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Nobiletin (NBT), a citrus flavonoid, has been associated with various health benefits. Herein, we investigated the chemopreventive actions of NBT and its metabolites in a pulmonary carcinogenesis mouse model and human lung cancer cells. In 4-(methylnitro-samino)-1-(3-pyridyl)-1-butanone (NNK)-treated mice, oral administration of NBT significantly suppressed lung tumorigenesis evidenced by reduced tumor volume compared to the control mice. NBT also greatly attenuated cell proliferation in the lung of NNK-treated mice. Our previous study has identified three major metabolites of NBT, namely, 3'-demethylnobiletin (M1), 4'- demethylnobiletin (M2), and 3',4' -didemethylnobiletin (M3). In this study, we further determined the inhibitory effects of NBT and its metabolites on human non-small cell lung cancer (NSCLC) cells and the underlying mechanisims of action. Interestingly, we found that M2 and M3 exerted much stronger growth inhibition on both H460 and H1299 cells, compared to their parent compound NBT. Flow cytometry and western blotting analysis revealed that M2 and M3 caused significant cell cycle arrest and cellular apoptosis, and profoundly modulated multiple proteins associated with cell proliferation and cell death, including p21, cyclin B1, CDK1, cyclin D1, CDK6, CDK4, Bax, cleaved caspase-1, cleaved PARP. Overall, our results demonstrated that oral administration of NBT significantly inhibited lung carcinogenesis in mice, and these chemopreventive effects could be attributed to its metabolites that showed potent anti-cancer effects.

1. Introduction

According to the Global Cancer Statistics report, lung cancer is the most common cause of cancer-related death in human globally, and was responsible for 18.4% of the total cancer deaths in 2018.[1, 2] The vast majority of the cases of lung cancer are due to long-term exposure to tobacco smoke. In order to prevent the occurrence and progression of lung cancer, except smoking cessation, some epidemiological studies suggested that people who eat diets with a higher proportion of vegetables and fruits tend to have a lower risk of lung cancer.[3-5] The protective effects of fruits and vegetables against lung cancer have been attributed to the bioactive phytochemicals present in these foods including flavonoids.[6-8]

As one of the most abundant polymethoxyflavones (PMFs), nobiletin (5,6,7,8,3',4'-hexamethoxyflavone, NBT, Fig. 1) is specifically found in the peel of *Citrus depressa* and *Citrus aurantium*, and functions as a natural resistance against pathogenic fungi.[9] Since it has been discovered in 1967, NBT has been demonstrated to exert a wide range of beneficial activities, including anti-inflammation,[10, 11] anti-carcinogenesis,[12-17] anti-dementia[18, 19] and anti-atherosclerosis.[20] Most notably, some previous studies had showed that treatment of NBT suppressed lung carcinogenesis both *in vitro* and *in vivo*.[21, 22] Luo et al. reported that NBT induced apoptosis and cycle arrest at G2/M phase in A549 cells, and suppressed the lung tumor growth in nude mice.[21] Gao et al. demonstrated that treatment of NBT significantly attenuated hypoxia-induced epithelial-mesenchymal transition (EMT) in H1299 cells, which was an early event in the process of tumor metastasis.[22] Moon et al. found that NBT could act as an effective chemosensitizer against Adriamycin (ADR) in the ADR resistant NSCLC A549/ADR cell line. The combination of NBT and ADR significantly reduced tumor volume in a xenograft mouse model.[23]

An increasing number of studies have documented that dietary bioactive components underwent extensive

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metabolism *in vivo*.[24-26] The biotransformation profile, including the bioavailability of the parent compound and its metabolites, could significant impact the *in vivo* biological activity of the ingested compound.[16, 27-29] Recently, we and others have identified three major metabolites of NBT in different rodent models, namely, 3'-demethylnobiletin (M1), 4'- demethylnobiletin (M2), and 3',4' -didemethylnobiletin (M3), as shown in Fig. 1.[16, 30] However, the biological activities of these metabolites on lung cancer cells remains unknown. In order to better understand the beneficial effects of NBT on lung carcinogenesis, and elucidate the underlying molecular mechanisms, herein, we investigated the chemopreventive effects of NBT in an 4-(methylnitro-samino)-1-(3-pyridyl)-1-butanone (NNK)-treated A/J mouse model, and determined

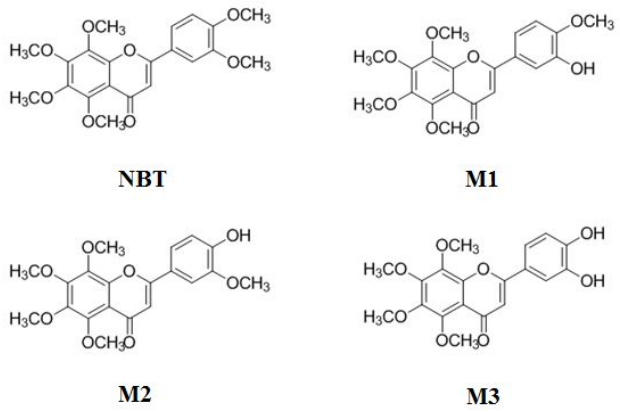

Figure 1. Chemical structures of NBT and its metabolites.

the growth inhibitory effects of NBT and its major metabolites in human NSCLC cells.

2. Materials and methods

2.1 Reagents and chemicals

NBT was purchased from Sigma-Aldrich. 3'-Demethylnobiletin (M1), 4'-demethylnobiletin (M2) and 3',4'-demethylnobiletin (M3) were synthesized following previously published methods.[31, 32] The purity of them were >98%, and their chemical structures have been confirmed by MS and NMR. All other organic solvents were of HPLC grade and obtained from Fisher Scientific.

2.2 Animals and experimental procedure

The protocol (#2014-0079) for the animal experiment was approved by Institutional Animal Care and Use Committee of the University of Massachusetts Amherst. Female A/J mice (5-week of age) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Upon arrival, the mice were kept in a temperature-controlled animal room (23°C), humidity (65-70%) and alternating 12 h light/dark cycle with free access to water and AIN-76A diet for 1 week for acclimation. Mice were then randomly divided into three groups (negative control, positive control and NBT-fed group) as shown in Fig. 2. Positive control and NBT group received an intraperitoneal injection of saline containing NNK (100 mg/kg body weight); meanwhile the mice in negative control group were given same volume of saline. After 2 days, the mice in NBT group were provided with the AIN-76A diets supplemented with 0.05% (w/w) NBT. The mice in both positive and negative control group were kept with standard diet during the entire experiment. Body weights, food and fluid consumption as well as general health status were monitored weekly.

All the mice were sacrificed by CO_2 asphyxiation at 16 weeks after NNK injection. The liver and spleen were collected and weighted. Lung of each mouse was inspected under a dissection microscope. Number and size of tumors were measured using an ocular micrometer. The sizes of tumors were determined by the following formula: tumor volume (mm^3) = $L \times W^2 / 2$, where L is the length and W is the width of the tumor. Then the lung was fixed with 10% neutral buffered formalin (pH 7.4) for 24 hours for further histopathological and immunohistochemical analysis.

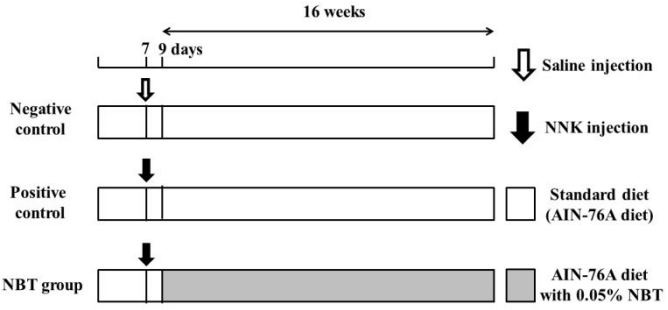

Figure 2. Experimental design.

2.3 Histopathological and immunohistochemical analysis

For histopathological examination, haematoxylin and eosin (H&E) staining were performed on formalin fixed, paraffin-embedded lung tissues by a routine procedure.[33, 34] Based on H&E staining, histological alterations such as hyperplasia, adenoma and neoplasm were evaluated according to the criteria previously reported.[35] For immunohistochemistry, cell proliferation in the lung was measured by staining with the antibodies against proliferating cell nuclear antigen (PCNA).[33]

2.4 Cell cultures and treatments

H460 and H1299 human NSCLC cell lines were obtained from American Type Cell Collection (ATCC, Manassas, VA, USA), and maintained in RPMI 1640 media (Mediatech, Herndon, VA, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 U/mL of penicillin and 0.1 mg/mL

Table 1. Final body weight, relative organ weights, and lung assessment of mice

Group	Negative control	Positive control	NBT treated
Treatment	Saline	NNK	NNK + 0.05% NBT
Number of mice	10	20	20
Body weight (g)	23.83±3.02	23.53±2.01	24.61±2.13
Liver weight (g)	1.04±0.22	0.92±0.11	1.03±0.14
Spleen weight (mg)	78.12±18.23	70.43±18.31	68.46±12.08
Tumor volume (mm^3)	0 [a]	0.42±0.01 [b]	0.25±0.02 [c]
Tumor incidence (%)	0 (0/10) [a]	100 (20/20) [b]	100 (20/20) [b]
Tumor multiplicity	0 [a]	14.91±1.04 [b]	14.51±1.10 [b]

Tumors > 0.1 mm were scored under a dissecting microscope. Tumor volume (mm^3) were measured using the formula $V = 4/3\pi r^3$, where r is the radius of the tumor determined by the mean values of the longest and shortest diameters. Values are the mean ± SD. Different superscripts indicate statistical significance for the comparison of difference among three groups ($p<0.05$) by ANOVA.

of streptomycin at 37°C with 5% CO_2 and 95% air. Cells were kept sub-confluent and media were changed every 3-4 days. All cells used in experiments were between 4 and 25 passages. DMSO was used as vehicle to deliver NBT 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 Cell viability assay

H460 or H1299 (2,000 cells/well) cells were seeded in 96-well plates. After 24 hours, cells were treated with serial concentrations of treatments in 200 µL of complete media. After reached desired treatment times, the media were replaced with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich)-containing medium for viability test as we previously reported.[36]

2.6 Flow cytometric analysis of cell cycle distribution and apoptosis

H460 or H1299 (75×10^4 cells/well) cells were seeded in 6-well plates. After 24 hours of incubation for cell attachment, cells were treated with serial concentrations of NBT and its metabolites in 2 mL of serum complete media. Media containing any floating cells were harvested and combined with adherent cells that were detached by brief trypsinization (0.25% trypsin-EDTA, Mediatech). Cell pellets were washed with 1 mL of ice-cold PBS then subject to cell cycle and apoptosis analysis as we described previously.[37]

2.7 Immunoblotting

H460 cells were seeded in 150 mm culture dishes. After 24 hours of incubation, cells were treated with NBT (50 µM), M2 (50 µM) and M3 (25 µM). After another 24 or 48 hours of incubation, cells were washed with ice-cold PBS, and collected with cell scrapers. Whole cell lysates were prepared and subjected to western blotting analysis as we previously reported.[38] Antibodies for $p21^{Cip1/Waf1}$, cyclin B1, cyclin D1, CDK-1, CDK-4, CDK-6, Bax, cleaved caspase-3, and cleaved PARP were purchased from Cell Signaling Technology (Beverly, CA, USA). Anti-β actin antibody was from Sigma-Aldrich (St. Louis, MO, USA).

2.8 Statistical analysis

All data were presented as mean ± SD. Student's *t*-test was used to test the mean difference between two groups. ANOVA model followed by Tukey's HSD test was used for the comparison of differences among three or more groups. A *p* value < 0.05 was considered to be statistically significant.

3. Results

3.1 Oral administration of NBT decreased the tumor volume in NNK-treated mice

NNK is a carcinogenic tobacco-specific nitrosamine. NNK-induced lung tumorigenesis model is a well-established model that has been utilized to determine the chemopreventive effects of dietary bioactive compounds against lung cancer.[39] Therefore, we used this model to investigate the *in vivo* anti-cancer effects of NBT on lung tumorigenesis. As shown in Table 1, during the 16-week experimental period, no significant differences were found in the body, liver or spleen weights between three groups, suggesting that no noticeable toxic effect was caused by long-term feeding of 0.05% NBT to the mice.

Injection of NNK in both positive and NBT-treated group resulted in a 100% incidence of lung tumors, while negative control group had no tumor. The tumor multiplicity of NBT-treated group (14.51±1.10) was also similar to positive control group (14.91 ± 1.04). However, NBT treatment significantly reduced the average volume per tumor by 40.5%, compared to

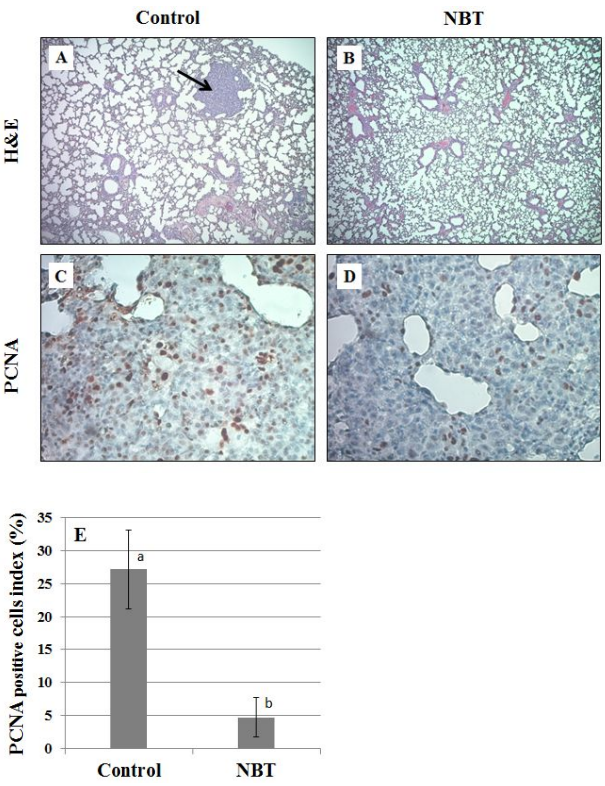

Figure 3. Histological characterization of lung tumors of NNK-treated mice. (i) H&E staining of lung tissues of the positive control (A) and NBT-treated (B) groups (magnification: 60×); (ii) PCNA staining of the positive control (C) and NBT-treated (D) groups (magnification: 300×). Quantification of PCNA-positive cells in lung tumors (E). Different letters in the bar charts indicated statistical significance ($p < 0.01$, n=6) by ANOVA.

positive control group (0.25 ± 0.02 mm^3 versus 0.42 ± 0.01 mm^3).

3.2 NBT attenuated cell proliferation in NNK-treated mice

H&E staining showed that the lung segment of the positive control group (Fig. 3A) presented typical solid adenoma (arrow), which according to established criteria represented collapse of alveolar areas containing tumor cells and some extension into the adjacent alveoli.[35] In contrast, the lung segment of NBT-fed group preserved normal histological appearance to a certain extent (Fig. 3B). Moreover, cell proliferation was determined by immunohistochemical analysis with anti-PCNA antibody. As shown in Fig. 3C, 3D and 3E, a 5.8-fold reduction in the number of PCNA positive cells was observed in the carcinoma section of NBT-fed group (4.7%) than the positive control group did (27.2%).

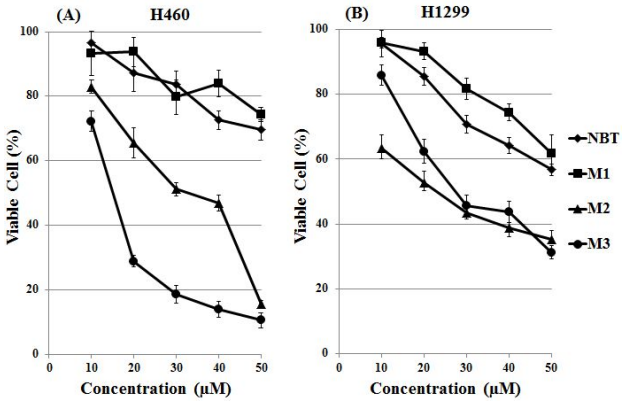

Figure 4. Growth inhibitory effect of NBT and its metabolites on H460 (A) and H1299 (B) human lung cancer cells. Cells were seeded in 96-well plates, and after 24 h of incubation, cells were treated with serial concentrations as indicated in the figure for 72 h. Growth inhibition was measured by MTT assay. Data represent mean ± SD (n=6).

3.3 Metabolites of NBT had stronger inhibitory effects than NBT on the growth of human NSCLC cells

Next, we compared the anti-cancerous potential of NBT and its metabolites on the growth of two NSCLC cell lines, H460 and H1299, using a cell viability (MTT) assay. H460 and H1299 cells were treated with a series concentration of NBT, M1, M2 and M3 (from 10 μM to 50 μM) for 72 hours. As shown in Fig. 4, all compounds showed dose-dependent inhibition on the growth of both H460 and H1299 cells. Overall, M2 and M3 had much stronger inhibitory effects than NBT and M1**.** The IC_{50} of NBT was about 78.44 and 54.33 μM in H460 and H1299 cells, respectively. The IC_{50} of M1 was 73.69 μM in H1299 cells, and higher than 100 μM in H460. The IC_{50} of M2 were 27.46 and 21.71 μM in H460 and H1299 cells, respectively. The IC_{50} of M3 were 14.77 and 29.97 μM in H460 and H1299 cells, respectively.

3.4 Metabolites of NBT were more efficacious in inducing cell-cycle arrest and apoptosis than NBT

Triggering cell cycle arrest and cellular apoptosis are effective strategies in preventing and treating cancer. To elucidate the mechanism of inhibition on NSCLC cells, the effects of NBT, M2 and M3 on cell cycle progression and apoptosis were determined using flow cytometry. As shown in

Fig. 5A, treatments with NBT (50 μM) or M2 (50 μM) for 24 hours caused cell-cycle arrest at G0/G1 phase in H1299 cells, and the effect of M2 were stronger than that of NBT. M2 (50 μM) also caused G0/G1 phase arrest in H460 cells, but NBT (50 μM) had no effect on cell cycle distribution in H460 cells. M3 (25 and 50 μM) led to cell-cycle arrest at S phases in both H460 and H1299 cells.

As shown in Fig. 5B, in H460 cells, NBT (50 μM) slightly increased late apoptotic cell population after 48 hours of treatment, compared to the control cells, but did not achieve statistical significance. Treatments with M2 (50 μM) and M3 (25 μM) increased early apoptotic cell population by 2.0- and 3.4-fold compared to the control, respectively; while they increased late apoptotic cell population by 1.6- and 1.5-fold, respectively. In H1299 cells, NBT (50 μM) did not cause significant apoptosis. Both M2 (50 μM) and M3 (50 μM) increased early apoptotic cell population by 3.7-fold compared to the control, while they increased late apoptotic cell population by 1.9- and 1.8-fold, respectively. Overall, in H460 and H1299 cells, M2 and M3 were more efficacious in inducing both early and late apoptosis than NBT.

3.5. NBT and its metabolites modulated the expression of key signaling proteins related to cell proliferation and death

In order to further elucidate the molecular mechanism underlying the inhibitory effects of NBT and its metabolites on lung cancer cells, several key signaling protein related to the cell cycle and apoptosis pathways were examined by immunoblotting analysis in H460 cells. Cell cycle-related proteins p21, cyclin B1, CDK1, cyclin D1, CDK6 and CDK4 were quantified after 24 hours of treatment, and pro-apoptotic proteins Bax, cleaved caspase-3 and cleaved PARP were evaluated after 48 hours of treatment. As shown in Fig. 6, immunoblotting results revealed that treatments of NBT (50 μM), M2 (50 μM) and M3 (25 μM) profoundly modulated the expression levels of these signaling proteins in an anti-cancer direction. All compounds significantly upregulated the expression of Bax, and downregulated the expression of cyclin D1, CDK1 and CDK6. M2 and M3 caused evident increases in $p21^{Cip1/Waf1}$, cleaved caspase-3 and cleaved PARP expression levels. NBT and M2 also led to a reduction in cyclin B1 expression level. While only M2 decreased the level of CDK4 significantly.

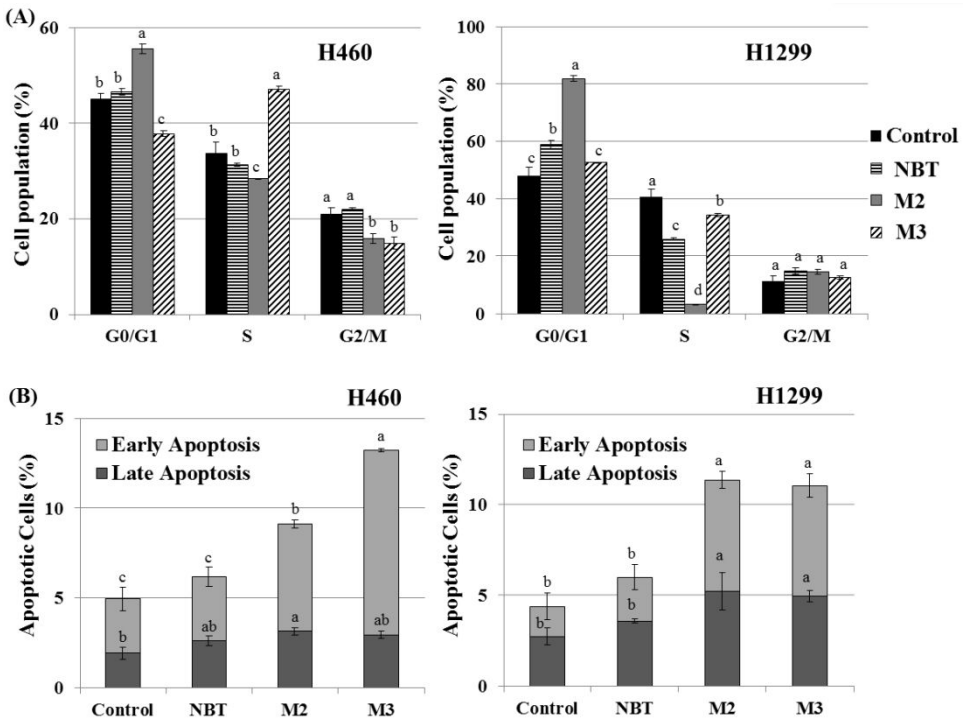

Figure 5. Effects of NBT and its metabolites on cell cycle progression (A) and apoptosis (B) of H460 and H1299 human lung cancer cells. The cells were seeded in 6-well plates for 24 hours, and then treated with serial concentrations of NBT and its metabolites. After 24 or 48 hours of treatment, cells were collected and subjected to cell cycle analyses or apoptosis analyses described in methods section. Different letters indicate significant differences ($p < 0.01$) between groups by ANOVA. All data represent mean ± SD (n=3).

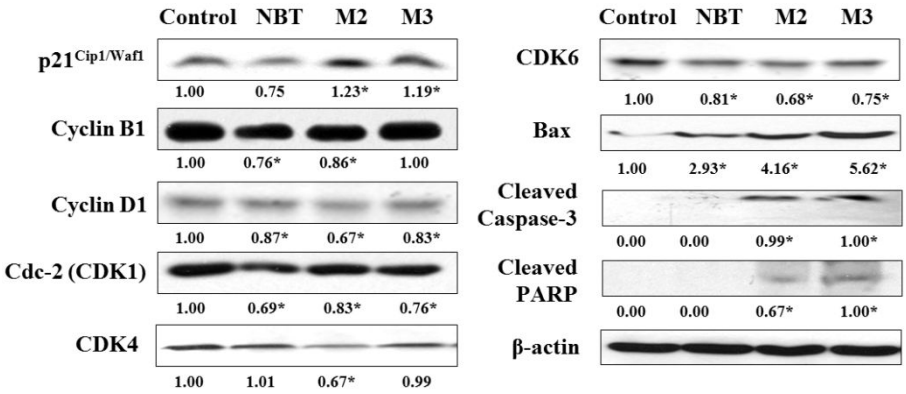

Figure 6. Effects of NBT and its metabolites on cell cycle and apoptosis related signaling proteins in H460 human NSCLC cell line. H460 cells were seed into 15 cm culture dishes for 24 hours, and then cells were treated with serial concentrations of NBT and its metabolites. After another 24 or 48 hours of incubation, cells were collected for immunoblotting analysis as described in methods section. The number underneath of the blots represents band intensity (normalized to β-Actin loading control, means of three independent experiments) measured by Image J software. The SDs (all within 15% of the means) were not shown. β-Actin was served as an equal loading control. * Indicates statistical significance in comparison with the control (p < 0.05, n=3).

Discussion

Diet-based chemoprevention is considered a promising strategy to reduce cancer risk in the long run. An accumulating number of studies have suggested that bioactive phytochemicals extracted from fruits and vegetables could prevent the development of human cancers in various experimental models.[4, 5, 40] In recent decades, naturally occurring PMFs have attracted growing attention due to their broad range of health-promoting effects.[7, 8] In this study, we focused on NBT, which is a common PMF found in orange peel.[41]

The use of tobacco is one of the strongest environmental risk factor for lung cancer, as it has been estimated that smoking causes about 90% cases of lung cancer.[42] Cigarette smoke contains various carcinogens, including NNK, benzo(a)pyrene, cadmium, formaldehyde and ethylcarbamate.[39] Therefore, in this study, we first determined the chemopreventive effects of NBT in an NNK-induced lung cancer mouse model. We found that the long-term feeding of 0.05% NBT significantly reduced the average size of lung tumor in NNK-treated mice, with no obvious adverse effects (Table 1). Histopathological assessment revealed that NBT treatment appeared to largely maintain the histological characteristics of normal lung tissue (Fig. 3A, 3B).

Out-of-control cell proliferation is an important hallmark of lung cancer. PCNA is a cofactor of DNA polymerase, which increases during G1 phase through S phase in the cell cycle. PCNA plays an essential role in DNA replication; thus, it is often used as a marker for cell proliferation measurement.[33] Immunohistochemical analysis showed that high number of PCNA-positive cells was found in the lung of NNK-treated positive control mice. In contrast, NBT treatment greatly decreased the number of PCNA-positive cells by 5.8-fold (Fig. 3C, 3D, 3E). Together, these results suggested that long-term dietary intake of NBT might inhibit the development of lung cancer to some extent.

Biotransformation of dietary components has been found to dictate their biological activities *in vivo*. Three major metabolites of NBT, M1, M2 and M3 have been identified *in vivo*.[30, 43] M2 and M3 showed stronger anti-cancer effects in human colon cancer cells[16] and anti-inflammatory effects both *in vitro* and *in vivo*.[30, 44] Others have also shown that the metabolites of NBT were often more active than the prototype. Su et al. found that both NBT and M3 attenuated cell death in serum withdrawal and H_2O_2-treated PC12 cells, whereas only M3 suppressed the accumulation of intracellular reactive oxygen species (ROS) and showed better activity.[45] In this study, for the first time, we investigated the inhibitory efficacy of NBT metabolites in H460 and H1299 human NSCLC cells, and the mechanisms involved. Our results demonstrated that M2 and M3 had much stronger growth inhibitory effects than NBT did in both H460 and H1299 cells (Fig. 4). For example, in H460 cells, the IC_{50} concentration of M2 and M3 were approximately 2.9- and 5.3- fold lower than that of NBT. This finding is consistent with what we previously found in human colon cancer cells, that the number and location of demethylated group have profound influence on the bioactivities of PMFs.[16] The higher activity exhibited by NBT's metabolites might be in part due to the higher ability of the phenolic hydroxyl group form a hydrogen bond connected with the corresponding active protein, thereby reducing the binding energy.[46]

Induction of cell cycle arrest and apoptosis in lung cancer cells can effectively prevent the abnormal cell proliferation and in turn slow down cancer progression.[47] NBT and other PMFs have been found to induce cell cycle arrest and cell death in multiple cancer cells.[16, 48, 49] Therefore, we further investigated the mode of action of NBT and its metabolites in inhibiting NSCLC cell growth by determining the effects of NBT and its metabolites on cell cycle progression and cellular apoptosis. Our results showed that NBT and its metabolites had distinct effects on cell-cycle progression of human NSCLC cells (Fig. 5A). NBT slightly caused G0/G1 phase arrest in H1299 cells, but had no effect on cell cycle distribution in H460 cells. M2 and M3 led to cell-cycle arrest at G0/G1 and S phases in human lung cancer cells, respectively. The result suggested that different molecular mechanisms were involved in their actions.

The loss of regulated cell cycle is one of the important hallmarks of cancer. The regulation of cell cycle progression is governed by cyclins, cyclin dependent kinases (CDKs) and CDK inhibitors, i.e. cyclin-CDK complexes promote cell cycle progression whereas CDK inhibitors block cell cycle.[47, 50, 51] Progress in the eukaryotic cell cycle is driven by protein kinase complexes consisting of a cyclin and a CDK. During G1 phase progression, the complexes cyclin D-CDK4, cyclin D-CDK6 and cyclin E-CDK2 are activated and promote the cell cycle from the G1 phase to the S phase.[52, 53] It could explain the down-regulation of cyclin D1, CDK4 and CDK6 after M2 treatment as it caused G0/G1 phase arrest. As a crucial CDK inhibitor, p21 often binds to and inhibits the activity of cyclin-CDK complexes, and thus functions as a regulator of cell cycle progression at G1 and S phase.[54] Both M2 and M3 affected the protein level of $p21^{Cip1/Waf1}$, whereas NBT showed no effect. This might partially explain why NBT had no effect in inducing cell-cycle arrest in H460 cells. The transition of cell cycle from G1 phase to S phase is mainly regulated by cyclin D-CDK4/6 complexes. Induction of p21 can in turn cause the degradation of cyclin D1, leading to cell-cycle arrest in G0/G1 or S stage.[54] Our observation that M3-induced S-phase arrest was partly in agreement with our published report that treatment of M3 in HCT116 cells resulted in S-phase arrest, whereas M3 led to cell-cycle arrest at G0/G1 phases in HT29 cells [16]. Therefore, the effects of M3 on the expression of cell cycle control proteins appeared to vary considerably between cell systems.

Cellular apoptosis (programmed cell death) is another principal approach to control cancer cell proliferation. During the development of cancer, cancerous cells are able to evade apoptosis and survive because of aberrations of the apoptotic signaling pathway.[55] Dietary bioactive compounds which can induce apoptosis by re-regulating these signaling proteins may have the potential for preventing cancers.[56] It was observed that M2 or M3 treatments significantly increased the apoptotic cell population in both H460 cells and H1299 cells, while NBT had no suppressive effect (Fig. 5B). These findings were consistent with previous report showing that NBT and its metabolites induced cellular apoptosis in colon cancer cells.[16] We also found that M2 and M3 treatment increased the levels of cleaved caspase-3 and cleaved PARP in lung cancer cells (Fig. 6). Activation (cleavage) of caspase-3 (a critical executioner of apoptosis) and its downstream target PARP triggers apoptosis through interfering chromatin condensation and DNA fragmentation.[57] These results were consistent with that from Annexin-V/PI double staining assay showing the treatment of M2 and M3 resulted in both early and late apoptosis in lung cancer cells (Fig. 5B). We also observed that M2 and M3 treatment increased the expression levels of Bax (Fig. 6). Bax, a Bcl-2 family proapoptotic protein, is responsible for the mitochondrial damage that can lead to the activation of caspase cascade.[58] The results suggested that mitochondria-mediated intrinsic apoptosis was involved in the apoptosis induced by M2 and M3 treatments.

Conclusions

Taken together, our results showed that oral administration of NBT effectively inhibited pulmonary tumorigenesis in NNK-treated A/J mice. For the first time, we demonstrated that metabolites of NBT had even stronger anti-cancerous effects than NBT did, and these effects were associated with superior cell cycle arrest and apoptosis caused by the metabolites as the result of the modulation of critical oncogenic signaling pathways. Overall, our results suggested NBT and its bioactive metabolites may serve as potential chemopreventive agents on human lung cancer.

Acknowledgements

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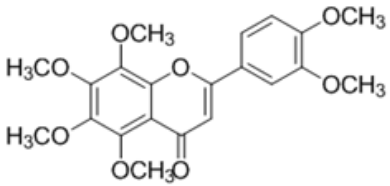
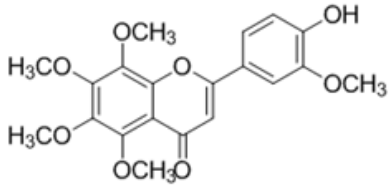
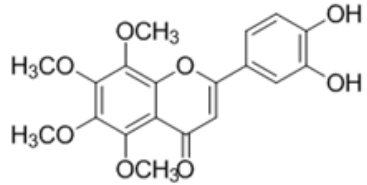


Nobiletin

Phase I metabolism

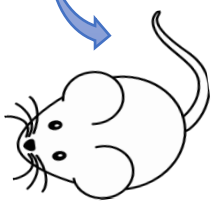


M2

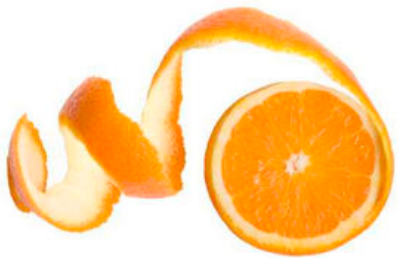


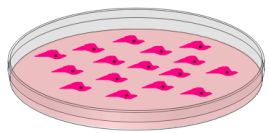
M3

Metabolites



NNK-induced lung tumorigenesis





human non-small cell lung cancer (NSCLC) cells

↓ Tumor volume
↓ Cell proliferation

↓ Cell growth
↑ Cell cycle arrest
↑ Apoptosis