

Regular article

Chemopreventive Effects of Nobiletin against Genotoxicity Induced by 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in the Lung of *gpt* delta Transgenic Mice

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Nobiletin, a major component of citrus polymethoxyflavones, possesses anticancer, antiviral and anti-inflammatory activities. To evaluate the chemopreventive potential against lung cancer induced by cigarette smoke, we examined suppressive effects of nobiletin against genotoxicity induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), the most carcinogenic tobacco-specific nitrosamine, in the lung of *gpt* delta transgenic mice. Male and female *gpt* delta mice were fed nobiletin at a dose of 100 or 500 ppm in diet for seven days and treated with NNK at a dose of 2 mg/mouse/day, i.p. for four consecutive days. Dietary administration of nobiletin continued at the doses during the NNK treatments and in the following period before sacrifice at day 38. NNK treatments enhanced the *gpt* mutant frequency (MF) in the lung 19- and 9-fold, respectively, over the values of untreated female and male mice. Interestingly, nobiletin reduced the NNK-induced MFs by 25–45% in both sexes and the reduction at a dose of 100 ppm in females and 500 ppm in males was statistically significant ($P < 0.05$). To further characterize the suppressive effects, we conducted bacterial mutation assay with *Salmonella typhimurium* YG7108 to examine whether nobiletin inhibits S9-mediated genotoxicity of NNK. Nobiletin as well as 8-methoxypsoralen, an inhibitor of CYP2A, reduced the genotoxicity of NNK by more than 50%. These results suggest that nobiletin may be chemopreventive against NNK-induced lung cancer and also that the chemopreventive efficacy may be due to inhibition of certain CYP enzymes involved in the metabolic activation of NNK.

Key words: nobiletin, NNK, chemoprevention, cigarette smoking, *gpt* delta transgenic mice

Introduction

Humans are exposed to a variety of exogenous and endogenous genotoxic agents. Of various hazardous environmental factors, cigarette smoke may be the most

causative factor associated with the incidence of human cancer (1). Although cigarette smoke contains more than 4,000 compounds including 40 known human carcinogens, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (nicotine-derived nitrosamino ketone, NNK) is the most carcinogenic tobacco-specific nitrosamine (2,3). NNK is estimated to be present at levels of 17–430 and 390–1,440 ng, respectively, per cigarette in mainstream and sidestream of cigarette smoke (3). NNK induces lung tumors in rats, mice and hamsters and is classified into Class 2B carcinogen (possibly carcinogenic to humans) by International Agency for Research on Cancer (4). NNK is metabolically activated by CYP (P-450) enzymes, and the metabolites generate methylated and pyridyloxobutylated DNA, which can induce G:C-to-A:T and G:C-to-T:A mutations, respectively. *O*⁶-Methylguanine in the lung may be a causative lesion of NNK leading to activation of *Ki-ras* proto-oncogene, an initiation of tumor development (5,6).

With smoking the major etiological factor for lung cancer, a number of naturally occurring and synthetic chemicals have been proposed as candidates of chemopreventive agents to protect smokers who are unwilling or unable to quit smoking. Examples of the candidates include inhibitors of metabolic activation of NNK, e.g., phenethyl isothiocyanate and curcumins (7–10), enhancers of detoxication enzymes, e.g., prodrugs of L-selenocystein (11), antioxidants, e.g., vitamin E and carotenoids (12,13) and inhibitors of signal transduction downstream from the activated oncogenes, e.g., perillyl alcohol and deguelin (14,15).

Nobiletin (5,6,7,8,3',4'-hexamethoxyflavone) is a

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polymethoxyflavone found in *Citrus depressa* Rutaceae, a popular citrus fruit in Okinawa, Japan (16). Interestingly, nobiletin seems to possess anticancer activities by inhibiting critical steps of carcinogenesis, i.e., initiation (13,17), promotion (18,19) and metastasis (16,20,21). In addition, nobiletin inhibits the P-glycoprotein drug efflux transporter, suggesting the ability to reverse multi-drug resistance of tumor cells (22).

To evaluate the chemopreventive efficacy against lung cancer induced by cigarette smoke, we examined suppressive effects of dietary administration of nobiletin in the lung of *gpt* delta mice treated with NNK. In this mouse model, base substitutions such as G:C-to-A:T or G:C-to-T:A can be detected by *gpt* selection. In fact, Miyazaki *et al.* (23) have employed the mice to demonstrate the chemopreventive effects of 8-methoxypsoralen against NNK-induced mouse lung adenoma. Besides *in vivo* genotoxicity assays, we conducted a bacterial mutation assay with *Salmonella typhimurium* YG7108 to examine whether nobiletin inhibits the genotoxicity of NNK in the presence of S9 metabolic activation system. The bacterial strain lacks *O*⁶-methylguanine methyltransferase activity, so that it is highly sensitive to base substitution mutations by NNK and other alkylating agents (24,25). The results suggest that nobiletin clearly suppresses the genotoxicity of NNK *in vivo* and *in vitro*. We discuss the mechanisms underlying the suppressive effects and the possible usage of nobiletin as a chemopreventive agent against lung cancer induced by cigarette smoke.

Material and Methods

Materials: Nobiletin (>99.9% purity) was chemically synthesized according to the method described by Tsukayama *et al.* (26) with slight modifications. Sources of other chemicals used in this study are as follows: NNK, Toronto Research Chemicals (Toronto, Canada); benzo[a]pyrene (BP), Wako Pure Chemicals (Osaka, Japan); 8-methoxypsoralen and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), Sigma-Aldrich Japan K. K. (Tokyo, Japan). S9 prepared from male Sprague-Dawley rats pretreated with phenobarbital and 5,6-benzoflavone was purchased from Kikkoman Cooperation, Chiba, Japan.

Treatment of *gpt* delta mice: Male and female *gpt* delta C57BL/6J transgenic mice, obtained from Japan SLC, Inc. (Shizuoka, Japan), were maintained in Animal Facility of Kanazawa Medical University, according to the institutional animal care guidelines. The animals were housed in plastic cages with free access to tap water and powdered basal diet CRF-1 (Oriental Yeast, Tokyo, Japan) under controlled conditions of temperature at $23 \pm 2^\circ\text{C}$, humidity of 10% and lighting (12 h light-dark cycle). Twenty female and 25 male *gpt* delta mice were each divided into four

experimental and one control groups (Fig. 1). When the mice were 8 weeks of age, they were fed diet supplemented with nobiletin at a concentration of 100 ppm (Group 2) or 500 ppm (Groups 3 and 4) for 38 days. Groups 1 through 3 were treated with a single i.p. injection of NNK dissolved in saline at a dose of 2 mg/mouse/day for four consecutive days from day 7 through day 10. Groups 4 and 5 were treated with saline as vehicle. Mice were sacrificed under ether anesthesia at day 38. The lung was removed, placed immediately in liquid nitrogen, and stored at -80°C until analysis.

DNA Isolation, *in vitro* packaging and *gpt* mutation assay: High-molecular-weight genomic DNA was extracted from the lung using the RecoverEase DNA Isolation Kit (Stratagene, La Jolla, CA). λ EG10 phages were rescued using Transpack Packaging Extract (Stratagene, La Jolla, CA). The *gpt* mutation assay was performed according to previously described methods (27,28). *gpt* MFs were calculated by dividing the number of colonies growing on agar plates containing chloramphenicol and 6-thioguanine by the product of the number of colonies growing on plates containing chloramphenicol and the dilution factor.

Bacterial mutation assay: The mutagenicity assay was carried out with a pre-incubation method with modifications (29). Nobiletin or 8-methoxypsoralen was dissolved in DMSO and the solution (50 μL) was mixed with S9 mix (0.5 mL). They were kept on ice for 5 min and mixed with the solution (50 μL) of chemicals, i.e., NNK, BP or MNNG, dissolved in DMSO. Then, they were mixed with overnight culture (0.1 mL) and incubated for 20 min at 37°C . When the mutagenicity of MNNG was assayed, 1/15M phosphate buffer pH7.4 (0.5 mL) was added instead of S9 mix. The reaction mixture containing bacteria, nobiletin (or 8-methoxypsoralen) and the chemical with or without S9 mix was poured onto agar plates with soft agar and incubated for two days at 37°C . Each chemical was assayed with 6–8 doses on triplicate or duplicate plates. Tester strains for the mutation assays were *S. typhimurium* YG7108 for NNK and MNNG, and *S. typhimurium* YG5161 (30) for BP. Relevant genotypes of the strains are as follows: YG7108 (24,25) as *S. typhimurium* TA1535 but is Δ ada_{ST} Δ ogt_{ST}; YG5161 (30) as *S. typhimurium* TA1538 harboring plasmid pYG768 carrying the *dinB* gene of *Escherichia coli*.

Statistical analysis: All data are expressed as mean $\pm$ standard deviations. Differences between groups were tested for statistical significance using a Student's *t*-test. A *P* value less than 0.05 denoted the presence of a statistically significant difference.

Results

Dietary administration of nobiletin suppresses mutations induced by NNK in the lung of *gpt* delta mice:

To examine the suppressive effects of nobiletin against genotoxicity induced by NNK, female and male *gpt* delta mice were fed nobiletin in diet at a dose of 100 or 500 ppm for a week and treated with NNK (Fig. 1). Dietary administration of nobiletin continued during

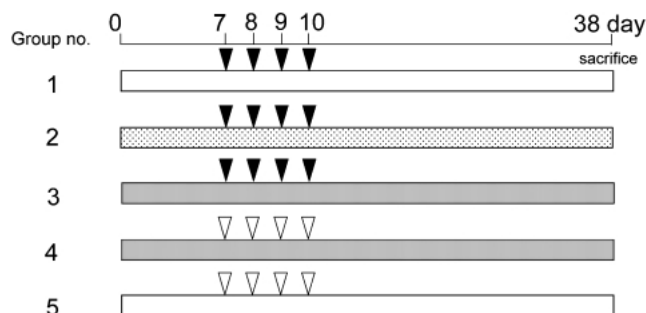


Fig. 1. An experimental design to examine chemopreventive effects of nobiletin against genotoxicity of NNK in the lung of *gpt* delta mice. Twenty female and 25 male eight-week-old *gpt* delta mice were each divided into five groups. Groups 1 through 3 were treated with a single i.p. injection of NNK at a dose of 2 mg/mouse/day for four consecutive days from day 7 through day 10. Groups 2 and 3 were fed diet supplemented with nobiletin at doses of 100 ppm and 500 ppm, respectively, for 38 days. Groups 4 and 5 were treated with saline as vehicle, and Group 4 was fed diet with nobiletin at a dose of 500 ppm for 38 days. Mice were sacrificed at day 38, and the *gpt* MF in the lung were determined. □, basal diet; ▨, nobiletin in diet at a dose of 100 ppm; ▩, nobiletin in diet at a dose of 500 ppm; ▼, NNK (2 mg/mouse/day, i.p.); ▽, saline.

the NNK treatments and in the following period before sacrifice at day 38. NNK treatments enhanced *gpt* MF in the lung 19 times in females and 9 times in males over the control levels (Tables 1 and 2). Since the MFs ($\times 10^{-6}$) of untreated controls were similar between females and males (3.0 ± 1.3 versus 3.1 ± 2.0), NNK-induced MF was higher in females (58.1 ± 16.7) than in males (26.5 ± 11.8). Nobiletin itself was non-genotoxic (Group 4). Nobiletin appeared to reduce the MFs in both sexes. In females, the dietary administration of nobiletin at 100 and 500 ppm (Groups 2 and 3) reduced the NNK-induced MF by 34 and 32%, respectively, and the reduction at 100 ppm was statistically significant ($P < 0.04$). In males, nobiletin at 100 and 500 ppm reduced the MF by 25 and 45%, respectively, and the reduction at 500 ppm was statistically significant ($P < 0.04$). These results indicate that nobiletin suppresses NNK-induced genotoxicity in the lung of *gpt* delta mice.

Nobiletin inhibits genotoxicity of NNK in the presence of S9 activation in *S. typhimurium* YG7108:

To further characterize the suppressive effects of nobiletin against genotoxicity of NNK, we conducted bacterial mutation assays to examine whether nobiletin inhibits genotoxicity of NNK in the presence of S9 activation enzymes (Fig. 2A). NNK at a dose of $500 \mu\text{g}/\text{plate}$ induced mutations in *S. typhimurium* YG7108 and produced about 900 His⁺ revertants/plate, which was 40–50 times higher than the value of spontaneous mutations. Nobiletin itself was non-genotoxic either with or without S9 activation (Fig. 2A, C and D).

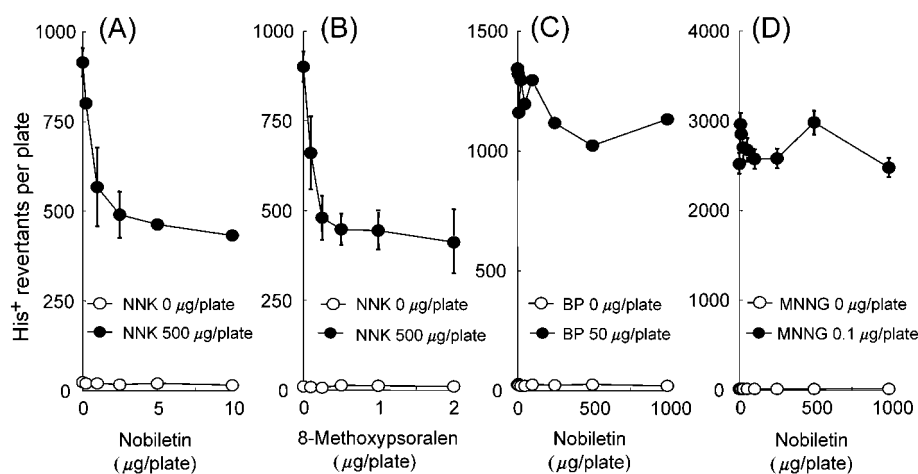


Fig. 2. Suppressive effects of nobiletin against genotoxicity of NNK in the presence of S9 mix in *S. typhimurium* YG7108. Closed circles represent the numbers of His⁺ revertants/plate induced by the following compounds: NNK (500 μg/plate) in the presence of S9 mix along with the increasing doses of nobiletin (A), NNK (500 μg/plate) in the presence of S9 mix along with the increasing doses of 8-methoxypsoralen (B); BP (50 μg/plate) in the presence of S9 mix along with the increasing doses of nobiletin (C); MNNG (0.1 μg/plate) in the absence of S9 mix along with the increasing doses of nobiletin. Open circles represent the numbers of His⁺ revertants/plate when the non-genotoxicity of nobiletin (A, C and D) and 8-methoxypsoralen (B) were confirmed. Strains used are *S. typhimurium* YG7108 (A, B and D) and *S. typhimurium* YG5161 (C). Averages and standard deviations are presented in A, B and D where three plates were used for the assays. Averages are presented in C where two plates were used for the assay.

Table 1. Suppressive effects of nobiletin against genotoxicity of NNK in the lung of female *gpt* delta mice

Group number*	Animal I.D.	Total colonies	No. of mutants	<i>gpt</i> MF ($\times 10^{-6}$)	Average $\pm$ S.D. [†]	P-value [‡]
1 NNK alone	F001	898,500	68	75.7	58.1 $\pm$ 16.7	
	F002	1,017,000	57	56.1		
	F003	1,464,000	53	36.2		
	F004	1,054,500	68	64.5		
		4,434,000	246	55.5		
2 NNK + Nobiletin (100 ppm)	F005	1,134,000	36	31.8	38.6 $\pm$ 6.6	0.036 [§]
	F006	1,353,000	48	35.5		
	F007	1,152,000	54	46.9		
	F008	916,500	37	40.4		
		4,555,500	175	38.4		
3 NNK + Nobiletin (500 ppm)	F009	1,369,500	33	24.1	39.3 $\pm$ 10.4	0.052
	F010	798,000	36	45.1		
	F011	1,606,500	66	41.1		
	F012	1,027,500	48	46.7		
		4,801,500	183	38.1		
4 Nobiletin (500 ppm) alone	F013	1,059,000	3	2.8	4.5 $\pm$ 1.9	<0.001
	F014	1,377,000	4	2.9		
	F015	1,092,000	6	5.5		
	F016	900,000	6	6.7		
		4,428,000	19	4.3		
5 No treatments	F018	2,856,000	6	2.1	3.0 $\pm$ 1.3	<0.001
	F019	1,560,000	4	2.6		
	F020	1,809,000	9	5.0		
	F021	2,013,000	5	2.5		
		8,238,000	24	2.9		

*Group 1, mice treated with NNK (2 mg/mouse/day $\times$ 4 days) alone; Group 2, mice treated with NNK plus nobiletin at a dose of 100 ppm in diet; Group 3, mice treated with NNK plus nobiletin at a dose of 500 ppm in diet; Group 4, mice fed nobiletin at a dose of 500 ppm in diet without NNK treatments; Group 5, mice without treatments with NNK or nobiletin. The Group No. corresponds with group No. in Fig. 1.

[†]Average $\pm$ standard deviation of *gpt* MF of four mice.

[‡]Differences between *gpt* MF of each group and that of Group 1 were tested for statistical significance using a Student's *t*-test.

[§]Statistically significant ($P < 0.05$) against Group 1. The values in Groups 4 and 5 are also statistically significant. But the mice in Groups 4 and 5 are not treated with NNK so that the values are not marked with §.

An addition of nobiletin in the reaction mixture containing NNK and S9 mix reduced the genotoxicity of NNK in a dose-dependent manner, and the number of His⁺ revertants/plate decreased by more than 50% at the highest dose of nobiletin, i.e., 10 μ g/plate. There was no obvious reduction of background lawn of bacteria at any dose of nobiletin, suggesting that nobiletin was not very much toxic under the experimental conditions. Similar dose-dependent reduction of the genotoxicity of NNK was observed with 8-methoxypsoralen (Fig. 2B). An addition of 8-methoxypsoralen into the reaction mixture containing NNK and S9 mix reduced the number of His⁺ revertants/plate by more than 50%. Despite the similar inhibitory effects, the dose necessary to reduce the genotoxicity of NNK by 50% was 5- to 10-fold higher with nobiletin than with

8-methoxypsoralen (2.5 μ g/plate for nobiletin versus 0.25–0.5 μ g/plate for 8-methoxypsoralen). In contrast, nobiletin exhibited weak or virtually no inhibitory effects on the genotoxicity of BP or MNNG, respectively (Fig. 2C and D). An addition of nobiletin reduced the genotoxicity of BP in the presence of S9 activation by 20%, while it did not modulate the genotoxicity of MNNG in the absence of S9 enzymes.

Discussion

Lung cancer continues to be the leading cause of cancer death in developed countries. Dietary compounds with potential to inhibit lung cancer may be a promising and practical approach for reducing the risk of lung cancer caused by smoking. In this study, we examined the chemopreventive efficacy of nobiletin

Table 2. Suppressive effects of nobiletin against genotoxicity of NNK in the lung of male *gpt* delta mice

Group number*	Animal I.D.	Total colonies	No. of mutants	<i>gpt</i> MF ($\times 10^{-6}$)	Average $\pm$ S.D. [†]	<i>P</i> -value [‡]
1 NNK alone	M001	960,000	21	21.9	26.5 $\pm$ 11.8	
	M002	987,000	32	32.4		
	M003	1,320,000	57	43.2		
	M004	876,000	20	22.8		
	M005	1,892,000	23	12.2		
		6,035,000	153	25.4		
2 NNK + Nobiletin (100 ppm)	M007	1,156,000	16	13.8	19.9 $\pm$ 6.1	0.147
	M008	991,000	19	19.2		
	M009	828,000	20	24.2		
	M010	828,000	23	27.8		
	M011	840,000	12	14.3		
		4,643,000	90	19.4		
3 NNK + Nobiletin (500 ppm)	M013	700,000	16	22.9	14.4 $\pm$ 5.4	0.035
	M014	1,404,000	11	7.8		
	M015	1,052,000	14	13.3		
	M016	760,000	10	13.2		
	M017	1,000,000	15	15.0		
		4,916,000	66	13.4		
4 Nobiletin (500 ppm) alone	M019	1,028,000	4	3.9	3.5 $\pm$ 1.0	0.003
	M020 [§]	388,000	4	10.3		
	M021	1,640,000	6	3.7		
	M022	708,000	3	4.2		
	M023	972,000	2	2.1		
		4,348,000	15	3.5		
5 No treatments	M024 [§]	705,000	14	19.9	3.1 $\pm$ 2.0	0.003
	M025	1,410,000	8	5.7		
	M026	1,410,000	5	3.6		
	M027	1,928,000	3	1.6		
	M028	2,032,000	3	1.5		
		6,780,000	19	2.8		

*Group 1, mice treated with NNK (2 mg/mouse/day $\times$ 4 days) alone; Group 2, mice treated with NNK plus nobiletin at a dose of 100 ppm in diet; Group 3, mice treated with NNK plus nobiletin at a dose of 500 ppm in diet; Group 4, mice fed nobiletin at a dose of 500 ppm in diet without NNK treatments; Group 5, mice without treatments with NNK or nobiletin. The Group No. corresponds to Group No. in Fig. 1.

[†]Average $\pm$ standard deviation of *gpt* MF of four or five mice.

[‡]Differences between *gpt* MF of each group and that of Group 1 were tested for statistical significance using a Student's *t*-test.

[§]Two unusually high *gpt* MF of M020 and M024 were excluded for the calculation of average by the Smirnov-Grubb's outlier test.

^{||}Statistically significant ($P < 0.05$) against Group 1. The values in Groups 4 and 5 are also statistically significant. But the mice in Groups 4 and 5 are not treated with NNK so that the values are not marked with ||.

against genotoxicity of NNK in the lung of *gpt* delta mice. NNK exposure significantly enhanced the *gpt* MFs in the lung of mice (Tables 1, 2). There was a marked sex difference in the genotoxicity of NNK where females exhibited about twice higher sensitivity than males. This may be due to gender-related differences in the metabolic activation enzymes for NNK (31). The high sensitivity in female than in male mice may be relevant in humans because women are more sensitive to the genotoxic effects of NNK than men (32). Interestingly, dietary administration of nobiletin substantially reduced the

gpt MFs in both sexes, and the reduction at a dose of 100 ppm in females and 500 ppm in males was statistically significant ($P < 0.05$). Administration of nobiletin at 500 ppm also reduced the genotoxicity in females at a similar extent to that observed with nobiletin at 100 ppm. Ikeda *et al.* reported that NNK induces G:C-to-A:T, G:C-to-T:A, A:T-to-T:A, A:T-to-G:C in the lung of *gpt* delta mice (unpublished observations). Since G:C-to-A:T can activate *Ki-ras* oncogene, the reduction of *gpt* MF may correlate with the reduction of lung tumors (5). Thus, we suggest that nobiletin may be a

chemopreventive agent against NNK-induced lung tumorigenesis in mice. Nobiletin inhibits metastasis (20,21) and suppresses inflammation and promotion (18,33–36). Hence, it may prevent events that occur in multi-step of lung carcinogenesis, i.e., initiation, promotion and progression/metastasis, induced by cigarette smoke. However, certain compounds that can reduce NNK-induced tumors do not necessarily reduce lung tumors in smoke-exposed animals (37). Thus, further examination is needed to evaluate the chemopreventive efficacy of nobiletin against lung tumors induced by cigarette smoke.

In addition to *in vivo* results, we observed reduction of NNK-induced mutations by nobiletin in the present of S9 activation enzymes *in vitro*. Interestingly, nobiletin exhibited a specificity inhibiting the genotoxicity of chemicals in *S. typhimurium*. Although nobiletin inhibited the genotoxicity of NNK, it inhibited the genotoxicity of BP with S9 activation only slightly and did not inhibit the genotoxicity of MNNG without S9 activation. Since MNNG induces *O*⁶-methylguanine leading to G:C-to-A:T mutations (38), we suggest that nobiletin may not enhance the repair activity against *O*⁶-methylguanine or promote error-free translesion bypass across the lesion. Instead, we suggest that nobiletin may suppress the genotoxicity of NNK by inhibiting the activity of CYP (P-450) enzymes involved in the metabolic activation of NNK (39–41). In fact, 8-methoxypsoralen, a specific-inhibitor of CYP2A, similarly suppressed the genotoxicity of NNK in the presence of S9 enzymes (23). The inhibitory effect of nobiletin may be specific to certain CYP enzymes including CYP2A because the genotoxicity of BP, which is activated *via* CYP1A1 (42), was weakly inhibited by nobiletin. However, since both nobiletin and 8-methoxypsoralen inhibited the genotoxicity of NNK only by 50%, we suggest that other CYP enzymes may be responsible for the remaining genotoxicity of NNK in the S9 enzymes. Although nobiletin did not effectively affect the genotoxicity of BP in the present study, Conney *et al.* (43) observed that nobiletin stimulates human liver microsomes and activates both the hydroxylation of BP and the metabolism of aflatoxin B₁ to mutagens. Nobiletin also stimulates oxidative metabolism of zoxazolamine by rat liver microsomes (44) and acetaminophen by human liver microsome (45). These reports suggest that nobiletin has a potential to modulate CYP enzyme activities.

In summary, we examined the chemopreventive efficacy of nobiletin against the genotoxicity of NNK in the lung of female and male *gpt* delta mice. Dietary administration of nobiletin significantly reduced the genotoxicity of NNK in both sexes. In addition, the chemical was able to reduce NNK-induced genotoxicity in *S. typhimurium* YG7108 in the presence of S9 activat-

ing enzymes. Our findings suggest that nobiletin could inhibit the activities of certain CYP enzymes involved in the metabolic activation of NNK, thereby suppressing the genotoxicity in the lung of mice.

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