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Dietary 5-Demethylnobiletin Inhibits Cigarette Carcinogen NNK-Induced Lung Tumorigenesis in Mice

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Abstract

5-Demethylnobiletin (5DN) is a unique citrus flavonoid with various beneficial bioactivities. In this study, we determined the inhibitory effects of 5DN and its two major metabolites in the 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumorigenesis mouse model as well as in human and mouse lung cancer cell models. In NNK-treated female A/J mice, dietary administration of 5DN (0.025% or 0.05% w/w in diet) significantly decreased both lung tumor multiplicity and tumor volume. Immunohistochemical analysis showed strong anti-proliferative effect of 5DN in lung tumors. Two major metabolites of 5DN, named 5,3'-didemethylnobiletin (M1) and 5,4'-didemethylnobiletin (M2), were found in the lung tissue of 5DN-fed mice. Cell culture studies demonstrated that 5DN, M1 and M2 significantly inhibited the growth of human and mouse lung cancer cells by causing cell cycle arrest, inducing apoptosis and modulating key signaling proteins related with cell proliferation and cell death. Interestingly, the metabolites of 5DN, especially M1 produced much stronger inhibitory effects on both human and mouse lung cancer cells than those produced by 5DN itself. Our results demonstrated that dietary administration of 5DN significantly inhibited NNK-induced tumorigenesis in mice, and this effect may be partially associated with the metabolites of 5DN in lung tissues.

Graphical Abstract

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M.S., X.W., and H.X. have conceived and designed experiments, M.S., X.W., N.C., M.W., J.Z., Z.G., F.X., Z.L., F.L., and J.Z. have performed the experiments, M.S. and H.X. have analyzed the data and written the manuscript. All authors read and approved the final manuscript.

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Our results showed that dietary administration of 5-demethylnobiletin (5DN, a unique citrus flavonoid) inhibited cigarette carcinogen-induced lung tumorigenesis in mice. Two major metabolites of 5DN, named 5,3'-didemethylnobiletin (M1) and 5,4'-didemethylnobiletin (M2), were found in the lung tissue of 5DN-fed mice. It was found that 5DN, M1 and M2 significantly inhibit the growth of human and mouse lung cancer cells by inducing cell cycle arrest and apoptosis, with M1 and M2 being much stronger in producing these effects than 5DN.

Keywords

5-Demethylnobiletin; Lung tumorigenesis; Metabolites; Chemoprevention

1. Introduction

Lung cancer, a major public health problem, is the leading cause of cancer mortality in both men and women in the United States.¹ However, human trials on the prevention and treatment of lung cancer have not produced satisfactorily promising results. The 5-year survival rate for lung cancer patients (18.1%) is significantly lower than many other leading cancer sites, including the colon and rectum (65.1%), breast (89.8%) and prostate (98.7%).² Therefore, there is an urgent need of effective strategies for the prevention of lung cancer. Nearly 90% of all lung cancers are directly linked to cigarette smoking.³ Among more than 60 carcinogens in cigarette smoke,⁴ 4-methylnitrosamino-1- β -pyridyl-1-butanone (NNK) is considered to be the most potent etiological factor in the initiation and progression of lung carcinogenesis in human.⁵⁻⁷ Lung tumors induced by NNK in the female A/J mice share many histopathologic and molecular characteristics with human lung carcinogenesis, and therefore provides an excellent model for lung cancer chemoprevention study.⁸

Polymethoxyflavones (PMFs) are a unique class of flavonoids specifically found in the citrus fruits such as sweet oranges (*Citrus sinensis*) and mandarin oranges (*Citrus reticulata*).⁹ Studies have shown that PMFs exhibit a series of biological activities including anti-carcinogenic, anti-inflammatory, and antiviral effects.¹⁰⁻¹³ Among more than 20 different citrus PMFs identified, 5-demethylated PMFs are a unique subclass that has been associated with many beneficial biological activities. For example, potent inhibitory effects of 5-demethylated PMFs have been demonstrated by us and others on multiple types of human cancer cells.¹⁴⁻²¹ 5-Demethylnobiletin (5DN) is one of the most abundant 5-demethylated PMFs from citrus fruit. Studies have showed strong inhibitory effects of 5DN on human

lung cancer in both *in vitro* cell culture models and an *in vivo* xenograft model^{14,21,22}. However, no information is available on the effects of 5DN on cigarette carcinogen-induced lung tumorigenesis.

Biotransformation of dietary bioactive components is crucial for their *in vivo* biological effects. Studies have showed that the metabolites of flavonoids may have significant bioactivities after oral administration.^{23,24} In the previous study, we identified three major metabolites of 5DN in the urine of 5DN-fed mice (Fig. 1), i.e. 5,3'-didemethylnobiletin (M1), 5,4'-didemethylnobiletin (M2) and 5,3',4'-tridemethylnobiletin (M3), and they were mainly in the conjugated forms as glucuronides and/or sulfates.²⁵ Interestingly, these metabolites in free forms were found to be more potent in inhibiting the growth of human colon cancer cells in culture than their parent compound 5DN.²⁵ In this study, for the first time, we investigated the inhibitory effects of 5DN on the NNK-induced lung tumorigenesis in female A/J mice. Furthermore, we identified the major metabolites of 5DN in the lung tissue of 5DN-fed mice, and examined their effects on the growth of both human and mouse lung cancer cells.

2. Materials and Methods

2.1. Animals, diets, and experimental design

The protocol of this animal study was approved by Institutional Animal Care and Use Committee of University of Massachusetts Amherst (#2014-0079). Five-week-old female A/J mice (18–22g) were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in plastic cages (5 mice/cage) with free access to water and a basal diet, under controlled conditions of 22±2 °C and 50±10% humidity in a 12-h light-dark cycle. The mice were randomly divided into four groups as detailed in fig. 2, and maintained on AIN-76A diet (Dyets, INC., Bethlehem, PA), ad libitum, for 1 week. Studies have showed that AIN-76A diet provide more numerous and reproducible lung tumors in response to NNK than other commercial rodent diets in A/J mouse strain.^{26,27} After 1-week acclimation, the mice in positive control group and in 5DN treatment group received an i.p. injection of saline containing NNK (100mg/kg body weight). Mice in negative control received an equal volume of saline (vehicle control). Two days after NNK injection, the mice in treatment groups were fed with the AIN-76A diets supplemented with 5DN (0.025% or 0.05% w/w), and the mice in vehicle control group and NNK positive control group were maintained on the standard diet. Sixteen weeks after NNK injection, all mice were sacrificed. Liver and spleen were harvested and weighted. Intact lungs were excised and analyzed for tumor number and size under dissection microscope. A part of the lungs without tumors were carefully dissected and rapidly frozen for metabolites analysis, and the rest of lungs were infused with 10% neutral buffered formalin for 48 hours, then transferred to 80% alcohol. The results were present as the percent of mice with lung tumors (incidence), the mean number of tumors per mouse ± SEM (multiplicity) and the mean volume of tumors ± SEM (calculated according the formula: $V=4/3\pi r^3$; r=radius, the average of the longest and shortest diameters).

2.2. Tumor Histology and Immunohistochemical analysis

The fixed lung tissue were embedded in paraffin, sectioned (5 μ m) and stained with hematoxylin and eosin (H&E) for histopathological analysis by a trained pathologist blinded to the study design. Lung proliferative lesions were diagnosed as alveolar cell hyperplasia and adenoma according to established criteria²⁸. The lung tumors were not sub-classified into adenoma and adenocarcinoma because it was impractical to evaluate malignancy at 16 weeks after NNK injection.^{29,30} Immunohistochemical analysis was performed in the lung tissue sections^{31–33}. Cell proliferation in the lung tissue was determined by positive staining of proliferating cell nuclear antigen (PCNA) and p21. Briefly, paraffin sections were deparaffinized in xylene, rehydrated through graded ethanol solutions, and washed in PBS. Antigen retrieval was carried out by heating the slides in 0.01 mol/L citrate buffer (pH 6.0) for 20 minutes in a PT Module antigen retrieval device (Thermo Fisher Scientific, Agawam, MA, USA). Endogenous peroxidase was quenched in 0.3% hydrogen peroxide. Nonspecific binding was blocked by incubation with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA). Then, sections were incubated overnight at 4 °C with antibodies against PCNA (1:1000, Dako, Denmark) or p21 (1:200, Cell Signaling Technology, Danvers, MA, USA). After thorough washes with PBS, sections were incubated with a biotinylated secondary antibody for 30 minutes at the room temperature and subsequently incubated with an avidin-biotin complex reagent (Dako, Denmark) and then with the chromogen 3-diaminobenzidine (DAB) for 5 minutes. Sections were then counterstained with hematoxylin for 3 minutes. Positive staining was observed using a Nikon E400 microscope, and digital computer images were recorded with a SPOT Idea 1.3 Mp camera.

To calculate the proliferation index and p21-positive index, five random microscopic fields of 300 $\times$ magnification were selected from each sample slide. Proliferation index/p21-positive index is the percentage of PCNA/p21 positive staining cells in 500 cells counted in each field.

2.3. Quantification of 5DN and its metabolites in lung tissues

Lung tissues (500 mg for each sample) were homogenized in methanol (50% in phosphate buffered saline, pH=5.00) by an OMNI bead rupter (OMNI International, NW Kennesaw, GA, USA) and then extracted with ethyl acetate for three times. Pooled ethyl acetate fractions were dried under vacuum and reconstituted in 50% methanol. Identification and quantification of 5DN and its metabolites were carried out by using an HPLC with an electrochemical detector as we previously described.^{25,34} Synthesized compounds M1, M2, and M3, with purity greater than 98% were used as standards to quantify the metabolites of 5DN in lung tissues. Standard curves of the standard compounds were constructed by plotting concentrations (x axis, μ M) versus peak areas (y axis, μ C). Quantification of the metabolite in lung tissues were performed by comparing their peak areas with the standard stock solutions of series of concentrations., Tangeretin (>98%) was used as an internal standard.

2.4. Analysis of cell viability, cell cycle and apoptosis

Assays for cell viability, cell cycle and apoptosis were conducted as we previously described.^{14,15,35} A549 human lung cancer cells were purchased from American Type Cell

Collection (ATCC, Manassas, VA, USA). CL13 mouse lung cancer cells were derived from NNK-induced lung tumors, and were from Dr. Steven A. Belinsky (Lovelace Respiratory Research Institute, Albuquerque, NM). Both cell lines were cultured in RPMI 1640 media. In brief, cancer cells were seeded in 96-well plates. After 24 h, cells were treated with 5DN or its metabolites, and the cell viability was quantified by MTT method. Cells were seeded in 6-well plates for cell cycle and apoptosis analysis. After 24 h of incubation for cell attachment, cells were treated with 5DN or its metabolites. Floating cells and adherent cells were combined to be subject to cell cycle and apoptosis analysis by flow cytometry as we described previously.^{14,15,21} DMSO was used as vehicle to deliver 5DN and its metabolites to the cells. The final concentration of DMSO in all experiments was 0.1% v/v in cell culture media.

2.5. Immunoblotting

Whole cell lysate were prepared following the procedure we described previously.^{14,15,21} Briefly, cells were seeded in 150mm cell culture dishes then treated with 5DN and its metabolites after 24 hours incubation. After another 24 or 48 hours, cells were collected with cell scrapers and subject to Western blotting analysis.

2.6. Statistical analysis

Data were presented as mean $\pm$ SEM or mean $\pm$ SD. Student's t-test was used to test the mean difference between two groups, whereas analysis of variance (ANOVA) followed by Tukey HSD test was used for the comparison of differences among three or more groups. Tumor incidence was analyzed by Fisher's exact probability test. Differences were considered statistically significant when $p < 0.05$.

3. Results and Discussion

3.1. Dietary 5DN did not exhibit toxicity in mice

To establish the chemopreventive effects of 5DN, NNK-treated female A/J mice were subjected to dietary treatment of 5DN (0.025% and 0.05% w/w in diet) for 16 weeks. By using the formula emanated from Reagan-Shaw *et al*, the human dose equivalent could be converted from the dose used in a mouse based on body surface area for a 60 kg person,³⁶ These two mouse doses were equivalent to 125 mg and 250 mg of 5DN per day in human approximately, which can be conveniently achievable through dietary supplementation. All mice survived the entire experimental period. During the treatment period, no significant difference was observed in the appearance or behavior of the mice between 5DN-treated groups and control groups. The body and selected tissue weights of the mice in all groups at the termination of the study were shown in Table 1. There was no significant difference in body weight, spleen weight or spleen relative weight (%RW) among all groups, suggesting that there is no apparent toxicity by dietary 5DN treatment. However, the liver weight of the mice from the positive control group was significantly lower than that of other groups. Similar results were observed by Kim *et al*²⁹ and Li *et al*,³⁷ suggesting that NNK injection had caused a decrease in liver weight, and 5DN treatment ameliorated this effect. Overall, dietary administration of 5DN did not exhibit noticeable toxicity in female A/J mice.

3.2. Dietary 5DN inhibited tumorigenesis in NNK-treated mice

After NNK or vehicle control injection, mice were maintained for 16 weeks. Our results (Tab.2) showed that lung tumors had developed in 100% of the mice in the positive control group, whereas no lung tumor was found in the negative control mice. All lung tumors were identified as solid adenomas based on the histological analysis, which was consistent with previous reports.^{29,33,38} Dietary 5DN treatments at 0.025% or 0.05% did not reduce the tumor incidence. However, 0.05% of 5DN significantly decreased the tumor multiplicity from 14.91 ± 1.04 to 11.72 ± 0.94 in comparison to the positive control group. Moreover, dietary administration of 5DN showed a dose-dependent inhibition of tumor volume, i.e. 0.025% and 0.05% of 5DN decreased the tumor volume by 33.6% (from 0.4220 ± 0.0255 to 0.2800 ± 0.0132) and 52.4% (from 0.4220 ± 0.0255 to 0.2800 ± 0.0132), respectively. Representative images of lung tumors from positive control group and 0.05% of 5DN treatment group have been shown in Fig 3. H&E staining showed that the solid adenomas in the positive control group (Fig 3A) arranged into clusters and had varying degrees of differentiation, and normal alveolar architecture was lost. In contrast, we observed that the lungs from 0.05% of 5DN treatment group (Fig 3A) had a moderate decrease in the development of solid adenoma, and were without significantly losing the alveolar architecture.

Uncontrolled cell proliferation is one of hallmarks of carcinogenesis. PCNA expression is increased during cell duplication and plays a critical role in regulating DNA replication, therefore it is often used as a key marker for cell proliferation.³⁹ We hereby determined the effects of 5DN on the expression level of PCNA in lung tissues (Fig. 3B and D). Our results showed a high PCNA labeling index in lung tumors of the mice in the positive control group, indicating a high cell proliferation rate (Figure 3B). However, the PCNA labeling index was significantly lower in the lung tumors of the mice treated with 5DN in comparison to that of positive control mice. Quantification of PCNA staining showed a labeling index of 46 ± 3.2 in tumors of mice in positive control group as compared with 23.6 ± 2.6 in mice treated with 5DN, reflecting a 48.7% decrease in the proliferation index (Figure 3D). p21 is a key cyclin-dependent kinase inhibitor involved in cell cycle progression and tumorigenesis.⁴⁰ In humans, loss or decrease of expression of p21 correlated with poor prognosis of lung cancer.⁴¹ We examined the effects of 5DN on p21 expression in NNK-induced lung tumors. As shown in figure 3C, the expression of p21 can be detected in the lung tumors of positive control mice. The expression level of p21 in the lung tumors of 5DN-treated mice was higher than that of the positive control mice. Quantification of p21 staining showed dietary administration of 5DN significantly elevated the p21 protein expression level in lung tumors by 41.2% in comparison to that of positive control mice. Increased p21 level may lead to lower cell proliferation by inducing cell cycle arrest.

3.3. Quantification of 5DN and its metabolites in the lung tissues of 5DN-fed mice

The metabolism of bioactive dietary compounds, including flavonoids, is crucial for their biological activities, because the metabolites produced from the parent compounds after oral ingestion may have different biological activities than the parent compounds. Therefore, metabolism may significantly alter the overall bioactivity of the parent compounds. Our previous study has identified three major metabolites of 5DN in the urine of 5DN-fed mice,

namely M1, M2 and M3 (Fig. 1), and these metabolites, especially M1 showed much stronger inhibitory effect on the growth of human colon cancer cells.²⁵ To better understand the chemopreventive effects of 5DN against lung tumorigenesis, we determined the levels of 5DN and its metabolites in the lung tissue of NNK-treated mice fed with 5DN, using HPLC method we established previously.^{25,34} This is because that metabolites found in the lung tissue are likely responsible for the biological activities observed in the lung. HPLC profiles of lung tissue samples from mice fed with or without 5DN were shown in figure 4. Lung tissue samples from the positive control group showed no apparent peaks, whereas there were three major peaks shown in the chromatogram of the lung tissue samples from mice fed 0.05% of 5DN. By comparing to the chromatogram of chemical standards of 5DN and its metabolites, the 3 peaks were identified as M1, M2, and 5DN at retention times of 11.9, 13.6 and 14.9 min, respectively. M3 was not detected in the lung tissue. The levels of 5DN, M1 and M2 in the lung tissue were shown in table 3. The results suggested that there were considerable amount of 5DN (1.56 nmol/gram of tissue) and its metabolites M1 (0.034 nmol/gram of tissue) and M2 (0.15 nmol/gram of tissue) distributed in lung tissue after dietary consumption of 5DN. This is very useful information because it demonstrated the presence of metabolites M1 and M2 in the lung tissues, indicating the potential role of these metabolites in inhibiting lung tumorigenesis. Based on their chemical structures, M1 and M2 could be formed from 5DN through demethylation at 3' and 4' positions, respectively. It is possible that there were other metabolites of 5DN in the lung tissue but they were too trace to be detected.

3.4. 5DN and its metabolites induce cell cycle arrest and apoptosis in lung cancer cells

Due to the fact oral administration of 5DN resulted in the presence of 5DN as well as its metabolites M1 and M2 in the mice lung tissues, we hypothesized that the metabolites might contribute to the inhibitory effects of dietary 5DN on lung tumorigenesis. Our previous studies have demonstrated that 5DN metabolites possessed potent inhibitory effects on the growth of two human lung cancer cell lines H1299 and H460. However, to better understand the roles of 5DN metabolites in inhibiting NNK-induced lung tumorigenesis, herein we studied the effects of 5DN and its metabolites (M1 and M2) on the growth of human (A549) and mouse (CL13) lung cancer cells. This is because that: (i) CL13 cells were derived from NNK-induced mouse lung tumors, therefore, serve as an excellent *in vitro* cell culture model closely related to NNK-induced mouse lung tumorigenesis; and (ii) A549, a human bronchioalveolar carcinoma cell line, was widely used in studies related to tobacco carcinogen NNK-induced lung tumorigenesis and associated carcinogenic mechanisms.^{42,43} Therefore, the effects of test compounds on A549 and CL13 cells may better represent what might occur during NNK-induced lung tumorigenesis. As shown in Figure 5, 5DN and its metabolites inhibited the growth of both human and mouse lung cancer cells in a dose-dependent manner. Interestingly, the metabolites M1 and M2 showed stronger inhibitory effect than 5DN. The IC₅₀ value of 5DN after 72 hours of treatment was 21.8 μ M in CL13 cells, however, the IC₅₀ values of M1 and M2 were 1.3 μ M and 5.6 μ M, respectively. The results indicated that M1 and M2 were 16.5-fold and 3.9-fold stronger than 5DN in inhibiting the growth of CL13 cells, respectively. The similar results were also observed in A549 cells. The much higher potency of M1 and M2 than 5DN suggested that they might play important roles in inhibiting lung tumorigenesis even their tissue levels in the lung were

lower than that of 5DN in mice. The effective concentrations of M1 and M2 in inhibiting the growth of lung cancer cells in culture used in this study were generally higher than the concentrations of M1 and M2 found in the lung tissue of 5DN-fed mice (if we assume 1 gram of tissue gives 1 mL in volume). However, it should be stressed that the tissue concentrations of M1 and M2 were achieved by an *ad libitum* dietary intake of 5DN by the mice throughout a 16-week of feeding period. It could be speculated that a long-term *in vivo* tissue exposure at the relatively lower concentrations might result in similar or even stronger biological effects caused by a short-term *in vitro* cellular exposure at the higher concentrations.

To further understand the mechanisms by which 5DN and its metabolites inhibit the cancer cell growth, cell cycle and apoptosis analysis was conducted on cancer cells treated with 5DN, M1 or M2 at concentrations that produced similar degree of growth inhibition on the cells. As showed in Figure 6, in A549 cells, 5DN at 20 μ M and M2 at 10 μ M significantly increased cell population in G1/G0 phase, and decreased cell population in S and G2/M phase, with M2 at much lower concentration led to higher G1/G0 cell population than 5DN. In contrast, M1 at only 2 μ M significantly increased G2/M cell population of A549 cells. In CL13 cells, treatment with 5DN at 20 μ M increased cell population in G2/M phase, while much more increase in G2/M cell population was caused by M1 at much lower concentration (1 μ M). M2 at 5 μ M modestly increased both G1/G0 phase and G2/M phase cell population. These results demonstrated that 5DN, M1 and M2 caused cell cycle arrest at different phases, and these effects were cell type specific as well. It is important to realize that small difference in the chemical structures of these compounds led to high degree of difference in their potency and mode of actions. Future research is warranted to investigate the combined effects of 5DN and its metabolites in tumor inhibition. This is because that the co-existence of these compound in the lung tissue may led to favorable interaction among them to produce enhanced tumor inhibition as they may have complementary mode of actions.

To determine the extent to which cellular apoptosis contributed to the growth inhibition by 5DN and its metabolites, we quantified apoptosis in lung cancer cells treated by these compounds by Annexin-V/PI double staining assay using flow cytometry. As shown in Figure 7, 5DN and its metabolites increased both early and late apoptotic cells in A549 cell line. However, M1 and M2 at lower concentrations showed much stronger effects 5DN 5DN. 5DN at 20 μ M increased early apoptotic cell population by 4-fold compared to the control. M1 at 2 μ M and M2 at 10 μ M dramatically increased early apoptotic cells by 42.8-fold and 24.2-fold compared to the control, respectively. Moreover, M1 and M2 also increased late apoptotic cell population by 5.5-fold and 9.5-fold compared to the control, respectively, whereas 5DN only increased it by 1.75-fold compared to the control. The results also showed that M1 at 2 μ M, M2 at 10 μ M induced total cellular apoptosis that were 22.2-fold and 5.9-fold stronger than that produced by 5DN at 20 μ M, respectively. Similar trend was also observed in CL13 mouse lung cancer cells. Overall, M1 and M2 showed much stronger potency in inducing cellular apoptosis in both human and mouse lung cancer cells than their parent compound 5DN. There were significant difference between M1 and M2 in terms of their inhibitory effects against cancer cells, which was apparently related to the difference in their chemical structures. However, it remains unknown why such small difference could

result in such big difference in biological activities between M1 and M2. Nevertheless, our findings suggested that M1 and M2 might play important roles in inhibiting lung tumorigenesis by inducing apoptosis.

3.5. 5DN and its metabolites modulated key signaling proteins related to cell proliferation and apoptosis.

To further elucidate the molecular mechanisms of the inhibitory effects caused by 5DN and its metabolites on lung cancer cells, we examined their effects on the expression of key proteins related to cell cycle progression and apoptosis in both human and mouse lung cancer cells. Cell cycle related proteins p21^{Cip1/Waf1} and p53 were analyzed after 24 hours of treatment while apoptosis related proteins cleaved caspase-3 and cleaved PARP were analyzed after 48 hours of treatment. Our results demonstrated that 5DN (20 μ M) and its metabolites M1 (2 μ M in A549 cells, 1 μ M in CL13 cells) and M2 (10 μ M in A549 cells, 5 μ M in CL13 cells) caused extensive changes in these signaling proteins (Fig. 8). In both cell lines, 5DN, M1 and M2 significantly increased expression levels of p21^{Cip1/Waf1} and p53, except that 5DN only caused moderate increase in p21^{Cip1/Waf1} expression level in A549 cells, and M2 slightly increased p53 expression level in CL13 cells. The loss of cell cycle regulation is one of distinct features of cancer cells. p21^{Cip1/Waf1} is a key inhibitory protein of Cip/Kip family which bind to CDK alone or to the CDK-cyclin complex to regulated CDK activity and thus control the cell cycle progression.⁴⁴ p53 is a tumor suppressor which has a broad range of cellular functions and plays important roles in inhibiting carcinogenesis⁴⁵ by inducing cell cycle arrest,⁴⁶ cellular apoptosis,⁴⁷ and DNA repair.⁴⁸ Increased expression of p53 can result in G2/M cell cycle arrest by downregulating of both cyclin B1 and Cdc2.^{46,49} Moreover, the expression of p21^{Cip1/Waf1} is under transcriptional control by the p53 tumor suppressor gene. The p21 gene promotor contains a p53-binding site which allows p53 to transcriptionally activate the p21 gene.⁵⁰ The p53-mediated upregulation of p21^{Cip1/Waf1} inhibits the activity of Cdk2- and Cdk4-cyclin complexes, thereby causing cell cycle arrest at G0/G1 phase. The p21^{Cip1/Waf1} can also inhibit Cdc2-cyclin B complexes and PCNA, interfering with PCNA-dependent DNA polymerase activity, thereby inhibiting DNA replication and modulating DNA repair processes.⁵¹ One or both of these actions can contribute to a G2/M cell cycle arrest following ectopic p21^{Cip1/Waf1} expression or DNA damage.^{52,53} Our immunoblotting results illustrated that the cell cycle arrest (G0/G1 or G2/M) in A549 and CL13 cells caused by 5DN, M1 and M2 were associated with p53-depended induction of p21^{Cip1/Waf1}. In A549 cells, 5DN treatment has moderated effects in increasing p21^{Cip1/Waf1} expression level, however it resulted in a significant increase of p53 expression. The induction of p53 will stimulate the transcription of different genes including Mdm2 and Bax along with p21^{Cip1/Waf1}, which may contribute to the G0/G1 cell cycle arrest caused by 5DN.

Research has shown that apoptosis and the genes that control it have a profound effect on the malignant type. It is now clear that disrupting apoptosis may lead to tumor initiation, progression and metastasis.⁵⁴ Thus triggering apoptosis has become one important strategy for cancer prevention. The central engines of apoptosis are the caspases, cascades of cysteine aspartyl proteases that implement cell death by cleaving a variety of intracellular substrates that trigger programmed cell death.⁵⁵ Activation (cleavage) of caspase-3 can lead to the

activation (cleavage) of other effector proteins, such as poly ADP ribose polymerase (PARP), which ultimately trigger apoptosis by interfering chromatin condensation and DNA fragmentation.⁵⁶ We here found that 5DN, and its metabolites M1 and M2 intensively activated caspase3 as well as its final protein target PARP in both A549 and CL13 cells. Moreover, M1 and M2 showed much stronger effect in terms of inducing activation of caspase-3 and PARP when compared to its parent compound 5DN. These results were consistent with the effects of 5DN, M1 and M2 on cellular apoptosis (Fig. 7). Overall our findings demonstrated that M1 and M2 at lower concentrations showed similar or stronger effects on key signaling proteins related with cell cycle progression and apoptosis in comparison with that of 5DN, suggesting their potential roles in inhibiting lung tumorigenesis.

4. Conclusion

In the present study, we showed that dietary administration of 5DN, a unique polymethoxyflavone from citrus fruit, significantly inhibited NNK-induced lung tumorigenesis in female A/J mice. To our knowledge this is the first demonstration of the chemopreventive effect of 5DN against lung tumorigenesis induced by cigarette carcinogen in mice. We also, for the first time, identified and quantified two major metabolites of 5DN in the lung tissue of 5DN-fed mice, namely M1 and M2. Furthermore, we demonstrated that these metabolites, M1 and M2 exhibited much stronger anti-cancer effects than 5DN. This was evidenced by their superior effects in inhibiting the growth of both human and mouse lung cancer cells, inducing cell cycle arrest, triggering apoptosis and modulating key signaling proteins. These findings strongly suggested that M1 and M2 could significantly contribute to the observed inhibitory effects of dietary 5DN on lung tumorigenesis in NNK-treated mice. In conclusion, this study provided a solid scientific basis for using 5DN as a potential chemopreventive agent against lung tumorigenesis in human.

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Abbreviations

5DN	5-demethylnobiletin
H&E	hematoxylin and eosin
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
PMFs	polymethoxyflavones
PCNA	proliferating cell nuclear antigen
PARP	poly ADP ribose polymerase

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Figure 1.

Chemical structures of 5-Demethylnobiletin and its three metabolites

Figure 2.

Experimental design to determine the inhibitory effect of 5DN on NNK-induced lung tumorigenesis in the A/J mouse model. Six-week-old mice were injected intraperitoneally with NNK (100mg/kg body weight) or saline after 1-week acclimation. 2 days after NNK injection, mice were fed with standard diet (AIN-76A) or standard diet containing 0.025% or 0.05% (w/w) 5DN till the end of the study. After 16 weeks on experimental diets, all mice were sacrificed and lungs were harvested for evaluation of tumor development. Detailed information was provided in Materials and methods.

Figure 3.

Effect of dietary 5DN on immunohistochemical staining of PCNA and p21 in NNK-induced lung tumors. Representative lung sections from positive control and 5DN treatment groups were shown. Data were presented as means $\pm$ SE and statistical significance was analyzed by Student's *t*-test. Proliferation index/p21-positive index was the average percentage of PCNA/p21 positive staining cells in 500 cells counted in five random microscopic fields of $300 \times$ magnification. (A) H&E stained lung sections from positive control and 5DN treatment groups. (B) PCNA staining of lung sections from positive control and 5DN

treatment groups. (C) p21 staining of lung sections from positive control and 5DN treatment groups. (D) Quantification of PCNA staining of lung sections from positive control and 5DN treatment groups. The asterisks in the bar charts indicated statistical significance in comparison with the control ($p < 0.05$, $n = 5$) by Student's-t test. (E): Quantification of p21 staining of lung sections from positive control and 5DN treatment groups. The asterisks in the bar charts indicated statistical significance in comparison with the control ($p < 0.05$, $n = 5$) by Student's-t test.

Figure 4.

Representative HPLC profile of lung tissue from positive control group (upper) and from 0.05% 5DN treated group (bottom). Lung tissue Samples were detected by electrochemical detector at 300mV. There is no apparent peak in chromatogram of positive control group. 3 major peaks in chromatogram of 0.05% 5DN treated group were identified as 5DN (retention times at 14.9 min) and its metabolites M1 (retention times at 11.9 min) and M2 (retention times at 13.6 min)

Figure 5.

Inhibitory effect of 5DN and its metabolites (M1 and M2) on the growth of A549 human (A) and CL13 mouse lung cancer cells (B). A549 and CL13 cells were seeded in 96-well plates and treated with serial concentrations of 5DN, M1 and M2. After 72 hours of treatments, cell viability was quantified by MTT assay as described in method section. Data represented mean $\pm$ SD (n=6), and asterisks indicated the statistical significance in comparison with the control cells (p<0.01)

Figure 6.

Effects of 5DN and its metabolites (M1 and M2) on cell cycle progression of A549 human (A) and CL13 mouse lung cancer cells (B). Cells were seeded in 6-well plates then treated with 5DN, M1 and M2. After 24h treatments, cells were harvested and subjected to cell cycle analysis as described in methods section. All data represented mean $\pm$ SD (n=3). Statistical analyses were conducted among control and treated groups in G0/G1, S and G2/M phases separately, and different notations in the bar charts indicated statistical significance ($p < 0.01$) according to ANOVA analysis followed by Tukey HSD test.

Figure 7.

Effects of 5DN and its metabolites (M1 and M2) on apoptosis of A549 human (A) and CL13 mouse lung cancer cells (B). Cells were seeded in 6-well plates then treated with different concentrations of 5DN, M1 and M2. After 48 hours of treatments, cells were collected and subjected to apoptosis analysis described in methods section. All data represented mean $\pm$ SD (n=3), and different notations in the bar charts indicated statistical significance ($p < 0.01$) according to ANOVA analysis followed by Tukey HSD test.

Figure 8.

Effects of 5DN, M1 and M2 on cell cycle and apoptosis related key proteins in human A549 (A) and mouse CL13 (B) lung cancer cells. Cells were seeded in 15cm culture dishes for 24 hours then treated with 5DN, M1 and M2 at different concentrations. After another 24 or 48 hours of incubation, cells were collected for immunoblotting as described in method section. The number underneath the blots represent band intensity (normalized to β -actin, means of three independent experiments) measured by Image Studio software. The SDs (all within $\pm 15\%$ of the means) were not shown. β -Actin was used as an equal loading control. Asterisks indicated statistical significance in comparison with control ($p < 0.05$, $n = 3$)

Table 1.

Final body, liver, relative liver, spleen and relative spleen weights

Group	Mice No.	Body weight (g)	Liver		Spleen	
			W (g)	% RW	W (g)	% RW
Negative control	10	23.8±3.0	1.04±0.22 ^a	4.38±0.56 ^a	0.078±0.018	0.32±0.08
Positive control	20	23.5±2.0	0.92±0.11 ^b	3.91±0.45 ^b	0.070±0.018	0.30±0.08
0.025% of 5DN	20	24.3±2.9	1.02±0.16 ^a	4.21±0.65 ^a	0.078±0.015	0.31±0.09
0.05% of 5DN	20	23.5±2.8	1.04±0.12 ^a	4.43±0.36 ^a	0.075±0.013	0.32±0.06

All values represented mean ± SEM, and different notation (a or b) indicated statistically significant difference (P<0.05) according to ANOVA analysis followed by Tukey HSD test. 5DN: 5-demethylnobiletin; W: weight; RW: relative weight

Table 2.

Effects of dietary 5DN treatment on NNK-induced lung tumorigenesis in mice

Group	Mice No.	Tumor incidence (%)	Tumor multiplicity	Tumor Volume (mm ³)
Negative control	10	0 (0/10)	0	0
Positive control	20	100 (20/20)	14.91±1.04 ^a	0.422±0.026 ^a
0.025% of 5DN	20	100 (20/20)	14.73±0.93 ^a	0.280±0.013 ^b
0.05% of 5DN	20	100 (20/20)	11.72±0.94 ^b	0.201±0.010 ^b

Tumor multiplicity and volume were presented as mean ± SEM, and different notation (a or b) indicated statistically significant difference (P<0.05) according ANOVA analysis followed by Tukey HSD test.

Table 3.

Quantification of 5DN and its metabolites in the lung tissue of 5DN-fed mice

Compound	Concentration (nmol/gram tissue)	Percentage among compounds (%)
5DN	1.56 ± 0.26	89.3
M1	0.034 ± 0.018	1.9
M2	0.15 ± 0.076	8.8

5DN and its metabolites were extracted by ethyl acetate from lung tissue homogenate of mice fed with 5DN (0.05% w/w in diet), then quantified by HPLC. Concentration values were presented as mean ± SEM (n=5). M1: 5,3'-didemethylnobiletin; M2: 5,4'-didemethylnobiletin