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A metabolite of nobiletin, 4'-demethylnobiletin and atorvastatin synergistically inhibits human colon cancer cell growth by inducing G0/G1 cell cycle arrest and apoptosis

Xian Wu^{#,a}, Mingyue Song^{#a,b}, Peiju Qiu^{a,c}, Fang Li^a, Minqi Wang^a, Jinkai Zheng^{a,d}, Qi Wang^a, Fei Xu^a, and Hang Xiao^{*,a}

^aDepartment of Food Science, University of Massachusetts, Amherst, MA, USA.

^bCollege of Food Science, South China Agricultural University, Guangzhou, China.

^cSchool of Pharmacy, Ocean University of China, Qingdao, Shandong, P. R. China

^dInstitute of Agro-Products Processing Science and Technology, Chinese Academy of Agricultural Sciences, Beijing, China.

[#] These authors contributed equally to this work.

Abstract

Combining different chemopreventive agents is a promising strategy to reduce cancer incidence and mortality due to potential synergistic interactions between these agents. Previously, we demonstrated that oral administration of nobiletin (NBT, a citrus flavonoid) at 0.05% (w/w, in diet) together with atorvastatin (ATST, a lipid-lowering drug) at 0.02% (w/w, in diet) produced much stronger inhibition on colon carcinogenesis in rats in comparison with that produced by NBT (at 0.1% w/w in diet) or ATST (at 0.04% w/w in diet) alone at higher doses. To further elucidate the mechanism of this promising synergy between NBT and ATST, herein, we measured the levels of NBT, its major metabolites and ATST in the colonic tissue of rats fed NBT (0.05% w/w, in diet) + ATST (0.02% w/w, in diet), and determined the mode of interaction between the major NBT metabolite and ATST in inhibiting colon cancer cell growth. HPLC-MS analysis showed that 4'-demethylnobiletin (4DN) is the most abundant metabolite of NBT with a level about 5-fold as high as that of NBT in the colonic tissue, which indicated potential significance of 4DN in mediating biological effects of NBT in the colon. We found that co-treatments of 4DN/ATST at 2: 1 concentration ratio produced much stronger growth inhibitory effect on human colon cancer HT-29 cells than 4DN or ATST alone, and isobologram analysis confirmed that this enhanced inhibitory effect by 4DN/ATST combination was highly synergistic. Co-treatment of 4DN/ATST led to G0/G1 cell cycle arrest and induced extensive apoptosis in HT-29 cells. Furthermore, 4DN/ATST co-treatment profoundly modulated key signaling proteins related with regulation of cell cycle and apoptosis. Our results demonstrated a strong synergy produced by 4DN/ATST co-

*Corresponding Authors: Hang Xiao: hangxiao@foodsci.umass.edu; Xian Wu: xianwu@alumni.umass.edu.

Conflict of interest

The authors have declared no conflict of interest.

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treatment in inhibiting colon cancer cell growth, which provided a novel mechanism by which NBT/ATST in combination synergistically inhibit colon carcinogenesis.

Graphic Abstract

Introduction

At present, colon cancer is one of the leading causes of human death in the United States³. Many of the current treatment modalities for colon cancer are limited due to undesirable side effects and complications associated with their long-term use. Thus, it underscores the need of novel strategies to prevent and treat colon cancer. Recently, combination regimens of using food bioactive components with pharmacologic agents have gained growing attention. Accumulating evidence suggests that the combination of different chemopreventive agents that have complementary mechanism of actions may produce a synergistic interaction thus an enhanced inhibition against carcinogenesis. Moreover, the enhanced efficacy can reduce the dosage required for each agent in the combination, which may lower potential adverse effects associated with long-term high-dose administration of a certain agent⁴⁻⁶.

Polymethoxyflavones (PMFs) is a unique group of flavonoids found in citrus fruits. There have been more than 20 PMFs being isolated and identified from citrus fruits, such as sinensetin (5,6,7,3',4'-pentamethoxyflavone), nobiletin (5,6,7,8,3',4'-hexamethoxyflavone, NBT), and tangeretin (5,6,7,8,4'-pentamethoxyflavone). PMFs are the secondary compounds located in the peel, especially in the flavedo that act as antifungal agents⁷. NBT has been shown to be a potent anti-carcinogenic agent against different cancers in animal models, including colon⁸⁻¹¹, prostate¹² and lung¹³ cancer. Previously, we found that oral administration of NBT in combination with ATST synergistically suppressed colon carcinogenesis in azoxymethane (AOM)-treated rats via modulating multiple pathways associated with cell cycle progress, apoptosis, inflammation, angiogenesis and metastasis process of colon cancer¹¹.

ATST (commercially sold as Lipitor® or Torvast®) is an important member of statin drugs that are mainly used to lower blood cholesterol and prevent cardiovascular disease. Besides their lipid-lowering effects, statins have been reported to reduce the risk of colon cancer in observational, preclinical and clinical studies¹⁴. The chemopreventive property of statins is mainly due to their inhibitory activity on HMG-CoA reductase, which thereby reduces the levels of mevalonate and its associated products. It in turn inhibits the membrane localization and activation of some small G-proteins, such as RhoA and RhoC, and suppresses epidermal growth factor-induced invasion of cancer cells. RhoA and RhoC have

been found overexpressed in many human cancers, and they are related uncontrolled cell cycle regulation and increased immigration and metastasis of cancer cells⁴.

Biotransformation plays essential roles in the bioactivities of dietary compounds. The metabolites generated after biotransformation in the body may have different biochemical and pharmacological properties. Oral administration of a dietary compound may result in higher levels of metabolites than the parent compound in certain tissues. NBT undergoes extensive biotransformation leading to its structural modifications after oral consumption^{9, 11, 15}. We previously reported that NBT was transformed to its demethylated metabolites such as 4'-demethylnobiletin (4DN), 3'-demethylnobiletin (3DN) and 3',4'-didemethylnobiletin (34DN) in the colon of mice after long-term feeding of NBT⁹. Interestingly, it was found that 4DN was the most abundant colonic metabolite of NBT. The colonic level of 4DN was about 12-fold higher than that of NBT and 2-fold higher than that of 34DN, the second most abundant metabolite. More importantly, 4DN exhibited stronger bioactivities than NBT, such as anti-cancer and anti-inflammatory effects^{9, 16, 17}. Under this circumstance, 4DN, rather than the ingested parent compound NBT, may exert more important roles in the anti-carcinogenic activities of NBT in the colon.

Previously, the synergistic colon cancer preventive effects of NBT and ATST in combination observed in animal model were ascribed to NBT itself¹¹. There is no detailed information available about the role of biotransformation of NBT in the chemopreventive effect of the NBT/ATST co-treatment in the colon. To better understand the possible mechanisms involved in the synergistic interaction between NBT and ATST, in this study, we determined the colonic levels of ATST, NBT, 4DN, 3DN and 34DN in the rats fed with NBT/ATST co-treatment. Moreover, we determined the growth inhibitory effects of the combination of 4DN and ATST in HT-29 human colon cancer cells, and elucidated the underlying molecular mechanisms.

Materials and methods

2.1 Animals, diets, and experimental procedure

NBT and ATST were purchased from Bepharma Ltd. (Shanghai, China) with purity greater than 98%. 4DN, 3DN and 34DN were chemically synthesized as we previously described^{18, 19}. Chemical structures of ATST, NBT and its metabolites were illustrated in Figure 1. The protocol (#2011-0066) for the animal experiment was approved by the Institutional Animal Care and Use Committee of the University of Massachusetts.

Twelve male F344 rats (5 weeks of age) were obtained from Charles River Laboratories (Wilmington, MA, USA). Upon arrival, the rats were kept in a temperature-controlled animal room (23°C), humidity (65–70%) and day/night cycle (12 h light, 12 h dark) with free access to water and a modified high-fat AIN-76A diet²⁰ for one week for acclimation. Rats were then randomly divided into two groups (6 animals each). The rats in the control group were fed basal diet (control group), while the NBT/ATST-treated group was fed with modified AIN-76A diet containing 0.05% NBT plus 0.02% ATST (w/w, in diet) (combination group). Both groups were allowed to eat and drink *ad libitum* during the whole

experiment period. After ten weeks of treatment, all rats were sacrificed by CO₂ asphyxiation. Colonic mucosa were collected and stored at -80 °C for LC-MS analysis.

2.2 Determination of colonic levels of ATST, NBT and NBT metabolites

Colonic mucosa samples from the rats fed with basal diet (control group) and basal diet containing NBT/ATST (combination group) were homogenized with 50% methanol using Bead Ruptor Homogenizer (Omni International, Kennesaw GA, USA). Samples were then extracted with equal volume of ethyl acetate for three times. The combined ethyl acetate extracts were dried under vacuum, and then dissolved in 50% methanol for LC-MS (Model 2020, Shimadzu, Kyoto, Japan) analysis. Identification and quantification of ATST, NBT and NBT metabolites (4DN, 3DN and 34DN) were carried out using previously published method²¹. ATST, NBT, 4DN, 3DN and 34DN, with purity greater than 98%, were used as external standards to identify and quantify their levels in the colonic mucosa of the rats.

2.3 Cell cultures and treatments

Human colorectal cancer cell line HT-29 was obtained from American Type Cell Collection (ATCC, Manassas, VA, USA). HT-29 cells were maintained in RPMI 1640 media (Mediatech, Herndon, VA, USA) supplemented with 5% heat-inactivated fetal bovine serum and 100 U/mL of penicillin and 0.1 mg/mL of streptomycin at 37°C with 5% CO₂ and 95% air. Cells were kept subconfluent and media were changed every 3–4 days. All cells used in experiments were between 4 and 25 passages. Atorvastatin (ATST) was purchased from Bepharma Ltd. (Shanghai, China). Dimethylsulfoxide (DMSO) was used as vehicle to deliver 4DN and ATST to the cells. The final concentration of DMSO in all experiments was 0.1% v/v in cell culture media.

2.4 Cell viability assay and analyses of synergy

HT-29 (2,000 cells/well) cells were seeded in 96-well plates. After 24 h, cells were treated with serial concentrations of 4DN, ATST and their half dose combinations in 200 µL of serum complete media. After 72 h, cells were subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Media were replaced by 100 µL of fresh media containing 0.5 mg/mL of MTT (Sigma-Aldrich, St. Louis, MO, USA). After 1 h incubation at 37°C, MTT-containing media were removed and the reduced formazan dye was solubilized by adding 100 µL of DMSO to each well. After gentle mixing, the absorbance was monitored at 570 nm using a plate reader (Elx800TM absorbance microplate reader, BioTek Instrument, Winooski, VT).

The analyses were based on Chou and talalay's empirical method¹ with modifications as described previously^{22–24}. It was assumed that the dose response model follow $\log [E/(1-E)] = \alpha(\log d - \log D_m)$, which is a linear regression model with the response $\log [E/(1-E)]$ and the regressor $\log (d)$. This model is used for 4DN, ATST and the co-treatment of 4DN/ATST with the fixed ratio of the doses of two agents. E is fraction of cell survived, d is the dose applied, D_m is the median effective dose of a drug (IC₅₀), and α is a slope parameter. On the basis of the regression model, median effect plots were constructed using data from MTT assay. Suppose that the combination (d_1, d_2) elicits the same effect x as 4DN alone at dose level $D_{x,1}$, and ATST alone at dose $D_{x,2}$, then the interaction index of 4DN/ATST = $(d_1/D_{x,1})$

$+(d_2/D_{x,2})$, ($D_{x,1}$ and $D_{x,2}$ were calculated from median effect models). The combination index was used to determine additivity, antagonism, and synergy of the combination treatment of 4DN and ATST at different dose pairs depending on the combination index = 1, > 1, and < 1, respectively. The delta method² was used to calculate the variance of the interaction index, which is given by $\text{var}(\text{combination index}) = \text{var}(D_{x,1})(d_1^2/D_{x,1}^4) + \text{var}(D_{x,2})(d_2^2/D_{x,2}^4)$. Data were further analyzed by using R program software.

2.5 Cell cycle analysis

HT-29 (7×10^4 cells/well) cells were seeded in 6-well plates. After 24 h of incubation for cell attachment, cells were treated with serial concentrations of 4DN, ATST, and their half dose co-treatment in 2 mL of serum complete media. After 24 h of treatment, 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 and then resuspended in 1 mL of 70% ethanol in -20°C overnight. After centrifugation (1600 g, 1 min), the supernatant was removed and cells were incubated with 0.5 mL of PBS containing 50 mg RNase (Sigma-Aldrich) and 5 mg propidium iodide (PI, Sigma-Aldrich) for 30 min at room temperature. Single-cell suspension was generated by gentle pipetting. Cell cycle was analyzed using a BD LSR II cell analyzer at the analytical cytometry facility (University of Massachusetts Amherst), and data were processed using Modifit software.

2.6 Detection of apoptosis

HT29 cells were seeded and treated exactly same as in cell cycle analysis described above. Apoptotic cells were quantified by Annexin V/PI double staining assay. Annexin V/PI staining was done using apoptotic detection kit (BioVision, Mountain View, CA, USA) following the manufacturer's instruction. After 48 h of treatment, cells were gently detached by brief trypsinization (any floating cells were also collected), and then washed with ice cold PBS. After another wash with binding buffer, cells were suspended in 300 mL binding buffer containing Annexin V and PI, and incubated for 5 min at room temperature. Early apoptotic cells were identified as Annexin V-positive/PI-negative cells, while late apoptotic/necrotic cells were identified as Annexin V-positive/PI-positive cells using a BD LSR II cell analyzer.

2.7 Immunoblot analysis

Whole-cell lysates were prepared as previously described^{22, 25}. For immunoblot analysis, equal amount of proteins (50mg) were resolved over 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membranes containing the transferred protein were blocked in blocking buffer (5% nonfat dry milk, 1% Tween-20 in 20 mM Tris-buffered saline, pH 7.6) for 2 h at room temperature, and then incubated with appropriate monoclonal primary antibody in blocking buffer overnight at 4°C . After incubation with appropriate secondary antibodies, the membranes were washed three times with Tris buffer with tween-20, and then visualized using enhanced chemiluminescence (Boston Bio Products, Ashland, MA, USA). Antibodies for CDK4, cleaved caspase 3 (Asp175), p53 and p21^{Cip1/Waf1} were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody for β -actin was from Sigma-Aldrich.

2.8 Statistical analysis

All data were presented as the mean $\pm$ standard deviation (SD) or standard error (SE). Student's *t* test was used to test the mean difference between two groups. An analysis of variance (ANOVA) model was used for the comparison of differences among more than two groups. A *P*-value <0.05 was considered statistically significant. The interaction between 4DN and ATST (synergistic, additive, or antagonistic) on cell cycle distribution and apoptosis in HT29 cells was analyzed based on the "simple designs and model free tests for synergy" by Laska *et al*²⁶.

Results

3.1 4DN was a major metabolite of NBT in the colonic tissue after oral administration of NBT/ATST in rats

In this study, we fed the F344 rats for ten weeks with the same doses of NBT/ATST co-treatment as we used in the previous rat study (0.05% NBT + 0.02% ATST w/w in diet)¹¹ to generate colonic mucosa samples for LC-MS analysis. We found that 4DN, 3DN and 34DN were present in the colonic mucosa of rats fed with NBT/ATST. It was observed that ATST, NBT and three colonic metabolites of NBT showed identical molecular weight and retention times as chemical standards of these compounds. As shown in Figure 2, the concentrations of ATST, NBT, 4DN, 3DN and 34DN in the rat colonic mucosa were 1.48 ± 0.34 , 0.59 ± 0.13 , 2.95 ± 0.38 , 1.82 ± 0.55 , and 2.06 ± 0.89 nmol/g of tissue, respectively. It is noteworthy that the level of 4DN was the most abundant among three major NBT metabolites, and was 4.95-fold as high as that of NBT. The high abundance of 4DN in the colonic tissue may lead to a significant role of 4DN in the anti-carcinogenic effects of NBT/ATST combination in rats.

3.2 4DN/ATST co-treatment synergistically inhibited the growth of human colon cancer cells

To establish the potential significance of 4DN in mediating synergistic inhibition on colon carcinogenesis in rats by NBT/ATST combination as we described previously¹¹, the inhibitory effects of 4DN, ATST and 4DN/ATST combination on human colon cancer HT29 cells were determined. HT-29 human colon cancer cells were treated with serial concentrations of 4DN (at 7.2, 14.4, 21.6, 28.8, or 36 μ M), ATST (at 3.6, 7.2, 10.8, 14.4, or 18 μ M), and 4DN/ATST combinations at a 2: 1 ratio (3.6/1.8, 7.2/3.6, 10.8/5.4, 14.4/7.2, or 18/9 μ M) for 72 h. The 2: 1 ratio between 4DN and ATST used in the cell culture experiment was based on the fact that the colonic level of 4DN was about 2-fold of that of ATST. As shown in Figure 3, single treatment with 4DN or ATST resulted in dose-dependent growth inhibition on HT-29 cells. However, at the highest concentration tested (36 μ M), 4DN only modestly inhibited cell growth by 25.89%. Similarly, at the highest concentration tested (18 μ M), ATST treatment showed a moderate 20.89% growth inhibition on HT-29 cells. In contrast, the half dose combinations of 4DN and ATST led to more potent growth inhibitory effects on cancer cells than the individual treatment with NBT or ATST at their full doses. For example, a combination of 14.4 μ M of 4DN with 7.2 μ M of ATST caused a 53.84% inhibition, while 28.8 μ M of 4DN alone and 14.4 μ M of ATST alone led to only 11.65 and 11.15% inhibition, respectively. Similarly, 18 μ M of 4DN combined with 9

μM of ATST caused a 68.89% inhibition, while 36 μM of 4DN alone and 18 μM of ATST alone led to only 25.89 and 20.89% inhibition, respectively. It is noteworthy that even 10.8 μM of 4DN plus 5.4 μM of ATST co-treatment produced slightly stronger inhibition than that produced by 36 μM of 4DN alone or 18 μM of ATST alone. These results demonstrated that 4DN/ATST combination led to much stronger inhibitory effects on human colon cancer cells in comparison with 4DN or ATST alone.

To determine whether the enhanced inhibitory effects on cancer cells from 4DN/ATST co-treatments was statistically synergistic, we next performed an isobologram analysis as we described previously^{22–24}. As shown in Figure 4A, the median effect plot verified that the linear regression model used in the isobologram analysis was well-fitted to the dose-response relationship of 4DN, ATST and their combinations within the concentration ranges exploited herein. The interaction index was used to determine additivity, synergy, or antagonism of the combination at different dose pairs depending on interaction index = 1, <1, or >1, respectively. An interaction index between 0.3 and 0.8 indicates moderate synergism, whereas an interaction index smaller than 0.3 indicates a strong synergism between two tested compounds. The interaction index of each co-treatment dose pair of 4DN/ATST was determined on the basis of the median effect plot. As shown in Figure 4B, all of the 4DN/ATST co-treatment dose pairs resulted in interaction indexes smaller than 0.7, suggesting synergistic interactions between 4DN and ATST in suppressing HT-29 cell growth within the concentration ranges tested. Furthermore, interaction indexes of 4DN/ATST combination treatments at the two higher dosed pairs (14.4 μM of 4DN + 7.2 μM of ATST, and 18 μM of 4DN + 9 μM of ATST) were 0.22 and 0.14, respectively, suggesting a strong synergistic effect between 4DN and ATST. Taken together, our results demonstrated a significant synergistic effect between 4DN and ATST in inhibiting the growth of human colon cancer cells.

3.3 4DN/ATST co-treatment synergistically induced G0/G1 cell cycle arrest in human colon cancer cells

Uncontrolled and disregulated cell proliferation is one of the most common features of cancer. To characterize the molecular mechanism underlying the synergistic anti-cancer effects of 4DN and ATST, we determined its effect on cell cycle progression by flow cytometric analysis. As shown in Figure 5, individual treatment with 4DN (36 μM) or ATST (18 μM) for 24 h significantly increased HT-29 cell accumulation in G0/G1 phase by 23.1 and 12.7%, respectively, compared to untreated control cells. However, their half dose co-treatment (4DN at 18 μM + ATST at 9 μM) resulted in 29.5%, in comparison to control cells, which was stronger than the effects of 4DN or ATST alone at their full doses. Using “simple designs and model free tests for synergy” established by Laska *et al.*²⁶, we analysed the cell cycle data and it was confirmed that the stronger effects produced by the 4DN/ATST co-treatment was synergistic in comparison with those of 4DN or ATST alone. This finding suggested that induction of cell cycle arrest might contribute to the enhanced growth inhibitory effects of 4DN/ATST co-treatment on cancer cells.

3.4 4DN/ATST co-treatment synergistically triggered cellular apoptosis in human colon cancer cells

Similar to abnormal cell cycle progression, evasion of apoptosis is another common feature of cancer. Thus, induction of apoptosis in cancer cells is an essential approach to prevent and treat cancer²⁷. Annexin-V/propidium iodine double staining assay was used to determine the pro-apoptotic activities of 4DN/ATST co-treatment. As shown in Figure 6, after 48 h of treatment, 4DN at 36 μ M did not cause a significant change in the apoptotic cell population, compared to control cells. ATST at 18 μ M led to a 2.3-fold increase in late apoptotic cell population, while it showed no effect on early apoptosis. On the other hand, the half dose co-treatment of 4DN/ATST (4DN at 18 μ M + ATST at 9 μ M) exerted a much stronger induction of early apoptosis of HT-29 cells. The co-treatment greatly increased early apoptotic cell population by 7.9-fold, in comparison to the control cells. Although 4DN/ATST co-treatment did not induce late apoptosis of HT-29 cells, compared to control cells, it elevated the total apoptotic cell population by 4.1-fold. Based on the statistical method established by Laska *et al*²⁶, the apoptosis-inducing efficacy by the 4DN/ATST co-treatment was verified to be synergistic. Therefore, these findings indicated that the growth inhibitory effects of 4DN/ATST co-treatment in human colon cancer HT-29 cells were associated with its ability to trigger cellular apoptosis synergistically.

3.5 4DN/ATST co-treatment modulated expression levels of key proteins related with cell cycle progression and apoptosis

To further understand the molecular mechanisms underlying involved in the synergistic chemopreventive activities of 4DN/ATST co-treatment, Western blot analysis was performed to evaluate the effects of this combination on the expression levels of some key signalling proteins related to cell cycle and apoptosis in HT-29 cells. As shown in Figure 7, 4DN at 36 μ M enhanced the protein levels of p21 by 1.53-fold and showed no effect on CDK4. ATST at 18 μ M even caused decreased the level of p21 and did not significantly change the level of CDK4. In contrast, 4DN/ATST co-treatment (4DN at 18 μ M + ATST at 9 μ M) showed the stronger effects on p21 and CDK4 than 4DN or ATST alone. It resulted in 1.84-fold increase in p21 level and 32% decrease in CDK4 level, compared to the control cells. All treatment groups increased the expression levels of tumor suppressor p53 and cleaved caspase-3. However, half dose co-treatment of 4DN/ATST led to much stronger increase than 4DN or ATST alone at full dose. It significantly elevated the protein levels of p53 and cleaved caspase-3 by 3.97- and 6.71-fold, respectively. This may explain why the co-treatment showed greater apoptosis-inducing and cell cycle arresting effects on colon cancer cells than 4DN or ATST alone.

Discussion

Combination regimens with multiple chemopreventive agents that show minimal risk of side effects during long-term treatment are of great interest to all in search of a solution to reduce the incidence and mortality of cancer. Numerous dietary bioactive compounds have been found to have cancer preventive potential but cause little or no adverse effects. A growing number of studies have suggested that combining chemopreventive dietary component with commonly used pharmaceutical drugs is a promising strategy for cancer prevention and

treatment^{4, 5, 28}. Previously, we have demonstrated the potential synergistic anti-cancer activities of NBT and ATST combination on colon carcinogenesis both *in vitro* and *in vivo*¹¹. Accumulating evidence has indicated that biotransformation dictates the biological fate and functions of PMFs including NBT^{9, 18, 29, 30}. We previously reported that 4DN was the most abundant metabolite of NBT in mouse colonic mucosa after long-term oral administration of NBT, and its colonic concentration was much higher than that of NBT⁹. Therefore, 4DN might play important roles in the biological activities of orally administered NBT in the colon. Based on our previous finding that oral administration of NBT and ATST resulted in strong synergy in inhibiting colon carcinogenesis in rats¹¹, we hypothesize that as a major NBT metabolite, 4DN may synergistically interact with ATST in the colon to produce enhanced anti-cancer effects. If this hypothesis is true it will provide a novel mechanism for the synergy between NBT and ATST in inhibiting colon carcinogenesis.

In this study, we fed rats with the identical doses of NBT and ATST as we used in the previous study¹¹ to generate colonic mucosa samples for LC-MS analysis to determine the levels of ATST, NBT, and major metabolites of NBT. Our results confirmed that 4DN was the major metabolite of NBT in the rat colon, and its level in colonic mucosa was 4.95-fold as high as that of NBT. This finding, supported the hypothesis that 4DN may significantly contribute to the synergistic anti-carcinogenic effects of NBT/ATST co-treatment. To test our hypothesis, herein, the inhibitory effects of the combination of 4DN and ATST were determined in human colon cancer HT-29 cells, and the underlying mechanisms were elucidated. It is noteworthy that the colonic level of ATST in the rats fed with 4DN and ATST was about half of that of 4DN, therefore, the concentrations of 4DN and ATST used in the cell culture study was at the ratio of 2: 1.

Using cell viability assay, we found that the half dose combinations of 4DN/ATST produced enhanced growth inhibitory effects on HT-29 cells in comparison with 4DN or ATST alone at their full doses. Moreover, the isobologram analysis verified that the enhanced bioactivity by the combination of 4DN and ATST was synergistic. All 4DN/ATST combination dose pairs resulted in interaction indexes lower than 0.7, and the two highest dose pairs tested even led to the interaction indexes lower than 0.3, indicating a strong synergy. This result demonstrated that there was a significant synergism between 4DN and ATST in inhibiting cancer cell growth. Considering the relatively high abundance of 4DN available in rat colon and the potent synergistic interactions produced by 4DN in combination with ATST in the cell culture, it is reasonable to state that the *in vivo* synergistic chemopreventive effects by NBT/ATST combination observed in the AOM-treated rats might be strongly dependent on the synergistic interactions between 4DN and ATST.

Uncontrollable cell cycle progression and evasion of programmed cell death (apoptosis) are common hallmarks of colon cancer. These molecular events leading to colon cancer are important targets for chemopreventive agents³¹. To further reveal the underlying molecular mechanisms, we utilized flow cytometric and Western blot analysis to determine the effects of 4DN/ATST co-treatment on cell cycle progression and apoptosis of HT-29 cells. Our results showed that 4DN/ATST half dose co-treatment markedly arrested HT-29 cell division in G0/G1 phase during cell cycle progression, and this effect was significantly stronger than those produced by 4DN or ATST alone at their full doses. Additionally, we investigated the

effects of 4DN/ATST on two key proteins associated with cell cycle that were p21 and CDK4. Critical signaling proteins related to cell cycle control are often deregulated during the promotion stage of carcinogenesis. Cyclin-cyclin-dependent kinase (CDK) complexes and checkpoints tightly regulate the cell cycle progression. P21 is an essential CDK inhibitor that can bind to multiple cyclin-CDK complexes, inhibit their activities, and in turn arrest cell cycle at G1 and S phase. In addition to the inhibition of Ras farnesylation in cancer cells, it has been suggested that the anti-proliferative effects of statins are also the consequence of p21 and p27 (another critical CDK inhibitor) stabilization³². The transition of cell cycle from G1 phase to S phase is precisely controlled by cyclinD-CDK4/6 complexes. Increased levels of p21 can lead to the inactivation and/or degradation of these complexes, thus resulting in G0/G1 phase cell cycle arrest^{33, 34}. As a major regulator of G1 phase progression, CDK4 is also an important target for anti-cancer drug research. Palbociclib, a recently FDA approved drug for the treatment of breast cancer, is a selective inhibitor of CDK4³⁵. Our results suggested that both individual treatment with 4DN and co-treatment of 4DN/ATST increased the expression levels of p21 by 1.53- and 1.84-fold, respectively, while only the co-treatment significantly decreased the levels of CDK4 in colon cancer cells. These results were consistent with the results from cell cycle analysis.

Our results showed that prolonged co-treatment with 4DN/ATST greatly elevated the levels of cellular apoptosis of colon cancer cells. In contrast, 4DN treatment alone did not cause significant change in apoptotic cell population, whereas ATST treatment alone only resulted in a modest induction in late apoptotic cells. We determined their effects on important proteins that have been found to play critical roles in the regulation of apoptosis, which are tumor suppressor p53 and cleaved caspase-3. P53 is one of the most potent suppressors of various types of cancer. Loss of p53 function can result in evasion of apoptosis, genomic instability, and promotion of angiogenesis. The expression of p21 is also mediated by p53, which can trigger G0/G1 phase cell cycle arrest in response to a range of stimuli^{34, 36}. Down-regulation of caspase-3 has been found in cancer patients and animal models. Caspase-3 is a member of caspase protein family. Activation (cleavage) of caspase-3 and its downstream target PARP play key roles in initiating cellular apoptosis³⁷. The mechanism of statin-induced apoptosis has been suggested to be mediated predominantly via the depletion of geranylgeranylated proteins. Statins are also capable of activating caspases pathways, such as caspase-3, -8 and -9³². Western blot analysis revealed that all the treatments induced the expression of p53 and cleaved capase-3, while 4DN/ATST co-treatment exhibited the strongest effects. The enhanced effects of 4DN/ATST combination were confirmed to be synergistic. HT-29 cells carry a mutant form of the p53 gene (R273H), which has been shown to increase cancer cell proliferation and migration, propagate cell cycle, and contribute to cancer cell survival³⁸. Nevertheless, our result suggests that the co-treatment of 4DN/ATST has the potential of up-regulating p53 gene expression in cancer cells, which in turn may contribute to its apoptosis-inducing effect. Future investigations using colon cancer cells and animal models with wild type p53 will provide definitive proof of this mechanistic scheme. Interestingly, 4DN seemed to play a more important role in arresting cell cycle progression than did ATST, while ATST exerted a stronger effect in inducing apoptosis than did 4DN, while they were used individually on the cancer cells. The results suggested that 4DN and ATST had distinct molecular target in colon cancer cells, and

may partially explain why these two compounds could act synergistically in inhibiting colon cancer.

Conclusions

This study demonstrated for the first time, the synergistic inhibitory effects on human colon cancer cells by the combination of 4DN and ATST. These effects were associated with the synergistic induction of cell cycle arrest at G0/G1 stage and cellular apoptosis of colon cancer cells by 4DN/ATST co-treatment. Further studies on (i) the interaction between other abundant metabolites of NBT (e.g. 3DN and 34DN) and ATST as well as its metabolites, and (ii) the combination of NBT, ATST and their major metabolites as a whole are important to provide mechanistic insights on the synergism between NBT and ATST in inhibiting colon carcinogenesis. Information obtained in these studies will provide a solid scientific basis for using NBT and ATST in combination to prevent colon cancer in humans.

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Abbreviations

PMF	Polymethoxyflavone
NBT	Nobiletin
4DN	4'-Demethylnobiletin
3DN	3'-Demethylnobiletin
34DN	3',4'-Didemethylnobiletin
ATST	Atorvastatin

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Figure 1.
Chemical structures of ATST, NBT, 4DN, 3DN and 34DN

Figure 2. Quantification of ATST, NBT, 4DN, 3DN and 34DN in the colonic mucosa of rats fed with NBT/ATST co-treatment by LC/ESI-MS analysis. Data were shown as mean $\pm$ SE (n = 6).

Figure 3.

Growth inhibitory effect of 4DN, ATST and their half dose combination on HT-29 human colon cancer cells. HT-29 cells were treated with serial concentration of 4DN, ATST, and their combinations for 72 h. Viable cells were measure by cell viability assay as described in Material and methods. The half-dose combination produced much stronger growth inhibition on HT-29 cells than those caused by full-dose individual treatments of 4DN and ATST. Data were shown as mean $\pm$ SD (n = 6), and viable cells of controls were used as 100%.

4DN (μ M)	3.6	7.2	10.8	14.4	18
ATST (μ M)	1.8	3.6	5.4	7.2	9
Interaction Index	0.54	0.67	0.41	0.22	0.14

Figure 4. Median effect (A) and interaction index (B) of 4DN, ATST, and their combination in HT-29 human colon cancer cells. Cells were treated with 4DN or ATST alone or in combination at serial concentrations for 72 h in 96-well plates. Cell viability was determined by MTT assay as described in Material and methods. The median effect plot and interaction index plot were constructed by using modified Chou and Talalay's method¹ as described in Materials and methods. Synergistic effect of two agents was defined as interaction index lower than 1.0.

Error bars in interaction index plots represented 95% confidence interval and were calculated using delta method².

Figure 5.

(A) Effects of 4DN (36 μ M), ATST (18 μ M), and their half dose combination (4DN at 18 μ M + ATST at 9 μ M) on cell cycle progression of HT-29 human colon cancer cells. Cells were seeded in 6-well plates for 24 h, and then treated with 4DN, ATST, and their half dose combination. After 24 h of treatments, cells were harvested and subject to cell cycle analysis, respectively. Data are shown as mean $\pm$ SD. Different notations in the bar charts indicate statistical significance ($p < 0.05$, $n = 3$) by ANOVA. (B) DNA histogram of cell cycle distribution of HT-29 cells.

Figure 6.

(A) Effects of 4DN (36 μ M), ATST (18 μ M), and their half dose combination (4DN at 18 μ M + ATST at 9 μ M) on apoptosis of HT-29 human colon cancer cells. Cells were seeded in 6-well plates for 24 h, and then treated with 4DN, ATST, and their half dose combination. After 48 h of treatments, cells were harvested and subject to apoptosis analysis. Data are shown as mean $\pm$ SD. Different notations in the bar charts indicate statistical significance ($p < 0.05$, $n = 3$) by ANOVA. (B) Dot plots of apoptotic cells of HT-29 cells.

Figure 7.

Effects of 4DN (36 μ M), ATST (18 μ M), and their half dose combination (4DN at 18 μ M + ATST at 9 μ M) on cell cycle and apoptosis related signaling proteins in HT-29 human colon cancer cells. HT-29 cells were seeded in 10-cm dishes for 24 h, and then cells were treated with 4DN, ATST, and their half dose combination. After another 24 or 48 h of incubation, cells were collected for Western blotting as described. The numbers underneath of the blots represent band intensity (normalized to β -actin, means of three independent experiments) measured by Image J software. The SDs (all within $\pm 20\%$ of the means) were not shown. β -Actin was served as an equal loading control. * indicates statistical significance in comparison with the control ($p < 0.05$, $n=3$).