Chemopreventive Effect of Kava on 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone plus Benzo[*a*]pyrene–Induced Lung Tumorigenesis in A/J Mice

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Abstract Lung cancer is the leading cause of cancer death, and chemoprevention is a potential strategy to help control this disease. Epidemiologic survey indicates that kava may be chemopreventive for lung cancer, but there is a concern about its potential hepatotoxicity. In this study, we evaluated whether oral kava could prevent 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) plus benzo[*a*]pyrene (B[*a*]P)–induced lung tumorigenesis in A/J mice. We also studied the effect of kava to liver. At a dose of 10 mg/g diet, 30-week kava treatment (8 weeks concurrent with NNK and B[*a*]P treatment followed by 22 weeks post-carcinogen treatment) effectively reduced lung tumor multiplicity by 56%. Kava also reduced lung tumor multiplicity by 47% when administered concurrently with NNK and B[*a*]P for 8 weeks. Perhaps most importantly, kava reduced lung tumor multiplicity by 49% when administered after the final NNK and B[*a*]P treatment. These results show for the first time the chemopreventive potential of kava against lung tumorigenesis. Mechanistically, kava inhibited proliferation and enhanced apoptosis in lung tumors, as shown by a reduction in proliferating cell nuclear antigen (PCNA), an increase in caspase-3, and cleavage of poly(ADP-ribose) polymerase (PARP). Kava treatment also inhibited the activation of nuclear factor κBNF-κB, a potential upstream mechanism of kava chemoprevention. Although not rigorously evaluated in this study, our preliminary data were not suggestive of hepatotoxicity. Based on these results, further studies are warranted to explore the chemopreventive potential and safety of kava.

Lung cancer is the leading cause of cancer death. Approximately 150,000 deaths and 160,000 new cases are expected in the United States in 2007 (1). The comparable rates of mortality and incidence illustrate the status of current treatment of lung cancer—almost all lung cancer patients die of this disease, and the 5-year survival rate is only 16.8% (2). There has been little improvement in lung cancer treatment over the past decades (3, 4), raising the need for alternative strategies to help control this disease. Because smoking is the major cause of lung cancer (5), the most effective approach to preventing the development of lung cancer would be tobacco control. This is underscored by the recent epidemiologic evidence that smoking cessation, even during middle age, can lead to a major reduction of lung cancer incidence (6, 7). However, even with all the goals of antismoking efforts attained, a large number of former smokers will remain at high risk for lung cancer for a significant number of years, who account for about half of the new cases of lung cancers in the United States. One complementary approach to helping control lung cancer is chemoprevention (8). However, clinical efforts in developing chemopreventive agents against lung tumorigenesis have thus far produced either neutral or harmful primary endpoint results (2, 9–11).

Epidemiologic studies by Henderson et al. (12) noted that several countries in the South Pacific islands, such as Fiji, Vanuatu, and Western Samoa, have very low cancer incidence rates including lung cancer. This difference is not due to varying degrees of cancer incidence registration, smoking rate, or race (12, 13), suggesting that there may be chemopreventive agents present in the diet of South Pacific islanders. Among the potential candidates, kava is promising because a negative correlation was observed between the amount of kava consumed and the cancer incidence rate among the populations in these countries—the more kava consumed by the population, the lower the cancer incidence (14). Kava has been reported to inhibit various cytochrome *P*450 enzymes, metabolic enzymes that activate chemical carcinogens to induce

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DNA damage and tumor initiation (15, 16). Kava has also been shown to inhibit the activation of nuclear factor κB (NF-κB; ref. 17), a transcription factor (18) that plays a central role in the regulation of carcinogenic responses (19–23). Inhibiting cytochrome *P*450s and suppressing NF-κB activation provide potential mechanisms of chemoprevention.

Kava is a traditional beverage in the South Pacific islands. Kava also had been used for the clinical treatment of anxiety (24, 25). It is not currently prescribed because of several reports of hepatotoxicity that led to kava being banned in Germany since 2002, and later on in Europe, Australia, and Canada (26). Whether the reported hepatotoxicity actually resulted from kava is still questionable. First, traditional kava has a long history of safe use in the South Pacific islands, where some kava users consume 300 to 600 g of kava root per week (12, 27–32). Of many safety surveys about traditional kava, only Russmann et al. (33) observed hepatotoxicity in two women in the islands of New Caledonia. Second, in response to the withdrawal of kava in Germany, the data leading to such a ban were reevaluated by several committees. These reviews found that many of the adverse events cited by the German Federal Institute for Drugs and Medical Products were reiterations of a small number of purported cases (34). Of the 30 to 40 cases of acute hepatitis between 1990 and 2002 with sufficient retrospective data, only one seemed to have a "possible connection" with kava use. In fact, many of the reported cases cannot be undoubtedly attributed to kava because most of the patients were using other medications that may have been responsible for the reported hepatotoxicity (35–38). Third, it has also been suggested that the hepatotoxicity of kava might be due to its improper preparation, which may have included leaves and stems; a recent study by Nerurkar et al. showed that pipermethystine, the major alkaloid in the leaves and stems, is more cytotoxic to liver cells compared with other alkaloids in the root (36, 39). Lastly, even based on the reported rate of hepatotoxicity, the incidence of kava-associated adverse hepatic events was ~0.25 case/1,000,000 daily doses. This compares extremely favorably with many daily-used drugs, such as the anxiolytic benzodiazepines, where the rate of hepatic adverse effects is 0.90 to 2.12 cases/1,000,000 daily doses (37). These data argue that kava has very low probability to cause hepatotoxicity and is very likely to be safe for daily usage with the proper preparation and appropriate administration.

Taken together, these epidemiologic and experimental data of kava prompted us to evaluate its chemopreventive activity against lung tumorigenesis. Because of the similarities in the pathology and progression stages between mouse and human lung cancers, a mouse lung tumorigenesis model would be useful to preevaluate the efficacy of putative lung cancer chemopreventive agents (40, 41). Among several strains of mice tested, the A/J mouse strain is very susceptible to develop lung tumorigenesis (42, 43). There are two general approaches to inducing lung tumorigenesis in the A/J mouse model (43). The first approach is exposing the A/J mouse to cigarette smoke, mimicking the natural smoking conditions (44). Whereas it could be argued that induction of lung cancer by tobacco smoke would be a better model, there are severe practical limitations of such an approach (43). The second approach is exposing the A/J mouse to selected model compounds from cigarette smoke, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo(*a*)pyrene (B[*a*]P), two carcinogens widely accepted as major causes of lung cancer in smokers (43). Both models have been widely used to search for chemopreventive agents against lung tumorigenesis (42, 45–50).

In this study, we evaluate the chemopreventive activity of kava against lung tumorigenesis induced by a mixture of NNK plus B[*a*]P in the A/J mouse model (47). To gain preliminary insight into the mechanism of the chemopreventive activity of kava, we evaluated kava with three treatment regimens (i.e., administered during carcinogen treatment, post-carcinogen treatment, or throughout the experiment). We also obtained preliminary data on potential hepatotoxicity of kava by measuring liver weights and serum levels of liver enzymes at the time of necropsy and by microscopic examination of liver.

Materials and Methods

Chemicals, reagents, and diets

HEPES, $MgCl_2$, NaCl, protease inhibitor cocktail, and B[*a*]P were purchased from Sigma. NNK was synthesized as previously described (51). Mouse diets (AIN-93G and AIN-93M) were purchased from Harlan Teklad. An ethanolic extract of kava standardized to 150 mg/mL total kavalactones was purchased from Gaia Herb.

Preparation of diet supplemented with kava at a dose of 10 mg/g diet

The ethanolic kava extract (70 mL; 360 mg residue/mL) was mixed with AIN-93 diet (300 g). The mixture was dried under vacuum to remove residual water and ethanol. This mixture was ground to a fine power and mixed with additional AIN-93 diet (2.2 kg). The dose of kava chosen was based on the results of a 4-wk pilot safety study carried out in-house. In the in-house pilot study, three groups of 6-wk-old female A/J mice (five per group) were fed diet supplemented with kava at doses of 0, 10, or 20 mg/g diet for 4 wk. No change in body weight or sign of toxicity was observed for either group that consumed kava compared with the control group (data not shown). Based on this study, we decided to evaluate the chemopreventive potential of kava at a dose of 10 mg/g diet, half of the maximal dose with no detectable side effects.

A/J mouse tumorigenesis experiment

Female A/J mice, 5 to 6 wk of age, were obtained from The Jackson Laboratory. On arrival, the mice were maintained on AIN-93G pelleted diet and housed in the pathogen-free animal quarters at the University of Minnesota Cancer Center. One week after arrival, the mice were switched to AIN-93G powdered diet. The powdered AIN-93G diet was administered using metal boxfeeders (Lab Products, Inc.). The metal boxfeeder allows easy monitoring of food consumption and minimizes diet waste. Fresh diet was provided every 3 d and water bottles were changed every week. After 1 wk of acclimation, the mice were randomized into six groups (groups 1 and 3-6, 20 mice per group; group 2, 30 mice per group). These mice were treated following the study design detailed in Fig. 1. Briefly, they were treated by gavage either with a mixture of B[*a*]P plus NNK (2 μmol each: groups 2, 4, 5, and 6) in 0.1-mL cottonseed oil or with 0.1-mL cottonseed oil alone (groups 1 and 3) once a week for 8 wk. Mice in groups 1 and 2 were maintained on standard AIN-93 powdered diet for 30 wk. Mice in groups 3 to 6 were given kava diet according to the experimental protocol (groups 3 and 4, kava diet for 30 wk; group 5, 8-wk kava diet during the 8 wk of carcinogen treatment; and group 6, 22-wk kava diet started after the last carcinogen treatment). Food consumption was monitored twice a week. Body weight and water consumption were recorded every week. The mice were euthanized 22 wk after the final carcinogen administration.

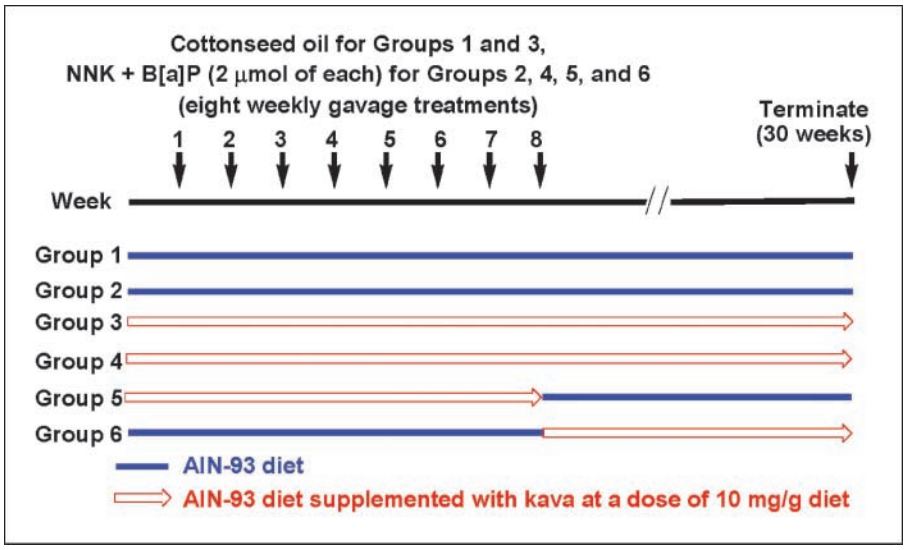

Fig. 1. Study design for evaluating the chemopreventive efficacy of kava against NNK plus B[*a*]P–induced lung tumorigenesis in the A/J mouse and the potential toxicity of kava. Beginning at age 6 to 7 wk, groups of female A/J mice (groups 2, 4, 5, and 6) were treated by gavage weekly for 8 wk with a mixture of NNK + B[*a*]P (2 μmol each) in 0.1-mL cottonseed oil. The mice in groups 1 and 3 were treated by gavage weekly for 8 wk with 0.1-mL cottonseed oil. The mice in groups 1 and 2 were maintained on standard diet treatment for the duration of the experiments. Mice in groups 3 to 6 were given a diet supplemented with kava at a dose of 10 mg/g diet according to the following schedules: groups 3 and 4, kava diet for 30 wk; group 5, 8-wk kava diet during the 8-wk carcinogen treatment; and group 6, 22-wk kava diet started 1 d after the last carcinogen treatment.

Lungs were perfused with cold PBS and harvested, and tumors were counted under a dissecting microscope by an American College of Veterinary Pathologists board-certified pathologist (M.G.O'S.) under unbiased conditions. During the tumor count, the lungs were protected from drying by moistening them with PBS. Subsequent to the tumor count, lung from each animal was frozen in liquid nitrogen for Western blot analyses. Blood from each mouse was collected by cardiac puncture at the time of euthanasia with samples pooled by cage (four to five animals per cage) and the enzyme levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyl transpeptidase (GGT) were determined at the Diagnostic Laboratories of Fairview Health Service using the Vitros 350 chemistry analyzer (Ortho-Clinical Diagnosis).

Liver from each mouse was collected, weighed, fixed overnight in 10% neutral formalin, and then transferred into 70% ethanol. Livers were examined by light microscopy from five mice chosen at random from groups 1, 2, 3, and 4; each mouse came from a different cage. Appropriately fixed tissues were processed into paraffin blocks using standard histologic techniques, and 5-μm sections were cut and stained with H&E. Histologic slides were examined using light microscopy by an American College of Veterinary Pathologists board-certified pathologist (M.G.O'S.).

Western blot analyses

We pooled tumors from the mice in the same group to prepare protein lysate for Western blot analyses because of the relatively small size and number of lung tumors in any individual animal (47). The pooling process also avoids any potential bias in selecting individuals for analyses. Due to the small number of lung tumors in group 1 (five total) and group 3 (seven total), normal lung tissues were used for protein sample preparation. In brief, after thawing, tumors from the frozen mouse lungs from groups 2, 4, 5, and 6 were collected and pulverized with a mortar and pestle on dry ice. For groups 1 and 3, samples of the frozen mouse lungs were collected and pulverized. The pulverized lung tissue (weighing 50-80 mg) was suspended in 100 μL of lysis buffer [15 mmol/L $MgCl_2$, 50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 8 mol/L urea, 0.1% Triton X-100] and a cocktail of protease inhibitors [10 μL/g tissue, leupeptin, pepstatin A, aprotinin, bestatin hydrochloride, *N*-(*trans*-epoxysuccinyl)-l-leucine 4-guanidinbutylamide, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride], sonicated for 1 min, and centrifuged at 15,000 rpm for 20 min. Recovered supernatants were frozen at −80°C. The protein concentration of the samples was determined according to the Bradford method. Five to twenty micrograms of the protein samples were loaded onto a Novex Tris-glycine gel (Invitrogen) and run for 100 min at 125 V. The proteins were then transferred onto a nitrocellulose membrane (Millipore) for 2 h at 25 V. Adequate transfer of proteins was confirmed by monitoring the colored molecular weight marker transfer. Subsequently, membranes were blocked in 5% Blotto nonfat dry milk in Tris buffer containing 1% Tween 20 for 1 h and probed overnight with the following primary antibodies (Cell Signaling Technology): proliferating cell nuclear antigen (PCNA), 1:1,000; poly(ADP-ribose) polymerase (PARP), 1:250; and NF-κB, 1:500. After incubating with secondary antibody (Sigma) for 1 h (1:2,000 rabbit anti-mouse IgG for PCNA and 1:2,000 goat anti-rabbit IgG for PARP and NF-κB), chemiluminescent immunodetection was used with the Supersignal chemiluminescence system (Pierce). The signal was visualized by exposure of membranes to HyBolt CL autoradiography film (Denville Scientific). The membranes were stripped and probed with anti–β-actin to check for differences in the amount of protein loaded in each lane. Relative band densities were quantified with the U-Scan-It software (Silk Scientific) and normalized relative to total protein to compensate for experimental variation. The level of cleaved caspase-3 in the protein lysates was measured by using Luminex beads specific for caspase-3 (AssayGate) following the manufacturer's recommendations.

Statistical analysis

Lung tumor multiplicity, body weight, liver weight, liver enzymes, PCNA, and caspase-3 comparisons were made using Student's *t* test. All statistical tests were two sided, and $P < 0.05$ was considered statistically significant. Several tumor multiplicity histograms showed evidence of right-skew, and thus all pairwise comparisons were followed up with the nonparametric Mann-Whitney test. The *t* test results were found to be conservative, with slightly larger *P* values for differences in tumor multiplicity than those obtained with the Mann-Whitney tests.

Results

The effect of kava treatment on food consumption and body weight in A/J mice

Over the experimental period, no difference in food consumption was observed among the mice in different groups during the study (Fig. 2A). Kava treatment induces slight reductions in body weight (Fig. 2B). The 30-week kava treatment alone induced a 5% reduction in body weight (group 1 versus group 3). In the presence of NNK + B[*a*]P, the 30-week kava treatment induced a 7% reduction in body weight (group 2 versus group 4). The other two kava temporal treatment

regimens also resulted in a 3% reduction in body weight (group 2 versus groups 5 and 6).

Kava reduces lung tumor multiplicity in A/J mice

Because the experiment was terminated 22 weeks after the last carcinogen treatment, the lung tumors formed should be mostly, if not all, adenoma with the size of 1 to 2 mm in diameter (46, 52). Our chemopreventive evaluation in this study, therefore, mainly focused on the effect of kava on lung tumor multiplicity instead of tumor burden, morphology, or pathology. The mice treated with NNK + B[*a*]P and given kava at a dose of 10 mg/g diet throughout the experiment had a statistically significant 55.9% reduction in lung tumor multiplicity than the mice treated with NNK + B[*a*]P (Table 1). The mice treated with NNK + B[*a*]P and given kava during the carcinogen treatment period had a statistically significant 47.1% reduction (Table 1). The mice treated with NNK + B[*a*]P and given kava after the last carcinogen treatment also had a statistically significant 48.7% reduction (Table 1).

Kava inhibits NNK and B[*a*]P–mediated activation of cell proliferation in A/J mice

To assess the effect of kava on the proliferation status of lung tumors, lung tumors were analyzed by immunoblotting for their relative levels of PCNA, a cell proliferation marker (Fig. 3). PCNA was quantified by densitometry analysis, adjusted with β-actin as the loading control, and then normalized to that of the control mice (group 1). The relative level of PCNA in the lung tumor samples from NNK + B[*a*]P–treated mice was substantially increased (group 2, 1.80 ± 0.20) compared with the control mice, indicating an increased rate of proliferation in the lung tumors compared with the normal lung tissues. The three kava treatment regimens all resulted in a statistically significant decrease of PCNA level in the tumor tissues, especially the whole-course treatment (group 4, 0.90 ± 0.09) and the post-carcinogen treatment (group 6, 0.85 ± 0.12) compared with the NNK + B[*a*]P–treated mice. Concurrent kava treatment with NNK + B[*a*]P only induced a moderate decrease of PCNA (group 5, 1.42 ± 0.12) compared with the mice treated with NNK + B[*a*]P alone.

Kava activates apoptosis in tumors induced by NNK and B[*a*]P in A/J mice

Lung tumors were analyzed for levels of caspase-3 and cleavage of PARP, characteristics of apoptosis (Fig. 4). The level of caspase-3 in lung tumor samples from NNK + B[*a*]P–treated mice was comparable to that in normal lungs from vehicle control mice (Fig. 4A). There were marked increases in caspase-3 in lung tumors from the mice given kava compared with NNK + B[*a*]P–treated mice, especially group 6. The cleavage of PARP was monitored by the disappearance of full-length PARP (Fig. 4B). The level of PARP in lung tumor samples from NNK + B[*a*]P–treated mice was comparable to that in normal lungs from vehicle control mice. Consistent with the increase in caspase-3, the levels of full-length PARP in lung tumors from mice treated with carcinogen and kava were all substantially decreased compared with the control group. The increase in caspase-3 and cleavage of PARP in the lung tumors with kava treatment indicate that kava induced apoptosis in lung tumors.

Kava inhibits NF-κB activation in response to carcinogen

The effect of kava on NF-κB in lung tumors was evaluated by immunoblotting of p65, the active form of NF-κB (53). As shown in Fig. 5, little, if any, p65 was detected in lung tissues from untreated mice (group 1). Kava administered alone did not affect the level of p65 (group 3). NNK and B[*a*]P treatment, on the other hand, resulted in an increase of p65 (group 2), but the levels of p65 decreased to control level on kava treatment (groups 4-6).

The effect of kava treatment on liver

The potential influence of kava on liver was examined in three different ways: liver weight, enzyme markers for liver damage, and liver pathology.

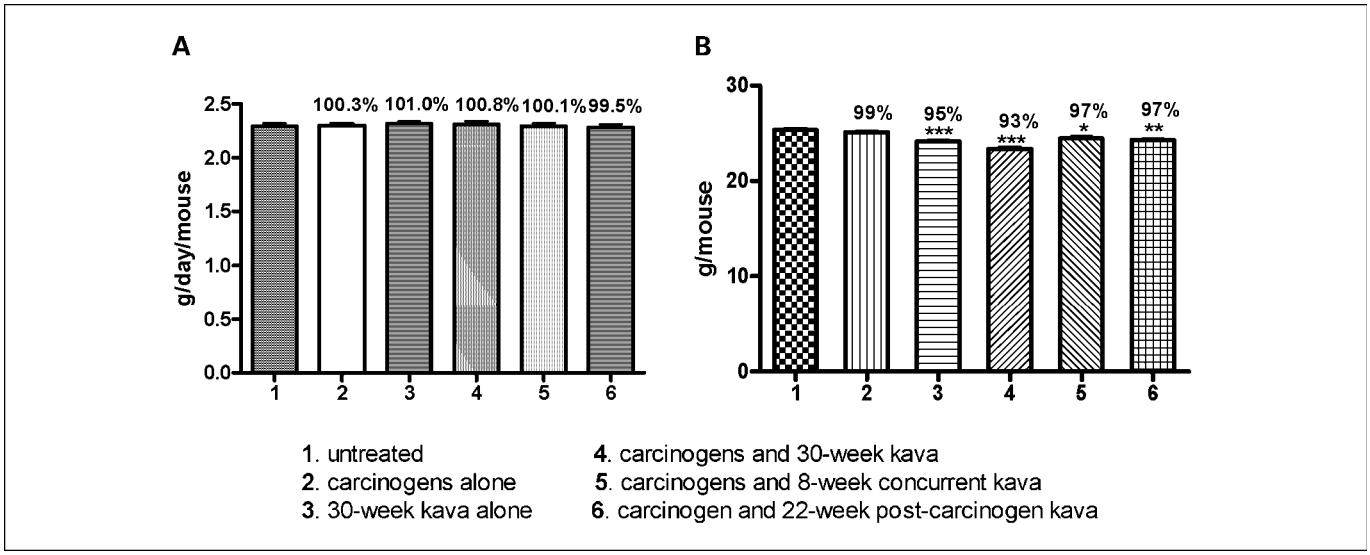

Fig. 2. Food consumption and body weight of mice. *A*, average food consumed by each mouse each day over the 30-wk period. *B*, average body weight of mice before termination. The comparison is between groups 1 and 2, groups 1 and 3, and group 2 with groups 4, 5, and 6. Groups 1 and 2 showed no statistically significant difference. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

Table 1. Effect of kava on lung tumor multiplicity by a mixture of NNK plus B[*a*]P in A/J mice

Group	No. mice at initiation	Body weight at initiation (mean ± SD, g/mouse)	No. mice at termination	Body weight at termination (mean ± SD, g/mouse)	Lung tumors			
					Mice with tumors (%)	Tumors/mouse (mean ± SD)	Reduction in tumor multiplicity (%)	*P**
1 (untreated control)	20	17.1 ± 0.7	20	25.3 ± 0.9	—	0.25 ± 0.5	—	—
2 (carcinogen alone)	30	17.0 ± 0.5	30	25.1 ± 1.0	100	12.8 ± 7.0	—	—
3 (30-wk kava alone)	20	17.0 ± 0.7	18	24.1 ± 0.7	—	0.33 ± 0.84	—	—
4 (carcinogen and 30-wk kava)	20	16.9 ± 0.7	20	23.3 ± 1.1	100	5.65 ± 3.25	56.0	<0.0001
5 (carcinogen and 8-wk concurrent kava)	20	16.9 ± 0.5	19	24.5 ± 0.9	100	6.79 ± 4.57	47.1	0.0018
6 (carcinogen and 22-wk post-carcinogen kava)	20	16.8 ± 0.4	17	24.3 ± 0.8	100	6.59 ± 3.45	48.7	0.0014

NOTE: Beginning at age 6 to 7 wk, female A/J mice in groups 2, 4, 5, and 6 were treated with gavage weekly for 8 wk with a mixture of NNK + B[*a*]P (2 μmol each) in 0.1-mL cottonseed oil. The mice were maintained on AIN-93G diet from age 5 to 6 wk until 1 wk after the end of carcinogen treatment and then shifted to AIN-93M diet for the duration of the experiment. The mice in groups 3 and 4 were maintained on a diet supplemented with kava at a dose of 10 mg/g diet throughout the duration of the experiment (30 wk). The mice in group 5 were maintained on a diet supplemented with kava at a dose of 10 mg/g diet 1 d before the first carcinogen treatment and 1 d after the last carcinogen treatment (8 wk). The mice in group 6 were maintained on a standard diet until 1 d after the last carcinogen treatment and then switched to a diet supplemented with kava at a dose of 10 mg/g diet for the rest of the experiment (22 wk).
*Compared with group 2.

The 30-week kava treatment induced no change in liver weight (both the absolute and the bodyweight corrected) compared with the control (Table 2). Although mice treated with NNK + B[*a*]P and kava had slightly smaller livers on average than mice treated with NNK + B[*a*]P alone (group 2), the liver weights of all kava-treated groups were comparable to that of untreated control mice (group 1), whereas mice treated with NNK and B[*a*]P (group 2) had slightly elevated liver weights (~16% increase) compared with control mice (group 1).

The enzymatic activities of serum ALT, AST, and GGT were comparable between mice administered kava alone and mice in the control group (group 3 versus group 1; Table 2). Although mice treated with NNK + B[*a*]P and kava had increased enzymatic activity for ALT when compared with the mice treated with NNK + B[*a*]P alone, there was considerable variability in the data and it was not statistically significant.

There were no specific microscopic lesions observed in group 3 or group 4 animals that might indicate kava toxicity.

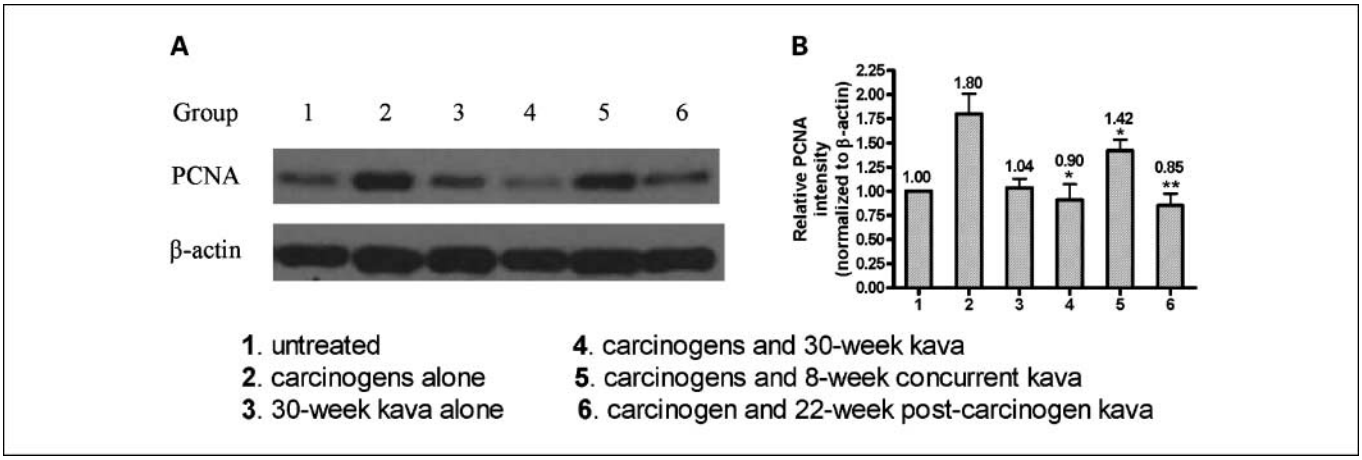

Fig. 3. *In vivo* antiproliferative effect of oral kava in lungs of A/J mice (lung tumor tissues in groups 2 and 4–6 and normal lung tissues in groups 1 and 2). *A,* immunoblot of PCNA. Reactive protein bands were visualized by enhanced chemiluminescence detection system, and membrane was stripped and probed with β-actin as loading control. *B,* densitometric analysis of PCNA; intensity was adjusted with β-actin and normalized to that of group 1. *Columns,* mean relative intensity of three replicates ($n = 3$); *bars,* SE. *, $P < 0.05$; **, $P < 0.01$.

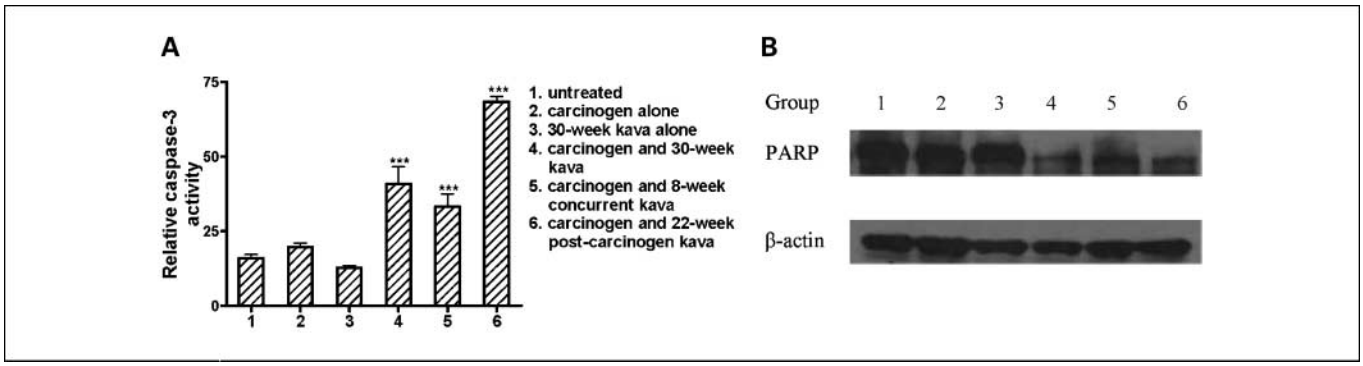

Fig. 4. *In vivo* apoptotic effect of oral kava in lungs of A/J mice (lung tumor tissues in groups 2 and 4–6 and normal lung tissues in groups 1 and 2). *A*, Luminex bead–based sandwich immunoevaluation of caspase-3 in lungs (AssayGate). *Columns*, mean of four replicates in each group ($n = 4$); *bars*, SE. ***, $P < 0.001$. *B*, immunoblot of full-length PARP in the lung tumors. Reactive protein bands were visualized by enhanced chemiluminescence detection system, and membrane was stripped and probed with β-actin as loading control.

Discussion

Lung cancer has high incidence rate and currently has no effective treatment. It is therefore imperative to develop alternative approaches, such as chemoprevention, to help control this disease.

Epidemiologic data suggest that kava could be a chemopreventive agent for lung cancer. As proposed by Gupta et al. (54), an ideal chemopreventive candidate for clinical evaluation should have a sound epidemiologic basis on one hand and proven efficacy in laboratory experiments on the other hand. The chemopreventive activity of kava, although supported epidemiologically, has not been established in an appropriate lung tumorigenesis model. The potential hepatotoxicity of kava is a concern as well. All of these issues need to be addressed before clinical evaluation of kava as a chemopreventive agent can proceed.

We evaluated the effect of kava on lung cancer development induced by NNK plus B[*a*]P in A/J mice following an established protocol (47). Three kava treatment regimens at a dose of 10 mg/g diet all resulted in a significant reduction of tumor multiplicity in NNK and B[*a*]P–treated mice (Table 1). These results clearly show a chemopreventive effect of kava against lung tumorigenesis in the A/J mouse model, and provide a basis for future evaluation of the potential of kava as a chemopreventive agent against lung cancer and possibly other malignancies. In particular, kava effectively reduced tumor number by 49% even with post-carcinogen treatment, showing its chemopreventive potentials to help former smokers prevent lung cancer development. Its chemopreventive efficacy compares fairly favorably over other chemopreventive candidates, such as phenethyl isothiocyanate (55) and polyphenon E (56), which reduce lung tumor multiplicity by ~45% with post-carcinogen treatment at doses of 25 and 20 mg/g diet, respectively.

Currently, we do not know the identity of the chemopreventive candidates in kava. Generally, six kavalactones, desmethoxyyangonin, dihydrokawain, dihydromethysticin, kawain, methysticin, and yangonin, are the major components in kava root, accounting for ~60% of the mass balance of the organic extract (26). Three chalcones, flavokawains A, B, and C, are minor components, accounting for ~1% of the organic extract (57). Among the different components in kava, the kavalactones seem to be the most potent inhibitors against cytochrome *P*450s (58). With respect to suppressing NF-κB activation, flavokawains A and B are the most potent compounds (18). The kava lactones and flavokawains are all potentially chemopreventive, some of which are under evaluation.

Based on the fact that all of the three kava treatment regimens significantly reduced lung tumor multiplicities, kava may prevent NNK and B[*a*]P–induced lung tumorigenesis through blocking the initiation phase and suppressing the promotion phase. To further explore the mechanisms of lung cancer chemoprevention by kava, we evaluated the effect of kava treatment on cancer cell proliferation because increased proliferation is a hallmark of tumor development and inhibition of proliferation is one way for assessing the efficacy of chemopreventive agents (59, 60). PCNA was selected to evaluate the proliferation status of the tumor tissues because PCNA is a cofactor of DNA polymerase that is required for DNA replication and DNA nucleotide excision repair, a well-known marker of cellular proliferation (61, 62). We observed that

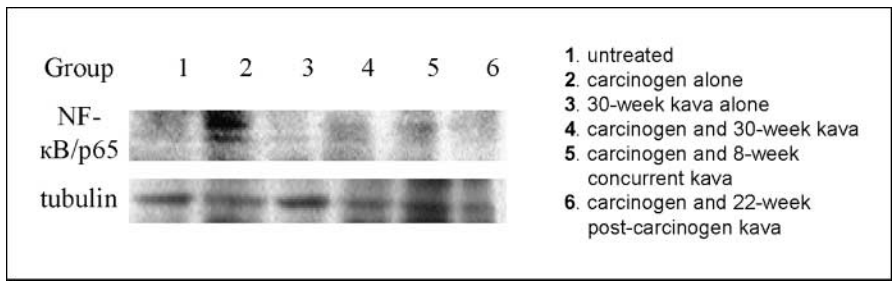

Fig. 5. *In vivo* inhibitory effect of oral kava on NF-κB in lungs of A/J mice (lung tumor tissues in groups 2 and 4–6 and normal lung tissues in groups 1 and 2). Immunoblot of p65, the active NF-κB, in the lung tumors. Reactive protein bands were visualized by enhanced chemiluminescence detection system, and membrane was stripped and probed with tubulin as loading control.

Table 2. Effect of kava on liver in A/J mice

Group	Absolute liver weight (g ± SD)	Relative liver weight (% of body weight, mean ± SD)	Liver enzymatic activities		
			ALT ± SD	AST ± SD	GGT ± SD
1 (untreated control)	1.50 ± 0.14	5.91 ± 0.61	89.8 ± 6.9	323 ± 48	7.0 ± 0
2 (carcinogens alone)	1.73 ± 0.28	6.93 ± 1.29*	99.7 ± 26.0	408 ± 66	7.0 ± 0
3 (30-wk kava alone)	1.46 ± 0.21	6.04 ± 0.86	83.1 ± 6.1	354 ± 12	7.0 ± 0
4 (carcinogens and 30-wk kava)	1.42 ± 0.20	6.07 ± 0.85†	192 ± 154	501 ± 201	7.0 ± 0
5 (carcinogens and 8-wk concurrent kava)	1.56 ± 0.27	6.38 ± 1.18	154 ± 123	439 ± 75	7.0 ± 0
6 (carcinogens and 22-wk post-carcinogen kava)	1.55 ± 0.15	6.38 ± 0.65	150 ± 143	385 ± 98	7.5 ± 1.0

NOTE: The liver weight and enzymatic activities were measured at the time of sacrifice. The comparison is between groups 1 and 2, groups 1 and 3, and group 2 with groups 4, 5, or 6. The only comparisons that showed statistically significant difference are the liver weights of group 1 versus group 2 and group 2 versus group 4. No other comparison showed any statistically significant difference, including liver weight and the enzymatic activities of the three enzymes.

*$P = 0.0019$.

†$P = 0.012$.

the lung tumors of the mice treated with NNK and B[*a*]P had significantly increased PCNA levels (Fig. 3), indicating elevated proliferation rate in lung tumor tissues compared with normal lung tissues. The lung tumors from kava-treated mice all had significantly reduced levels of PCNA, especially those mice with whole-course or post-carcinogen kava treatment. The relative small reduction in the 8-week concurrent kava treatment is expected because kava treatment was stopped 22 weeks before the time for tumor analyses. We expect the effect of kava on tumor growth to diminish over time. Nevertheless, the reduction of PCNA in lung tumors of kava-treated mice suggests that kava suppresses the proliferation of lung cancer cells.

Other than increased rate of proliferation, evasion of apoptosis is another hallmark of tumorigenesis, and induction of apoptosis is one mechanism whereby chemopreventive agents suppress tumorigenesis (52, 63, 64). Activation of caspase-3 and cleavage of PARP are two well-known markers of apoptosis (65, 66). The three kava treatment regimens all resulted in a marked increase in active caspase-3 and cleavage of PARP in the lung tumors (Fig. 4). Similar to the reduction of PCNA, relatively more increase of caspase-3 and cleavage of PARP were observed among the mice with whole-course or post-carcinogen kava treatment (groups 4 and 6) whereas those changes among the mice with carcinogen-concurrent kava treatment were relatively smaller (group 5). The kava-induced activation of caspase-3 and cleavage of PARP in lung tumor tissues indicate that kava induces apoptosis in lung cancer cells.

To explore potential upstream signaling pathways involved in the chemopreventive effect of kava, the effect of kava on p65 (the active form of NF-κB) was evaluated because activating the NF-κB pathway is one mechanism for carcinogen to induce tumorigenesis (19–23) and kava has been shown to inhibit NF-κB activation (17, 18). Consistent with the notion that activation of NF-κB is involved in tumorigenesis, NNK and B[*a*]P treatment greatly increased the level of p65 in the lung tumor tissues in the A/J mouse model (Fig. 5; group 2). All three kava treatment regimens effectively reduced the level of p65 to background level (Fig. 5; groups 4-6). The reduction of p65 implies that kava treatment inhibits the activation of NF-κB induced by NNK and B[*a*]P.

The reduction in cell proliferation and p65 and the increase in apoptosis in lung tumors seem to correlate with the reduction in tumor multiplicity induced by kava. Because promoting proliferation and suppressing apoptosis are two potential downstream responses of NF-κB activation (67, 70), our current data support the notion that one mechanism for kava to prevent lung tumorigenesis induced by NNK and B[*a*]P in A/J mice is to inhibit the activation of the NF-κB pathway, leading to inhibition of proliferation and activation of apoptosis. Future studies will be necessary to determine the potential involvement of other signaling pathways for the chemopreventive effect of kava.

To evaluate the safety of kava, especially potential hepatotoxicity, we monitored the mice for diet consumption; body and liver weight; enzymatic levels of ALT, AST, and GGT; and liver histopathology. Kava treatment has no effect on diet consumption by A/J mice, indicating that kava did not change the palatability of the diet. Kava treatment alone had a 5% body weight loss (Fig. 2; group 1 versus group 3), whereas the three kava treatment regimens among carcinogen-treated mice resulted in 3% to 7% body weight loss (group 2 versus groups 4-6). Such an extent of body weight reduction unlikely contributes to the observed reduction of tumor multiplicities because quite a few candidates, including sulforophane (52), benzyl isothiocyanate-*N*-acetylcysteine and phenethyl isothiocyanate-*N*-acetylcysteine (45), *N*-acetylcysteine (71), and isotretinoin (72), all could induce 5% to 20% body weight loss in A/J mice at specific dosages, whereas such treatments do not affect the lung tumor multiplicity induced by NNK, B[*a*]P, or tobacco smoke. Reduction in body weight is also not uncommon for chemopreventive candidates: Phenethyl isothiocyanate (52), selenium compounds (73, 74), indole-3-carbinol (47), budesonide (75), and dexamethasone (50) all induce >10% body weight loss.

Kava treatment alone also did not affect liver weight or enzymatic levels of ALT, AST, and GGT (Table 2; group 1 versus group 3). Although mice treated with NNK + B[*a*]P and kava had slightly smaller livers, on average, than mice treated with NNK + B[*a*]P alone (group 2 versus group 4-6), this is considered to be of dubious significance as liver weights for all kava-treated groups were comparable to the untreated control mice, whereas mice treated with NNK and B[*a*]P (group 2) had slightly elevated liver weights (~16% increase) compared with control mice (group 1). There was substantial variability in serum ALT and AST in the carcinogen-treated groups but the differences were not statistically significant. Microscopic examination of livers from mice treated with standard diet and mice treated with kava-supplemented diet (group 1 versus group 3 and group 2 versus group 4) did not reveal any lesions suggestive of kava toxicity.

Although hepatotoxicity was not rigorously evaluated in this study, our current data on kava were not suggestive of hepatotoxicity, consistent with the results of previous long-term animal studies and clinical studies of kava (24, 35, 76, 80). In contrast, a recent study by Clayton et al. (81) found evidence of modest increases in liver weight and hepatocellular hypertrophy in F344 rats at comparable dose of kava. These discrepancies may derive from species differences, kava sources, or administration regimens, highlighting the needs for further studies to more clearly define its safety. Given that there is controversy about the reported clinical cases of hepatotoxicity (e.g., the Traditional Medicines Evaluation Committee contended the withdrawal of kava was premature and proposed to reopen kava usage in the United Kingdom; ref. 36), there is an added impetus for definitive studies characterizing the safety of kava.

In conclusion, the results from this study clearly show that oral consumption of kava effectively reduced lung tumor multiplicity induced by NNK and B[*a*]P in the A/J mouse model, mechanistically through induction of apoptosis and suppression of proliferation possibly via inhibition of the NF-κB pathway. Furthermore, our preliminary data are not suggestive of hepatotoxicity. This study, for the first time, established the chemopreventive activity of kava against lung tumorigenesis. Combined with its strong epidemiologic data, kava is a promising chemopreventive agent against human lung cancer warranting further evaluations. At the same time, the safety of kava, especially with regard to hepatotoxicity, needs to be rigorously examined *in vivo* before any clinical evaluation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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