-epoxide as a standard [31].

2.6. Data and statistical analysis

The ic_{50} values of flavonoids for monooxygenase activities were calculated by curve fitting (Grafit, Erithacus Software Ltd.). The statistical significance of differences between the control and treated groups was evaluated by Student's *t*-test. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Inhibition of the genotoxicities of benzo[a]pyrene and AFB_1

Before assessing their protective effects, the genotoxicities of baicalein and wogonin were determined. Baicalein and wogonin at a concentration of 300 μM or less were not genotoxic in the presence of mouse liver microsomes (Fig. 1A). In addition, genotoxicity was not detected in the presence of cytosol or a mixture of cytosol and microsomes (data not shown). Baicalein and wogonin at 10, 50, and 100 μM had no effect on bacterial growth as monitored by measuring the absorbance of the bacterial suspension at 600 nm. However, 300 μM baicalein significantly decreased bacterial growth (Fig. 1B). In this test system, benzo[a]pyrene (100 μM) was mildly genotoxic, whereas AFB_1 (5 μM) was a relatively strong genotoxic agent (Fig. 2). The

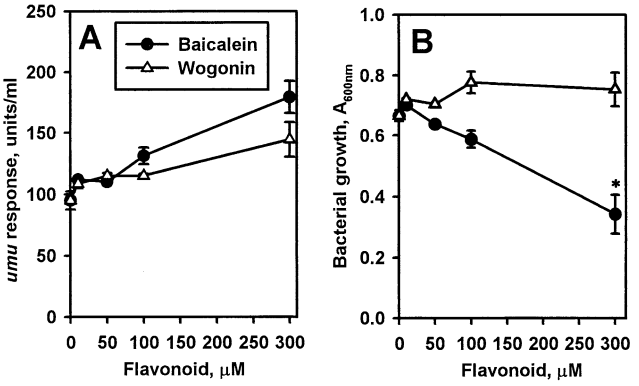

Fig. 1. Genotoxicities and effects of baicalein and wogonin on *S. typhimurium* growth in the *umu* test system. Genotoxicity (panel A) was determined in *S. typhimurium* TA1535/pSK1002 in the presence of liver microsomes prepared from untreated mice. Bacterial suspensions were incubated with chemicals, microsomes, and an NADPH-generating system at 37° for 2 hr. The same volume of DMSO was added in the incubation mixtures as was used in the controls. After incubation, bacterial growth (panel B) was monitored by measuring absorbance at 600 nm. Expressed β-galactosidase activity was determined using 2-nitro-β-d-galactopyranoside as a substrate. Data represent means ± SEM of three separate experiments with duplicates. Key: (*) significantly different from the control, $P < 0.05$.

AFB_1-induced expression of β-galactosidase was 11-fold higher than in the uninduced control. In the presence of 50 μM baicalein and wogonin, benzo[*a*]pyrene genotoxicity was suppressed to a level close to the control value obtained from the incubation in the absence of benzo[*a*]pyrene (Figs. 1 and 2). Baicalein at 50 μM dramatically decreased the genotoxicity of AFB_1 and at 300 μM decreased this toxicity to a level approaching the control value. Wogonin also decreased the genotoxicity of AFB_1, but showed less suppressive effects than baicalein (Fig. 2).

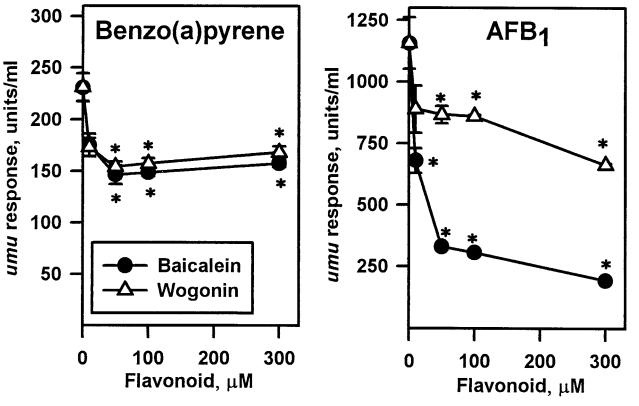

Fig. 2. Inhibitory effects of baicalein and wogonin on the genotoxicities of benzo[*a*]pyrene (left panel) and AFB_1 (right panel) as monitored by β-galactosidase expression in *S. typhimurium* TA1535/pSK1002. Bacterial incubation was carried out at 37° for 2 hr, and β-galactosidase activity was determined using 2-nitrophenyl-β-d-galactopyranoside as a substrate. Data represent the means ± SEM of three separate experiments with duplicates. Key: (*) significantly different from the control, $P < 0.05$.

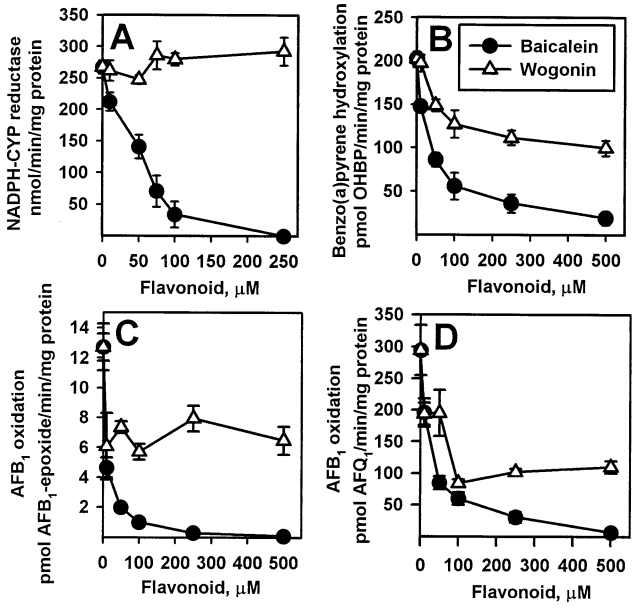

Fig. 3. In vitro effects of baicalein and wogonin on NADPH-cytochrome P450 reductase, and on AHH and AFO activities of mouse liver microsomes. AHH and AFO activities were determined using 100 μM benzo[*a*]pyrene and 50 μM AFB_1, respectively. Formation of AFB_1-epoxide was determined as the glutathione conjugate, using rat glutathione *S*-transferase. Data represent the means ± SEM of three mice.

3.2. Effects of baicalein and wogonin on NASPH-CYP reductase, AHH, and AFO activities of mouse liver microsomes in vitro

In vitro, the addition of baicalein inhibited NADPH-CYP reductase activity toward cytochrome *c* with an ic_{50} of 39 ± 9 μM, whereas wogonin had no effect (Fig. 3A). Both baicalein and wogonin decreased microsomal AHH and AFO activities, but baicalein had stronger inhibitory effects than wogonin (Fig. 3, B–D). Baicalein and wogonin at 500 μM inhibited AHH activity by 90 and 51%, respectively (Fig. 3B). Baicalein inhibited AHH activity with an ic_{50} of 34 ± 1 μM at 100 μM benzo[*a*]pyrene. AFQ_1 and the glutathione conjugate of AFB_1-epoxide were detected in the AFO assay of liver microsomes. The ic_{50} values of baicalein for AFQ_1 and AFB_1-epoxide were 23 ± 1 and 5 ± 1 μM at 50 μM AFB_1, respectively (Fig. 3, C and D). In contrast, the inhibition by wogonin, up to 500 μM, was not sufficient for ic_{50} estimation.

3.3. Dietary effects of baicalein and wogonin on AHH and AFO activities in mouse liver

In general, humans take herbal medicine orally. Therefore, mice were fed liquid diets containing 5 mM baicalein or wogonin. Flavonoid treatments had no effect on diet consumption and body and liver weights as compared to the control group (data not shown). There were no detectable baicalein and wogonin levels in the sera of flavonoid-treated mice. After the treatment of sera with glucuronidase/sulfatase, 45 ± 4 and 23 ± 7 μM concentrations of baicalein and wogonin were found in baicalein- and wogonin-treated groups, respectively. Treatment of mice with baicalein and wogonin resulted in 22 and 49% decreases in hepatic AHH activities, respectively (Table 1). Baicalein treatment resulted in 39 and 32% decreases in AFQ_1 and AFB_1-epoxide formation, respectively. Wogonin treatment resulted in 39 and 47% decreases in AFB_1-epoxide and AFQ_1 formation, respectively.

Table 1
Dietary effects of baicalein and wogonin on benzo[*a*]pyrene hydroxylation and AFB_1 oxidation activities in mouse liver

Treatment	Benzo[*a*]pyrene hydroxylation (pmol/min/mg protein)	AFB_1 oxidation (pmol product formation/min/mg protein)	
		AFQ_1	AFB_1-epoxide[a]
Control	380 ± 26	506 ± 63	10.9 ± 1.3
Baicalein	295 ± 14*	308 ± 15*	7.4 ± 0.5*
Wogonin	193 ± 19*	308 ± 28*	5.8 ± 1.1*

Mice were administered liquid diets containing 5 mM flavonoids for 1 week. Control mice received a control diet. Liver microsomes were prepared at 16 hr after the last feeding. Data represent means ± SEM of six mice.

[a]Formation of AFB_1-epoxide was traced as the glutathione conjugate using rat glutathione *S*-transferase.

*Significantly different from the control, $P < 0.05$.

3.4. Dietary effects of baicalein and wogonin on benzo[a]pyrene–DNA adduct formation in mouse liver

Due to the potent hepatotoxicity of AFB_1, we selectively determined the dietary effects of baicalein and wogonin on hepatic benzo[*a*]pyrene–DNA adduct formation. Chromatograms of ^{32}P-postlabeling analyses of DNA adducts are shown in Fig. 4. The control group received a control diet and then were treated with benzo[*a*]pyrene (Fig. 4B). One-week pretreatment of mice with wogonin significantly decreased the hepatic benzo[*a*]pyrene–DNA adduct level to 24% of the control (Fig. 4, B and D, Table 2). However, baicalein treatment had no effect on the benzo[*a*]pyrene–DNA adduct level (Fig. 4, B and C, Table 2).

4. Discussion

Flavonoids are widely distributed in the plant kingdom, including vegetables, fruits, and traditional Chinese herbal drugs. Although the bioavailability of flavonoids is low, the concentration *in vivo* is enough to evoke a pharmacological effect [32]. Baicalein and wogonin are the main active flavonoids of *Scutellariae radix*, which is one of the main constituents of a Kampo medicine, Sho-saiko-to. The water extract of *Scutellariae radix* suppressed the mutagenic activity of benzo[*a*]pyrene in *S. typhimurium* TA98 and

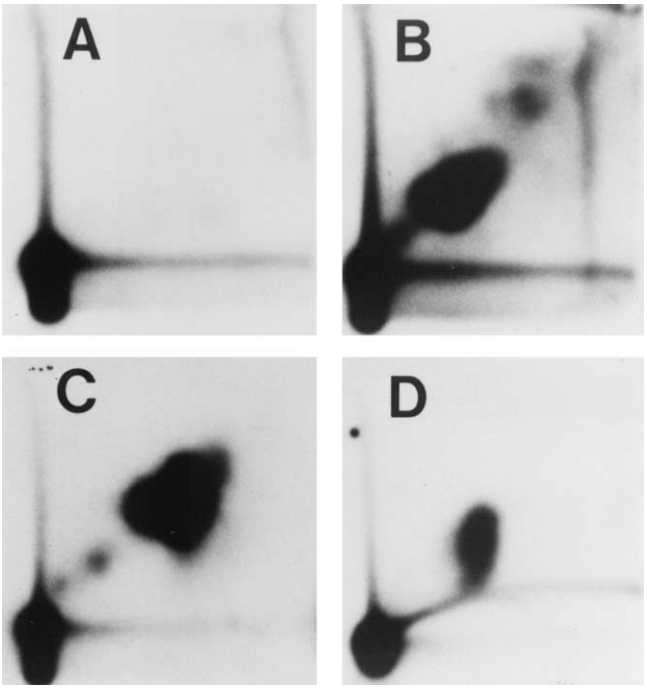

Fig. 4. Autoradiograms of benzo[*a*]pyrene–DNA adducts in mouse liver by ^{32}P-postlabeling assay. Panels (A) and (B) show the chromatograms of DNA isolated from mice fed a control diet for 1 week without and with 200 mg/kg benzo[*a*]pyrene treatment, respectively. Panels (C) and (D) show the chromatograms of DNA isolated from mice treated with benzo-[*a*]pyrene after 1-week treatments with diets containing 5 mM baicalein and wogonin, respectively. Autoradiography was carried out at –70° for 18 hr.

TA100 [33]. The water extract of *Scutellariae baicalensis* also decreased the DNA binding and metabolism of benzo-[*a*]pyrene and AFB_1 activated by Aroclor 1254-induced rat hepatic S9 [34]. These reports indicated the possible beneficial effects of the crude extract of *Scutellariae baicalensis.* Lee *et al.* [35] reported that baicalein suppresses the mutagenicity of AFB_1 in *S. typhimurium* and reduces chromosome aberration induced by AFB_1 in CHL cells. Our results showed that the addition of baicalein and wogonin decreased the genotoxicities of benzo[*a*]pyrene and AFB_1 as monitored by the *umu*C expression response in *S. typhimurium* TA1535/pSK1002 (Fig. 1). These results suggested that baicalein and wogonin produce protective effects against benzo[*a*]pyrene- and AFB_1-induced toxicities. Our results also showed that baicalein and wogonin decreased microsomal AHH and AFO activities *in vitro* and *in vivo* (Fig. 2 and Table 1). The DNA adduct formation induced by benzo[*a*]pyrene was reduced by the dietary intake of wogonin (Fig. 4D, Table 2). Lee *et al.* [18] reported that topical treatment of mouse skin with baicalein dramatically decreased the number of skin tumors in benzo[*a*]pyrene-initiated mouse skin. Although tissue-specific biotransformation and different treatment regimens may result in differential effects, this report demonstrates that baicalein has a chemopreventive role in benzo[*a*]pyrene carcinogenesis. Our *in vitro* and *in vivo* results together suggest that administration of baicalein and wogonin may possibly have beneficial effects against the carcinogenic risk caused by benzo-[*a*]pyrene and AFB_1. The daily dose of Sho-saiko-to in humans is 7.5 g. A 7.5-g sample of Sho-saiko-to contains the boiled water extract of 3 g *Scutellaria radix.* The amounts (w/w) of baicalin (baicalein-glucuronide), baicalein, and wogonin in Sho-saiko-to were 3.5, 0.3, and 0.04%, respectively [36]. The human daily intake (mg/kg/day) of baicalein and wogonin from Sho-saiko-to is relatively low (roughly about 0.2 and 0.01% of the dosage used in the present mouse study, respectively [14,36]). The serum concentrations of baicalein and wogonin in human-ingested Sho-saiko-to are unclear. In human urine, the accumulated excreted amounts of baicalein and wogonin were found to be 1.5 and 1.4 mg after the administration of a single dose of 5 g Sho-saiko-to [37]. Although the direct extrapolation from a mouse study to humans is difficult, our results, together with previous reports, indicate the contribution of baicalein and wogonin in the liver protective effects of *Scutellariae baicalensis.*

Table 2
Dietary effects of baicalein and wogonin on benzo[*a*]pyrene–DNA adduct formation in mouse liver

Treatment	Relative adduct labeling (^2N-guanyl adducts/10^7 nucleotides)
Control	8.8 ± 0.9(5)
Baicalein	12.2 ± 1.5(4)
Wogonin	2.1 ± 0.3*(4)

Mice were pretreated with either a control diet or liquid diets containing 5 mM baicalein or wogonin for 1 week. At the final treatment, mice were administered 200 mg/kg of benzo[*a*]pyrene for 16 hr. Livers were removed, DNA was isolated, and adduct formation was analyzed by a ^{32}P-postlabeling assay. Data represent means ± SEM; the number of mice per group is indicated in parentheses.

*Significantly different from the control, $P < 0.05$.

In comparison, baicalein had stronger inhibitory effects than wogonin on AHH and AFO activities *in vitro* (Fig. 3, B–D). Baicalein also had a stronger suppressive effect than wogonin on AFB_1 genotoxicity. However, the genotoxic response induced by benzo[*a*]pyrene was suppressed by baicalein and wogonin to similar extents (Fig. 2). The reason for the lack of difference in the inhibitory effects of baicalein and wogonin on benzo[*a*]pyrene genotoxicity is unclear, but the mild toxicity of benzo[*a*]pyrene detected in the *umu* test may limit the detection of differences (Fig. 2). To examine the differences of inhibition properties between these two flavonoids, CYP-flavonoid binding spectra and the effects of flavonoids on NADPH-CYP reductase activity were determined using untreated mouse liver microsomes. There were no binding spectra formed by CYP and these flavonoids (data not shown). Baicalein strongly inhibited NADPH-CYP reductase activity, whereas wogonin up to 250 μM had no effect (Fig. 3A). The potent inhibition of baicalein on reductase activity may contribute, at least in

part, to its stronger inhibitory effect on AHH and AFO activities *in vitro,* compared to wogonin. Buening *et al.* [12] reported that other flavonoids also showed inhibitory effects on benzo[*a*]pyrene and AFB_1 oxidations in human liver microsomes and the inhibition of NADPH-CYP reductase activity may be involved in their inhibitory action. However, Sato *et al.* [38] reported that wogonin could suppress the non-heme iron reduction by NADPH-CYP reductase in rat liver microsomes. The reduction of non-heme iron is different from the reduction of CYP or cytochrome *c* hemoproteins. The ferric chloride reduction of microsomes in the presence of NADPH needs a chelator and a microsomal protein(s) other than CYP and NADPH-CYP reductase [39]. Thus, the effects of wogonin on the reduction ability of NADPH-CYP reductase toward different substrates may be different. To clarify the effects of flavonoids on electron transfer in the CYP-catalytic cycle, further study on the effects of flavonoids on CYP reduction is required.

Consistent with the inhibitory effects *in vitro,* our *in vivo* study showed that baicalein and wogonin treatments significantly decreased AHH and AFO activities in mouse liver (Table 1). In contrast to the stronger inhibition by baicalein *in vitro,* baicalein had a smaller inhibitory effect than wogonin on AHH activity *in vivo.* Our results show that baicalein treatment caused a 22% decrease of AHH activity (Table 1). This decrease may not be strong enough to cause detectable changes in the number of hepatic benzo[*a*]pyrene–DNA adducts in mice treated with 200 mg/kg of benzo[*a*]pyrene (Fig. 4 and Table 2). In contrast, wogonin significantly reduced benzo[*a*]pyrene–DNA adduct formation. The actual reason for this discrepancy between the *in vitro* and *in vivo* effects is not clear. However, the influence of pharmacokinetic, pharmacodynamic, and other regulatory factors *in vivo* might be possible causes of this discrepancy. In general, the aglycone of flavonoids was thought to be their biological active form. After digestion, baicalein and wogonin are metabolized mainly to glucuronide and sulfate conjugates in rats and humans [37,40]. Our determinations show that there were no free baicalein and wogonin detected in sera from flavonoid-treated mice. The serum concentration of the conjugates of baicalein was 1-fold higher than the conjugates of wogonin. The potency of inhibition by flavonoids *in vivo* was not correlated with the serum concentration of metabolites. Baicalein and wogonin treatments suppressed hepatic AFO activity with similar potencies. AFB_1 can be oxidized by mouse liver microsomal CYP to form the detoxication metabolite, AFQ_1, and the potent mutagenic metabolite, AFB_1-epoxide. Although baicalein and wogonin decreased the formation of both AFQ_1 and AFB_1-epoxide, there is still a beneficial effect of ingestion of flavonoids. Since AFB_1-epoxide formation is the main cause of AFB_1-induced genotoxicity, the decreased formation rate of the epoxide may reduce the incidence of DNA adduct formation and possibly the subsequent tumorigenic effect. Reduction of AFO activity may also provide a better chance for the conjugation reaction with the glutathione pool.

There is controversy regarding the importance of human CYP1A2 and CYP3A4 in the activation of AFB_1 at the concentrations to which humans could possibly be exposed [5,41]. Using the CYP inhibitors troleandomycin and furafylline, Gallagher *et al.* [41] demonstrated that CYP1A2 was the main CYP responsible for the activation of 16 μM AFB_1 in human liver microsomes and lymphoblastoid cell lines expressing human CYPs. Using reconstituted systems of recombinant human CYPs, Ueng *et al.* [5] demonstrated that CYP3A4 appeared to be more active than CYP1A2 in the oxidation of AFB_1 to form the most potent mutagen, AFB_1 *exo*-epoxide. At 10 μM AFB_1, higher genotoxicity was activated by CYP3A4 than by CYP1A2 at various CYP concentrations. Our previous report showed that baicalein and wogonin decreased CYP3A-catalyzed nifedipine oxidation and erythromycin *N*-demethylation activities to a similar extent in mouse liver [14]. However, baicalein treatment increased CYP1A2-catalyzed oxidations. The significance of CYP1A2 induction by baicalein on AFB_1 toxicity needs further study. In mice, CYP2A is an important CYP in the activation of AFB_1 [2]. However, the effect of these flavonoids on CYP2A was not clear. For benzo[*a*]pyrene, CYP1A2 and CYP3A are the main hepatic CYPs involved in the oxidation of benzo[*a*]pyrene [4]. Our previous report showed that both baicalein and wogonin decrease the level of CYP3A protein. In contrast, CYP1A2 protein level is increased by baicalein but decreased by wogonin [14]. The up-regulation of CYP1A2 by baicalein may cause the smaller inhibition of microsomal AHH activity by baicalein than by wogonin *in vivo.* To better understand the differential modulatory effects of flavonoids, further studies are required to investigate the regulatory mechanism of flavonoids and the effects of flavonoids on human CYPs and NADPH-CYP reductase.

Herbal medicines have attracted great attention for their protective effects but several reports suggest the mutagenicity and hepatotoxicity of some natural products [21,42]. It is important to assess the toxicity when evaluating the protective effects of natural products. Elliger *et al.* [21] reported that wogonin showed mutagenic effects in the Ames test in the presence of liver S9 prepared from Aroclor 1254-treated rats. This mutagenic effect was not diminished when microsomes were removed from the S9 by centrifugation. Our results also showed that baicalein and wogonin were not genotoxic in *S. typhimurium* TA1535/pSK1002 in the presence of microsomes and NADPH (Fig. 1). However, there were also no genotoxicities detected in the presence of cytosol or a mixture of cytosol and microsomes instead of microsomes (data not shown). The difference in the toxicity detected might be due to the different bacterial test systems used. Our previous report showed that a 1-week treatment of a diet containing wogonin had no effects on mouse body and liver weights [14]. We have also determined mouse serum alanine aminotransferase activity, and there was no

difference between control (38 ± 4 IU/L) and flavonoid-treated groups (baicalein-treated group: 50 ± 5 IU/L; wogonin-treated group: 42 ± 6 IU/L). These results indicated that baicalein and wogonin were not hepatotoxic at the dosage for protection against benzo[*a*]pyrene and AFB_1 toxicities. However, baicalein at 300 μM showed an anti-bacterial effect (Fig. 1). This result was consistent with an earlier report that baicalin inhibits the growth of bacteria, including *Staphylococcus aureus* and *Pseudomonas aeruginosa*, at 474 μM [43]. The reason for the decreased bacterial growth is not clear, but possible causes include cytotoxicity, delayed growth, and arrested growth. In human colon cancer cell lines, baicalein was found to arrest cell growth without cytotoxicity [44]. However, the inhibition in the cell lines may be different from the inhibition of bacteria. To evaluate the anti-bacterial effect, the actual cause and the bacterial selectivity of this inhibition need further investigation. Although our determination of serum concentration of flavonoid metabolites was low, local high concentrations of baicalein in the intestine after digestion might cause anti-bacterial effects. In addition, our previous report suggested that ingestion of baicalein and wogonin affected the activities of phase I and phase II drug-metabolizing enzymes [14]. Therefore, attention should be paid to the possible adverse effects and flavonoid–drug interactions, particularly after long and continuous ingestion.

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References

[1] Collins JF, Brown JP, Dawson SV, Marty MA. Risk assessment for benzo(a)pyrene. Regul Toxicol Pharmacol 1991;13:170–84.
[2] Eaton DL, Gallagher EP. Mechanisms of aflatoxin carcinogenesis. Annu Rev Pharmacol Toxicol 1994;34:135–72.
[3] Qian GS, Ross RK, Yu MC, Yuan JM, Gao YT, Henderson BE, Wogan GN, Groopman JD. A follow-up study of urinary markers of aflatoxin exposure, and liver cancer risk in Shanghai, People's Republic of China, Cancer Epidemiol Biomarkers Prev 1994;3:3–10.
[4] Bauer E, Guo Z, Ueng YF, Bell C, Zeldin D, Guengerich FP. Oxidation of benzo(a)pyrene by recombinant human cytochrome P450 enzymes. Chem Res Toxicol 1995;8:136–42.
[5] Ueng Y-F, Shimada T, Yamazaki H, Guengerich FP. Oxidation of aflatoxin B_1 by bacterial recombinant human cytochrome P450 enzymes. Chem Res Toxicol 1995;8:218–24.
[6] Conney AH, Chang RL, Jerina DM, Wei S-JC. Studies on the metabolism of benzo[a]pyrene and dose-dependent differences in the mutagenic profile of its ultimate carcinogenic metabolite. Drug Metab Rev 1994;26:125–63.
[7] Bechtel DH. Molecular dosimetry of hepatic aflatoxin B_1-DNA adducts: linear correlation with hepatic cancer risk. Regul Toxicol Pharmacol 1989;10:74–81.
[8] Melendez-Colon VJ, Luch A, Seidel A, Baird WM. Cancer initiation by polycyclic aromatic hydrocarbons results from formation of stable DNA adducts rather than apurinic sites. Carcinogenesis 1999;20:1885–91.
[9] Wattenberg LW. Chemoprevention of cancer. Cancer Res 1985;45:1–8.
[10] Capasso F. Flavonoids: old, and new aspects of a class of natural therapeutic drugs. Life Sci 1999;65:337–53.
[11] Guengerich FP, Kim DH. *In vitro* inhibition of dihydropyridine oxidation and aflatoxin B_1 activation in human liver microsomes by naringenin, and other flavonoids. Carcinogenesis 1990;11:2275–9.
[12] Buening MK, Chang RL, Huang MT, Fortner JG, Wood AW, Conney AH. Activation and inhibition of benzo(a)pyrene and aflatoxin B_1 metabolism in human liver microsomes by naturally occurring flavonoids. Cancer Res 1981;41:67–72.
[13] Ueng YF, Chang YL, Oda Y, Park SS, Liao JF, Lin MF, Chen CF. *In vitro*, and *in vivo* effects of naringin on cytochrome P450-dependent monooxygenase in mouse liver. Life Sci 1999;65:2591–602.
[14] Ueng Y-F, Shyu C-C, Lin Y-L, Park SS, Liao J-F, Chen C-F. Effects of baicalein and wogonin on drug-metabolizing enzymes in C57BL/6J mice. Life Sci 2000;67:2189–200.
[15] Elangovan V, Sekar N, Govindasamy S. Chemopreventive potential of dietary bioflavonoids against 20-methylcholanthrene-induced tumorigenesis. Cancer Lett 1994;87:107–13.
[16] Lin CC, Shieh DE. *In vivo* hepatoprotective effect of baicalein, and wogonin from *Scutellariae rivularis.* Phytother Res 1996;10:651–4.
[17] Kubo M, Matsuda H, Tani T, Arichi S, Kimura Y, Okuda H. Studies on Scutellariae radix. XII. Anti-thrombic actions of various flavonoids from Scutellariae radix. Chem Pharm Bull (Tokyo) 1985;33:2411–5.
[18] Lee M-J, Wang C-J, Tsai Y-Y, Hwang J-M, Lin W-L, Tseng T-H, Chu C-Y. Inhibitory effect of 12-*O*-tetradecanoylphorbol-13-acetate-caused tumor promotion in benzo[a]pyrene-initiated CD-1 mouse skin by baicalein. Nutr Cancer 1999;34:185–91.
[19] Edenharder R, von Petersdorff I, Rauscher R. Antimutagenic effects of flavonoids, chalcones and structurally related compounds on the activity of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and other heterocyclic amine mutagens from cooked food. Mutat Res 1993;287:261–74.
[20] Ohtsuka M, Fukuda K, Yano H, Kojiro M. Effects of nine active ingredients in Chinese herbal medicine Sho-saiko-to on 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide mutagenicity. Jpn J Cancer Res 1995;86:1131–5.
[21] Elliger CA, Henika PR, MacGregor JT. Mutagenicity of flavones, chromones and acetophenones in *Salmonella typhimurium.* New structure-activity relationships. Mutat Res 1984;135:77–86.
[22] Tsai TH, Chou CJ, Tsai TR, Chen CF. Determination of wogonin in rat plasma by liquid chromatography and its pharmacokinetic application. Planta Med 1996;62:263–6.
[23] Shimada T, Oda Y, Yamazaki H, Mimura M, Guengerich FP. Gene and chromosome analysis. In: Adolph KW, editor. Methods in molecular genetics, vol. 5. Orlando, FL: Academic Press, 1994. p. 342–55.
[24] Reifferscheid G, Heil J. Validation of the SOS/*umu* test using results of 486 chemicals and comparison with Ames test and carcinogenicity data. Mutat Res 1996;369:129–45.
[25] Omura T, Sato R. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemeprotein nature. J Biol Chem 1964;239:2370–9.
[26] Phillips AH, Langdon RG. Hepatic triphosphopyridine nucleotide-cytochrome *c* reductase: Isolation, characterization, and kinetic studies. J Biol Chem 1962;237:2652–60.
[27] Nebert DW, Gelboin HV. Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. I. Assembly and properties of induced enzyme. J Biol Chem 1968;243:6242–9.
[28] Raney KD, Meyer DJ, Ketterer B, Harris TM, Guengerich FP. Glutathione conjugation of aflatoxin B_1 *exo*- and *endo*-epoxide by rat and human glutathione S-transferases. Chem Res Toxicol 1992;5:470–8.

[29] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[30] Gupta RC. Nonrandom binding of the carcinogen *N*-hydroxy-2-acetylaminofluorene to repetitive sequences of rat liver DNA *in vivo*. Proc Natl Acad Sci USA 1984;81:6943–7.
[31] Liu T-Y, Chao T-W, Chiang S-H, Chi C-W. Differential sensitivity of human hepatoma cell line and primary rat hepatocyte culture to benzo(a)pyrene-induced unscheduled DNA synthesis and adduct formation. Cell Biol Int 1993;17:441–7.
[32] Hollman PCH, Katan MB. Dietary flavonoids: intake, health effects, and bioavailability. Food Chem Toxicol 1999;37:937–42.
[33] Sakai Y, Nagase H, Ose Y, Sato T, Kawai M, Mizuno M. Effects of medicinal plant extracts from Chinese herbal medicines on the mutagenic activity of benzo[*a*]pyrene. Mutat Res 1988;206:327–34.
[34] Wong BYY, Lau BHS, Yamasaki T, Teel RW. Modulation of cytochrome *P*-450IA1-mediated mutagenicity, DNA binding and metabolism of benzo[*a*]pyrene by Chinese medicinal herbs. Cancer Lett 1993;68:75–82.
[35] Lee BH, Lee SJ, Kang TH, Kim DH, Sohn DW, Ko GI, Kim YC. Baicalein, an *in vitro* antigenotoxic compound from *Scutellaria baicalensis*, Planta Med 2000;66:70–1.
[36] Shimizu I. Current status of treatment of hepatobiliary disorders in Japan. Sho-saiko-to: Japanese herbal medicine for protection against hepatic fibrosis and carcinoma. J Gastroenterol Hepatol 2000;15: D84–90.
[37] Li C, Homma M, Oka K. Characteristics of delayed excretion of flavonoids in human urine after administration of Shosaiko-to, a herbal medicine. Biol Pharm Bull 1998;21:1251–7.
[38] Sato T, Kawamoto A, Tamura A, Tatsumi Y, Fujii T. Mechanism of antioxidant action of pueraria glycoside (PG-1) (an isoflavonoid) and mangiferin (a xanthonoid). Chem Pharm Bull (Tokyo) 1992;40:721–4.
[39] Tampo Y, Yonaha M. A microsomal membrane component associated with iron reduction in NADPH-supported lipid peroxidation. Lipids 1995;30:55–62.
[40] Abe K-i, Inoue O, Yumioka E, Biliary excretion of metabolites of baicalin and baicalein in rats. Chem Pharm Bull (Tokyo) 1990;38: 208–11.
[41] Gallagher EP, Wienkers LC, Stapleton PL, Kunze KL, Eaton DL. Role of human microsomal and human complementary DNA-expressed cytochrome P4501A2 and P4503A4 in the bioactivation of aflatoxin $B_{1.}$ Cancer Res 1994;54:101–8.
[42] Koff RS. Herbal hepatotoxicity: revisiting a dangerous alternative. JAMA 1995;273:502.
[43] Ng TB, Ling JML, Wang ZT, Cai JN, Xu GJ. Examination of coumarins, flavonoids and polysaccharopeptide for antibacterial activity. Gen Pharmacol 1996;27:1237–40.
[44] Kuntz S, Wenzel U, Daniel H. Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines. Eur J Nutr 1999;38:133–42.