RESEARCH ARTICLE

The p53-, Bax- and p21-dependent inhibition of colon cancer cell growth by 5-hydroxy polymethoxyflavones

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Scope: Previously, we reported that 5-hydroxy polymethoxyflavones (5OH-PMFs) isolated from orange, namely 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone, 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5HHMF) and 5-hydroxy-6,7,8,4′-tetramethoxyflavone (5HTMF), potently induced apoptosis and cell-cycle arrest in multiple human colon cancer cells. Herein, using isogenic variants of HCT116 human colon cancer cells, we investigated the effects of p53, Bax and p21 on the apoptosis and cell-cycle arrest induced by different 5OH-PMFs.
Methods and results: Annexin V/PI co-staining assay demonstrated that 5HHMF and 5HTMF significantly induced apoptosis in HCT116 ($p53^{+/+}$) cells but not in HCT116 ($p53^{-/-}$) cells. Furthermore, 5HHMF and 5HTMF significantly induced apoptosis in HCT116 ($Bax^{+/-}$) cells, whereas their pro-apoptotic effects on HCT116 ($Bax^{-/-}$) cells were marginal. All three 5OH-PMFs increased G0/G1 cell population of HCT116 ($p53^{+/+}$) cells, and these effects were abolished in HCT116 ($p53^{-/-}$) and HCT116 ($p21^{-/-}$) cells. Immunoblotting analysis showed that 5HHMF and 5HTMF increased the levels of cleaved caspase-3, cleaved PARP in both HCT116 ($p53^{+/+}$) and HCT116 ($Bax^{+/-}$) cells and these effects were much weaker in HCT116 ($p53^{-/-}$) and HCT116 ($Bax^{-/-}$) cells.
Conclusion: Our results demonstrated that 5OH-PMFs, especially 5HHMF and 5HTMF, induce apoptosis and cell-cycle arrest by p53-, Bax- and p21-dependent mechanism.

Received: June 11, 2010
Revised: September 5, 2010
Accepted: September 23, 2010

Keywords:
Bax / Colon cancer / 5-Hydroxy polymethoxyflavones / p21 / p53

1 Introduction

Many non-nutritive phytochemicals derived from commonly consumed fruits and vegetables have been associated with health-promoting and disease-preventing effects. As a major threat to human health, cancer has been listed as an important target for preventive intervention by diet-based strategies. This is due to the fact that many forms of cancer are associated with dietary pattern and lifestyles, and thus can be prevented. Furthermore, cancer prevention is considered to be a better and more effective way to decrease cancer-related death in comparison with traditional cancer chemotherapies.

An increasing number of studies have been conducted to investigate the cancer preventive effects and the mechanism of actions of dietary bioactive phytochemicals. We and others have reported that polymethoxyflavones (PMFs) isolated from orange peel can inhibit the growth of multiple human cancer cells [1–4]. Among these PMFs, 5-hydroxy PMFs (5OH-PMFs) have attracted more attention, because they have been shown to have much stronger anti-cancer

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Abbreviation: CDK, cyclin-dependent kinase; **HMF,** 5,6,7,8,3′,4′-heptamethoxyflavone; **5HHMF,** 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone; **5HPMF,** 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone; **5HTMF,** 5-hydroxy-6,7,8,4′-tetramethoxyflavone; **PMF,** polymethoxyflavone

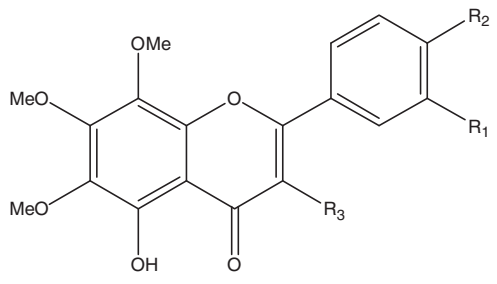
activities in comparison with their permethoxylated counterparts, *e.g.* nobiletin and tangeretin [1, 2].

We have studied the effects of three major 5OH-PMFs, namely 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (5HPMF), 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5HHMF) and 5-hydroxy-6,7,8,4′-tetramethoxyflavone (5HTMF), on human colon cancer cells (Fig. 1) [2]. Our results showed that these three 5OH-PMFs had profound effects on the cell cycle and apoptosis of multiple human colon cancer cells, including cell-cycle arrest at different phases and extensive apoptosis. We also demonstrated that the effects of 5OH-PMFs were associated with their ability in modulating key signaling proteins related to cell proliferation and apoptosis, such as $p21^{Cip1/Waf1}$, cyclin-dependent kinase (CDK)-2, CDK-4, phosphor-Rb, Mcl-1, caspases 3 and 8, and PARP. In this study, we continue our efforts in elucidating the mechanism of actions of 5OH-PMFs in inhibiting colon cancer cell growth. Using isogenic variants of human colon cancer cells HCT116, we investigated the effects of p53, Bax and p21 status on the apoptosis and cell-cycle arrest induced by three major 5OH-PMFs.

2 Materials and methods

2.1 Isolation and identification of PMFs

PMFs were isolated as previously described [1, 5, 6]. In brief, the sweet orange peel extract from Florida Flavors (Lakeland, FL, USA) (10 g) was dissolved in a mixture of methylene chloride and hexanes (1:1) and loaded onto a 120-g preconditioned silica gel flash column (Model Foxy 200, sg100, ISCO, Lincoln, NE, USA). The gradient was started with 10% ethyl acetate and 90% hexanes and reached 40% ethyl acetate and 60% hexanes within 35 min. Then the isocratic mobile phase (40% ethyl acetate–60% hexanes) was applied for another 15 min. The fractions that showed UV absorbance at 254 nm were analyzed by LC–ESI-MS and pooled based on their molecular weights. The pooled fractions containing PMFs of interest were concentrated, and



5HPMF	R1=OMe	R2=OMe	R3=H
5HHMF	R1=OMe	R2=OMe	R3=OMe
5HTMF	R1=H	R2=OMe	R3=H

Figure 1. Chemical structures of 5-hydroxy PMFs (5OH-PMFs).

the residue was dissolved in ACN and water. The dissolved solution was loaded onto a RP-C18 HPLC system. A gradient method was used from 25% ACN–75% water to 60% ACN–40% water in 25 min with a flow rate of 20 mL/min. The fractions were analyzed by LC–ESI-MS. Both the pure compounds and the mixtures were collected. To afford pure compounds, the mixture fractions were subjected to further purification using an HPLC system equipped with the Welk-O 1 (R,R) Regis column (mobile phase: 35% absolute ethanol and 65% hexanes). The fractions containing pure compounds analyzed by LC–MS were combined and concentrated or lyophilized to dryness. The dried compounds were analyzed by MS, UV and NMR for identification. Analytical data (MS, UV and NMR) of 5HPMF, 5HHMF and 5HTMF have been reported previously [5].

2.2 Cell culture and treatments with 5-hydroxyl PMFs

The isogenic lines of HCT116 human colon cancer cells, namely HCT116 ($Bax^{+/-}$), HCT116 ($Bax^{-/-}$), HCT116 ($p53^{+/+}$), HCT116 ($p53^{-/-}$) and HCT116 ($p21^{-/-}$), were kindly provided by Dr Bert Vogelstein (Johns Hopkins University, Baltimore, MD, USA). All HCT116 cell lines were maintained in McCoy's 5A media (ATCC, Manassas, VA, USA) supplemented with 5% heat-inactivated FBS (Mediatech, Herndon, VA, USA), 100 units/mL of penicillin and 0.1 mg/mL of streptomycin (Sigma-Aldrich) at 37°C with 5% CO_2 and 95% air. Cells were kept sub-confluent and media were changed every other day. All cells used were between 3 and 30 passages. DMSO was used as the vehicle to deliver 5-hydroxyl PMFs in the cell culture, and the final concentration of DMSO in all experiments was 0.1% v/v in cell culture media.

2.3 Cell cycle analyses

HCT116 ($p53^{+/+}$) (6×10^4 cells/well), HCT116 ($p53^{-/-}$) (6×10^4 cells/well), HCT116 ($Bax^{+/-}$) (6×10^4 cells/well), HCT116 ($Bax^{-/-}$) (6×10^4 cells/well) and HCT116 ($p21^{-/-}$) (12×10^4 cells/well) cells were seeded in six-well plates. Gentle mixing was required to avoid accumulation of cells in the center of each well. After 24 h of incubation for attachment, cells were treated with 5-hydroxyl PMFs in 2 mL of serum complete media. After another 24 or 48 h of incubation in the presence of 5-hydroxyl PMFs, media containing any floating cells were collected and combined with adherent cells that were detached by brief trypsinization (0.25% trypsin–EDTA; Sigma-Aldrich). Cell pellets were washed with 1 mL of ice-cold PBS, resuspended in 1 mL of 70% ethanol and then kept at −20°C overnight. After centrifugation ($1600 \times g$, 1 min), the supernatant was removed and the cells were incubated with 0.5 mL of PBS

containing 50 μg heat-treated RNase (Sigma-Aldrich) and 5 μg propidium iodine (Sigma-Aldrich) for 30 min at room temperature. Single-cell suspension was generated by gentle pipetting. Cell-cycle distribution 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.

2.4 Detection of apoptosis

HCT116 ($p53^{+/+}$) (6×10^4 cells/well), HCT116 ($p53^{-/-}$) (6×10^4 cells/well), HCT116 ($Bax^{+/-}$) (6×10^4 cells/well), HCT116 ($Bax^{-/-}$) (6×10^4 cells/well) and HCT116 ($p21^{-/-}$) (12×10^4 cells/well) cells were treated in the same manner as in Section 2.3. Apoptotic cells were quantified by Annexin V/PI double-staining assay. Annexin V/PI staining was conducted using apoptosis detection kit (BioVision, Mountain View, CA, USA) following manufacturer's instruction. 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 μL of binding buffer containing Annexin V and propidium iodide and incubated for 5 min at room temperature. Early apoptotic cells were identified as Annexin-V-positive/PI-negative cells, whereas late apoptotic/necrotic cells were identified as Annexin-V-positive/PI-positive cells using a BD LSR II cell analyzer at the analytical cytometry facility (University of Massachusetts, Amherst).

2.5 Preparation of whole cell lysate

Cells were washed with ice-cold PBS and collected with cell scrapers. The cells were combined with floating cells, if any, and incubated on ice in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 μg/mL leupeptin with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Boston Bioproducts, Boston, MA, USA) for 20 min. Cell suspensions were then subjected to sonication (5 s, three times). After further incubation for 20 min, supernatants were collected by centrifugation at $10\,000 \times g$ for 10 min. Protein concentrations were determined by BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA), following manufacturer's instruction.

2.6 Immunoblot analyses

For immunoblot analyses, equal amounts of proteins (20–50 μg, depending on the proteins of interest) was resolved over 12% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. 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 primary antibodies in blocking buffer overnight at 4°C. After incubation with appropriate secondary antibodies, the membranes were washed three times with Tris-buffered saline containing Tween-20 and then visualized using enhanced chemiluminescence (Boston Bioproducts, Ashland, MA, USA). Antibodies for p53, cleaved caspase-3 (Asp175), PARP, $p21^{Cip1/Waf1}$, Bax and Bcl-2 were purchased from Cell Signaling Technology (Beverly, CA, USA). Anti-β actin antibody was from Sigma-Aldrich.

2.7 Statistical analyses

All data were presented as mean ± SD. Student's *t*-test was used to test the mean difference between two groups. Analysis of variance (ANOVA) model was used for the comparison of the differences among more than two groups. A 1% significant level was used for all the tests.

3 Results

3.1 5HHMF and 5HTMF induced apoptosis through a p53-dependent mechanism

In order to investigate the role of p53 in the cellular apoptosis induced by different 5OH-PMFs, we studied the effects of 5HPMF, 5HHMF and 5HTMF on the apoptosis induction in different isogenic derivatives of HCT116 cells, namely HCT116 ($p53^{+/+}$) and HCT116 ($p53^{-/-}$). Previously, we have demonstrated that 5HPMF, 5HHMF and 5HTMF inhibited HCT116 human colon cancer cell growth with potency in the order of 5HPMF < 5HHMF < 5HTMF. On the basis of those results, we chose to use 5HPMF, 5HHMF and 5HTMF at concentrations of 15, 6 and 2 μM, respectively. At these concentrations (EC_{50}), 5OH-PMFs can cause about 50% growth inhibition after 48 h treatment. After treatments with different 5OH-PMFs, cells were subjected to Annexin V/PI double staining assay to quantify the apoptotic cell population. As shown in Fig. 2A, Annexin-V-negative and PI-negative cells were defined as live cells, whereas Annexin-V-positive and PI-negative cells were defined as early apoptotic cells. Annexin-V-positive and PI-positive cells were late apoptotic and/or necrotic cells. Figure 2B shows the histogram of Annexin-V-positive cell population. The results were quantified and summarized in the bar chart (Fig. 2C).

After 48 h treatment, all three 5OH-PMFs significantly induced apoptosis in HCT116 ($p53^{+/+}$) cells, which was evidenced by the increase in the Annexin-V-positive cell population. Specifically, 5HTMF increased early apoptotic cell population by 6.8-fold (from 2.7 to 18.4%) and late apoptotic/necrotic cell population by 3.2-fold (from 3.3 to

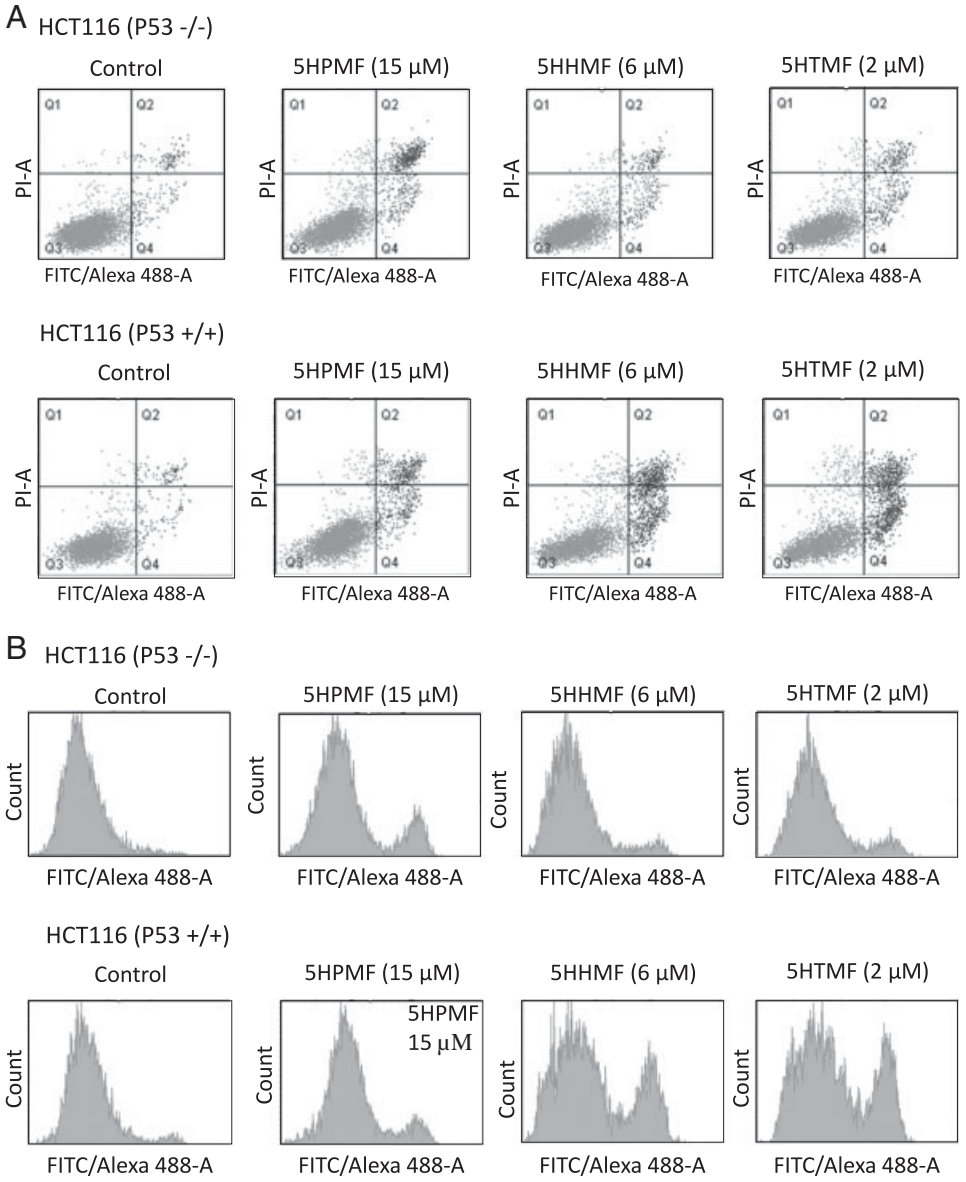


Figure 2. Effect of 5OH-PMFs on apoptosis of isogenic HCT116 ($p53^{+/+}$) and HCT116 ($p53^{-/-}$) human colon cancer cells. The cells were seeded in six-well plates for 24 h, and then treated with 5HPMF, 5HHMF or 5HTMF. After 48 h of treatments, cells were harvested and subjected to apoptosis analyses as described in Section 2. (A) Dot plots of Annexin V and PI co-staining. (B) Histogram of Annexin V staining. (C) Quantification of early and late apoptosis cells. All data represent mean ± SD, and the statistical significance ($p < 0.01$, $n = 3$) was indicated in the bar charts.

10.4%) (Fig. 2C). Similarly, 5HHMF increased early and late apoptotic cell population by 5.4- and 3.2-fold, respectively. 5HPMF also increased apoptotic cell population of HCT116 ($p53^{+/+}$) cells, but to much less extent in comparison with 5HHMF and 5HTMF. However, HCT116 ($p53^{-/-}$) cells showed very different responses to the same treatments with 5OH-PMFs. Among the three 5OH-PMFs, the 5HPMF increased the Annexin-V-positive HCT116 ($p53^{-/-}$) cell population the most, but mainly the late apoptotic/necrotic cell population. These effects were even stronger than those produced by 5HPMF in HCT116 ($p53^{+/+}$) cells, which suggested that 5HPMF-induced cell death was not through a p53-dependent mechanism. 5HTMF moderately increased the apoptotic cell population in HCT116 ($p53^{-/-}$) cells, whereas 5HHMF only slightly increased the apoptotic cell population in HCT116 ($p53^{-/-}$) cells. These effects were much weaker than those produced by 5HHMF and 5HTMF in HCT116 ($p53^{+/+}$) cells. Overall, these findings suggested that p53-dependent mechanism played an important role in apoptosis induced by 5HTMF and 5HHMF in colon cancer cells.

Western blot analysis confirmed that no p53 expression can be detected in HCT116 ($p53^{-/-}$) cells, and 5OH-PMFs treatment did not induce any expression of p53 (Fig. 3). For HCT116 ($p53^{+/+}$) cells, trace amount of p53 protein can be detected at 24 h in the control cells, whereas a significant increase in the expression level of p53 was observed in the cells treated with 5OH-PMFs with potency in the order of 5HHMF > 5HTMF > 5HPMF at the concentrations tested (Fig. 3). Similar trend was observed in the cells at 48 h after the treatment. To further confirm the apoptosis induced by 5OH-PMFs as measured by Annexin V/PI co-staining assay, the expression levels of cleaved caspase 3 and cleaved PARP proteins have been analyzed by Western blot (Fig. 4). The results demonstrated that 5HHMF and 5HTMF at the concentrations tested significantly increased the levels of cleaved caspase 3 in HCT116 ($p53^{+/+}$) cells at both 24 and 48 h, whereas 5HPMF only showed marginal effects. In contrast, only a slight increase in the cleaved caspase 3 can be observed in HCT116 ($p53^{-/-}$) cells after treatment with 5HHMF or 5HTMF for 24 or 48 h. Similarly, 5HHMF and 5HTMF showed much stronger effects in increasing the expression levels of cleaved PARP in HCT116 ($p53^{+/+}$) cells in comparison with HCT116 ($p53^{-/-}$) cells. These results convincingly demonstrated that apoptosis induced by 5HHMF and 5HTMF is mainly through a p53-dependent mechanism.

3.2 5HHMF and 5HTMF induced apoptosis through a Bax-dependent mechanism

To determine the role of Bax protein in the apoptosis induced by different 5OH-PMFs, we use Annexin V/PI co-staining assay to quantify the extent of apoptosis in two isogenic derivatives of HCT116 cells, namely HCT116 ($Bax^{+/-}$) and HCT116 ($Bax^{-/-}$) cells, after treatments with three 5OH-PMFs (Fig. 5). On the basis of the potency of three 5OH-PMFs in inhibiting the cell growth of HCT116 ($Bax^{+/-}$) cells, we chose to use 5HPMF, 5HHMF and 5HTMF at concentrations of 15, 8 and 1.5 μM, respectively.

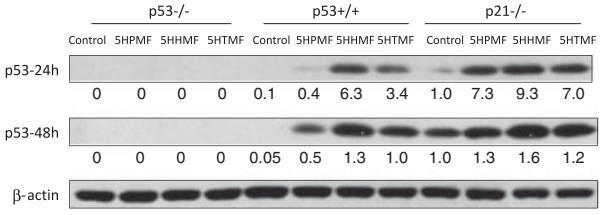


Figure 3. Effects of 5OH-PMFs on the expression levels of p53 in three isogenic human colon cancer cells, namely HCT116 ($p53^{+/+}$), HCT116 ($p53^{-/-}$) and HCT116 ($p21^{-/-}$) cells. Cells were seeded in 10-cm dishes for 24 h, and then treated with 5HPMF, 5HHMF and 5HTMF at 15, 6 and 2 μM, respectively. After 24 and 48 h of incubation, cells were harvested and equal amounts of cellular proteins were subjected to Western immunoblotting as described in Section 2. The numbers underneath the blots represent band intensities (normalized to β-actin, means of three independent experiments) measured by Image J software. The standard deviations (all within ±15% of the means) were not shown. β-Actin served as an equal loading control. The experiments were repeated for three times.

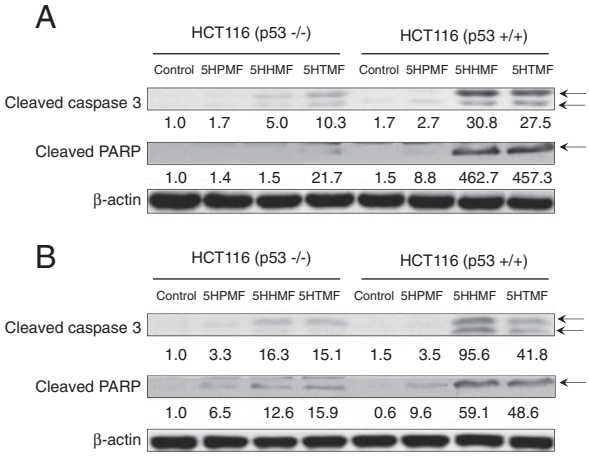


Figure 4. Effects of 5OH-PMFs on the expression levels of cleaved caspase 3 and cleaved PARP in isogenic human colon cancer cells, namely HCT116 ($p53^{+/+}$) and HCT116 ($p53^{-/-}$) cells. Cells were seeded in 10-cm dishes for 24 h, and then treated with 5HPMF, 5HHMF and 5HTMF. After (A) 24 and (B) 48 h of incubation, cells were harvested and equal amounts of cellular proteins were subjected to Western immunoblotting as described in Section 2. The numbers underneath the blots represent band intensity (normalized to β-actin, means of three independent experiments) measured by Image J software. The standard deviations (all within ±15% of the means) were not shown. β-Actin served as an equal loading control. The experiments were repeated for three times.

At these concentrations (EC_{50}), 5OH-PMFs can cause about 50% growth inhibition after 48 h treatment. Figure 5A shows the histogram of Annexin-V-positive cell population, and the overall results were quantified and summarized in the bar graph (Fig. 5B).

As shown in Fig. 5, all three 5OH-PMFs induced apoptosis in HCT116 ($Bax^{+/-}$) cells, but with marked difference in potency. Specifically, 5HPMF, 5HHMF and 5HTMF increased early apoptotic cell population of HCT116 ($Bax^{+/-}$) cells by 1.6-, 7.3- and 3.4-fold, respectively, in comparison with the control HCT116 ($Bax^{+/-}$) cells. The late apoptotic/necrotic cell population was not changed significantly by the treatments with three 5OH-PMFs in HCT116 ($Bax^{+/-}$) cells. In contrast, HCT116 ($Bax^{-/-}$) cells responded differently to the same treatments with three 5OH-PMFs. Treatment with 5HPMF did not cause any increase in the apoptotic cell population, and only a twofold increase was observed in 5HTMF-treated HCT116 ($Bax^{-/-}$) cells. Among three 5OH-PMFs, 5HHMF showed the strongest effect in inducing apoptosis in HCT116 ($Bax^{-/-}$) cells and it caused a fourfold increase in apoptotic cell population in comparison with the control HCT116 ($Bax^{-/-}$) cells. Overall, three 5OH-PMFs produced stronger pro-apoptotic effects on HCT116 ($Bax^{+/-}$) cells in comparison with HCT116 ($Bax^{-/-}$) cells.

Western blot analysis confirmed that there was no detectable Bax protein expressed in HCT116 ($Bax^{-/-}$) cells (Fig. 6), and treatments with 5OH-PMFs did not induce Bax expression. HCT116 ($Bax^{+/-}$) cells expressed abundant Bax protein, and treatments with 5OH-PMFs resulted in an increase in the expression levels of Bax at 24 and 48 h, especially with 5HHMF and 5HTMF. Treatments with 5HHMF and 5HTMF also significantly increased the levels of cleaved caspase 3 in HCT116 ($Bax^{+/-}$) cells at both 24 and 48 h. In contrast, much less increase in the levels of cleaved caspase 3 was observed in HCT116 ($Bax^{-/-}$) cells after same treatments with 5HHMF and 5HTMF. Consistent with these results, treatments with 5HHMF and 5HTMF caused a higher increase in the levels of PARP in HCT116 ($Bax^{+/-}$) cells than in HCT116 ($Bax^{-/-}$) cells.

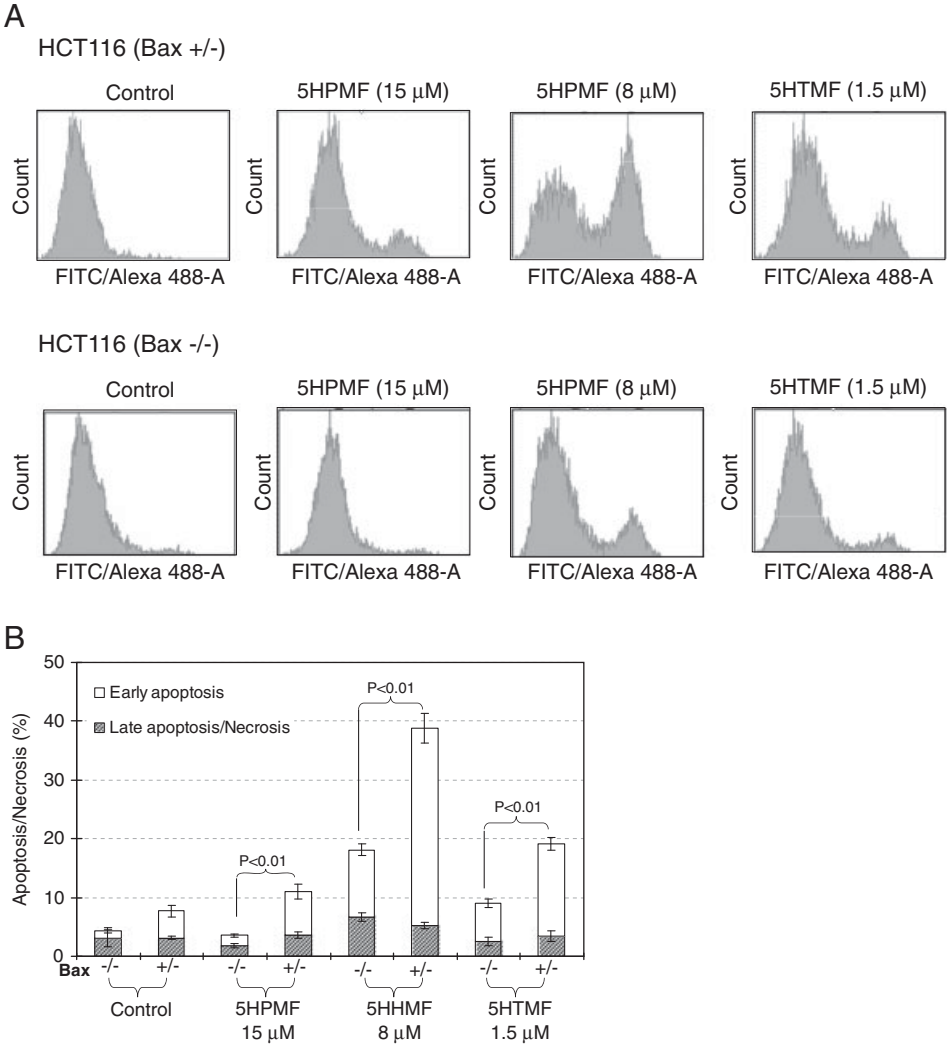


Figure 5. Effect of 5OH-PMFs on apoptosis of isogenic HCT116 ($Bax^{+/-}$) and HCT116 ($Bax^{-/-}$) human colon cancer cells. The cells were seeded in six-well plates for 24 h, and then treated with 5HPMF, 5HHMF or 5HTMF. After 48 h of treatments, cells were harvested and subjected to apoptosis analyses as described in Section 2. (A) Histogram of Annexin V staining. (B) Quantification of early and late apoptosis cells. All data represent mean ± SD, and the statistical significance ($p < 0.01$, $n = 3$) is indicated in the bar charts.

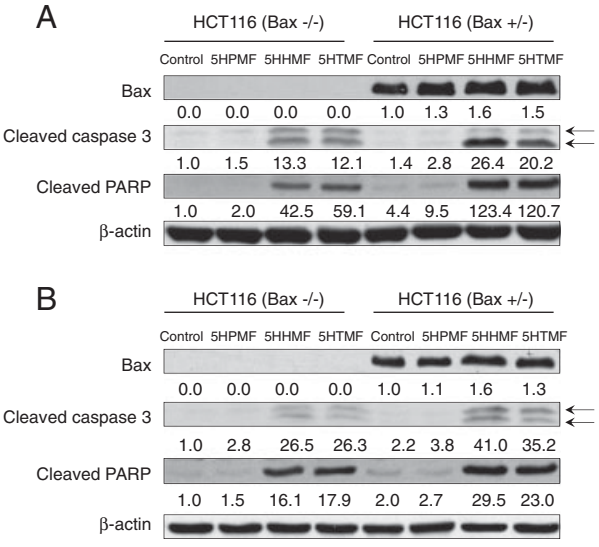


Figure 6. Effects of 5OH-PMFs on the expression levels of cleaved caspase 3 and cleaved PARP in isogenic human colon cancer cells, namely HCT116 ($Bax^{+/-}$) and HCT116 ($Bax^{-/-}$) cells. Cells were seeded in 10-cm dishes for 24 h, and then treated with 5HPMF, 5HHMF and 5HTMF at 15, 8 and 1.5 μM, respectively. After (A) 24 and (B) 48 h of incubation, cells were harvested and equal amounts of cellular proteins were subjected to Western immunoblotting as described in Section 2. The numbers underneath the blots represent band intensity (normalized to β-actin, means of three independent experiments) measured by Image J software. The standard deviations (all within ±15% of the means) were not shown. β-Actin served as an equal loading control. The experiments were repeated for three times.

3.3 5HPMF, 5HHMF and 5HTMF induced cell-cycle arrest at G0/G1 phase through a p53- and p21 *Cip1/Waf*-dependent mechanism

To investigate the role of p53 and $p21^{Cip1/Waf1}$ in the cell-cycle arrest produced by 5OH-PMFs, we studied the effects of three 5OH-PMFs on the cell-cycle distribution of isogenic human colon cancer cells, namely HCT116 ($p53^{+/+}$), ($p53^{-/-}$) and ($p21^{-/-}$) cells (Fig. 7D). The concentrations tested for the different 5OH-PMFs were the same as in Fig. 2. As shown in Fig. 7A, after 24 h treatment, all three 5OH-PMFs caused HCT116 ($p53^{+/+}$) cells to accumulate in both G0/G1 and G2/M phases. For example, treatments with 5HPMF, 5HHMF and 5HTMF increased HCT116 ($p53^{+/+}$) cell population at G0/G1 phase from 27.5% to 33.4, 35.4 and 36.4, respectively. At the same time, HCT116 ($p53^{+/+}$) cell population at G2/M phase was increased from 24.3% to 32.0, 46.6 and 51.6%, respectively. In contrast to the HCT116 ($p53^{+/+}$) cells, HCT116 ($p53^{-/-}$) cells did not respond to the 5OH-PMFs treatments in the same manner (Fig. 7B). Three 5OH-PMFs only caused HCT116 ($p53^{-/-}$) cells to accumulate at G2/M phase. Moreover, not only there was no increase in G0/G1 phase cell population but also there was a decrease in the G0/G1 cell population after treatment with all three 5OH-PMFs (Fig. 7B). Specifically, 5HPMF, 5HHMF and 5HTMF decreased HCT116 ($p53^{-/-}$) cell population at G0/G1 phase from 32.2% to 24.9, 28.7 and 18.2%, respectively. In HCT116 ($p21^{-/-}$) cells, similar trends were observed after treatments with three 5OH-PMFs, *i.e.* all three 5OH-PMFs caused a significant increase in G2/M cell population, but none of the three 5OH-PMFs caused cell population increase at G0/G1 phase (Fig. 7C). These results demonstrated that the cell-cycle arrest at G0/G1 phase induced by three 5OH-PMFs was mediated by a p53- and $p21^{Cip1/Waf1}$-dependent mechanism. We further characterized the status of p53 and $p21^{Cip1/Waf1}$ in isogenic derivatives of HCT116 cells. After treatments with 5OH-PMFs, the same amount of cellular protein from three different HCT116 cells was applied to Western blotting analysis. As shown in Fig. 3, HCT116 ($p21^{-/-}$) cells can express p53. The treatments with three 5OH-PMFs significantly increased the expression levels of p53 in HCT116 ($p21^{-/-}$) cells. For example, at 24 h, the treatments with 5HPMF, 5HHMF and 5HTMF increased the levels of p53 by 7.3-, 9.3- and 7.0-fold, respectively, in comparison with the control HCT116 ($p21^{-/-}$) cells. As shown in Fig. 8, HCT116 ($p21^{-/-}$) cells did not express $p21^{Cip1/Waf1}$ at a detectable levels, whereas only relatively small amount of $p21^{Cip1/Waf1}$ was detected in HCT116 ($p53^{-/-}$) cells. In contrast, HCT116 ($p53^{+/+}$) cells expressed high levels of $p21^{Cip1/Waf1}$ protein at both 24 and 48 h. Treatments with three 5OH-PMFs significantly increased the levels of $p21^{Cip1/Waf1}$ in HCT116 ($p53^{+/+}$) cells. An increase in the $p21^{Cip1/Waf1}$ level was also observed in HCT116 ($p53^{-/-}$) cells after the treatments with three 5OH-PMFs, although the total expression levels were still much lower than those of HCT116 ($p53^{+/+}$) cells.

4 Discussion

The p53 tumor suppressor protein is regarded as a major player in tumor suppression. It exerts its roles by inducing apoptosis, cellular senescence and growth arrest. Loss of p53 function can lead to increased cell proliferation, resistance to cell death stimuli, genomic instability and metastasis [7]. In this study, we investigated the role of p53 in the apoptosis induced by three different 5OH-PMFs isolated from orange peels. The results showed that treatments with 5HHMF and 5HTMF caused much more extensive apoptosis in HCT116 ($p53^{+/+}$) cells than in HCT116 ($p53^{-/-}$) cells, whereas treatment with 5HPMF caused less apoptosis in HCT116 ($p53^{+/+}$) cells than in HCT116 ($p53^{-/-}$) cells (Fig. 2C). HCT116 ($p53^{+/+}$) and HCT116 ($p53^{-/-}$) cells are isogenic cells, and the only difference between them is that HCT116 ($p53^{+/+}$) cells express wild-type p53 whereas HCT116 ($p53^{-/-}$) cells do not express p53, which the Western blotting data have confirmed in Fig. 3. Overall, our results clearly demonstrated that the apoptosis induced by 5HHMF and 5HTMF is mainly through a p53-dependent mechan-

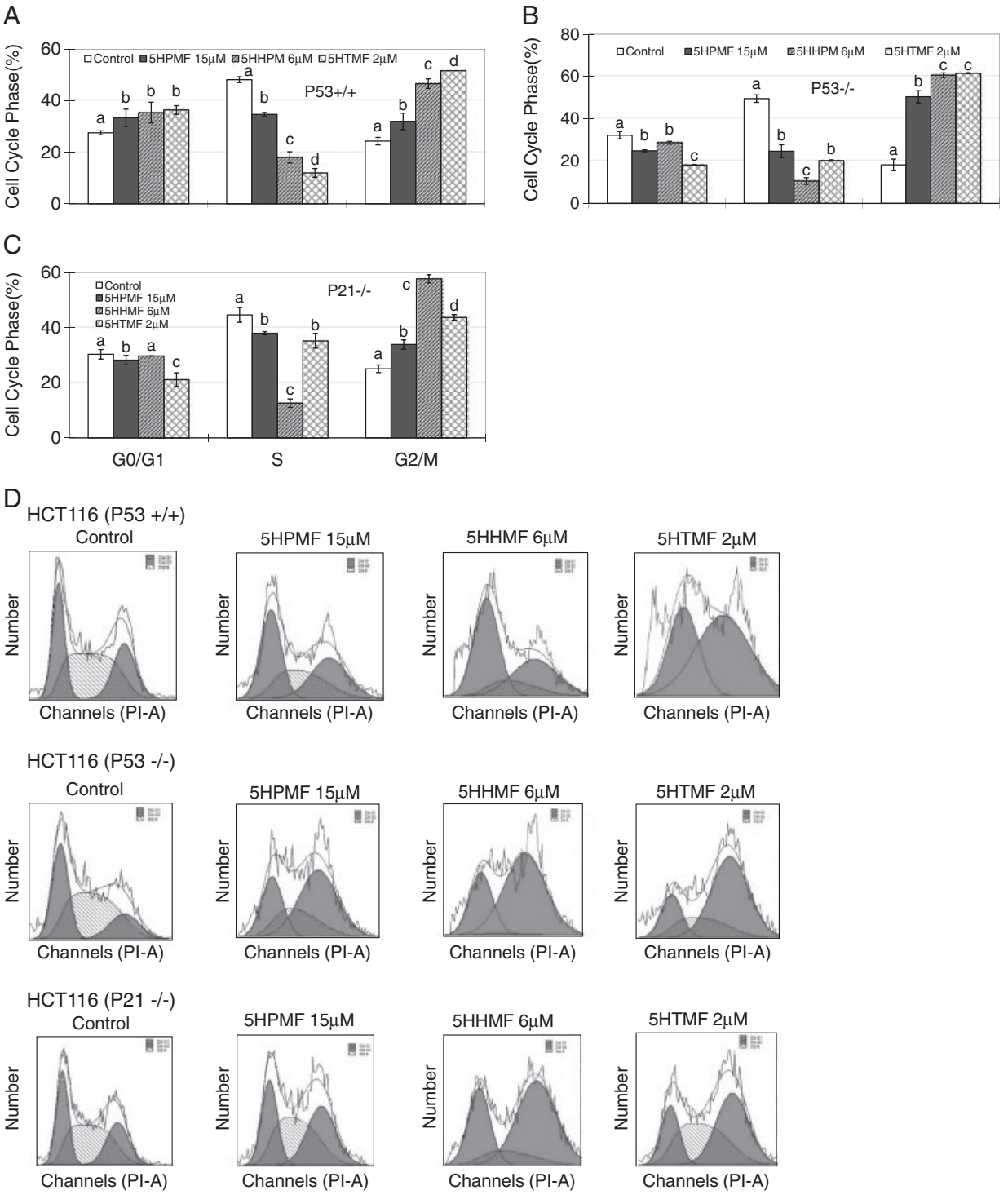


Figure 7. Cell-cycle distribution of (A) HCT116 ($p53^{+/+}$), (B) HCT116 ($p53^{-/-}$) and (C) HCT116 ($p21^{-/-}$) cells after treatments with different 5OH-PMFs. The cells were seeded in six-well plates for 24 h, and then treated with 5HPMF, 5HHMF and 5HTMF at 15, 6 and 2 μM, respectively. After 24 h of treatments, cells were harvested and subjected to cell-cycle analyses as described in Section 2. The DNA histograms are shown in (D). All data represent mean ± SD. 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 indicate statistical significance ($p < 0.01$, $n = 3$).

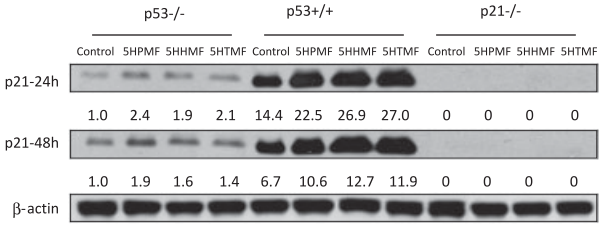


Figure 8. Effects of 5OH-PMFs on $p21^{Cip1/Waf1}$ in HCT116 ($p53^{+/+}$), HCT116 ($p53^{-/-}$) and HCT116 ($p21^{-/-}$) cells. HCT-116 cells were seeded in 10-cm dishes for 24 h, and then cells were treated with 5HPMF, 5HHMF and 5HTMF at 15, 6 and 2 μM, respectively. After 48 h of incubation, cells were harvested for Western immunoblotting as described in Section 2. The numbers underneath the blots represent band intensity (normalized to β-actin, means of three independent experiments) measured by Image J software. The standard deviations (all within ±15% of the means) were not shown. β-Actin served as an equal loading control. The experiments were repeated for three times.

ism. Furthermore, treatments with 5HHMF and 5HTMF significantly increased the expression levels of p53 in HCT116 ($p53^{+/+}$) cells. Treatment with 5HPMF also increased the p53 level, but to a much less extent, which may explain why 5HPMF caused much less apoptosis than did 5HHMF and 5HTMF in HCT116 ($p53^{+/+}$) cells. Pan *et al.* have reported that 5HHMF produced an oxidative cellular environment that induces DNA damage in HL-60 human leukemia cells [3]. It is well known that DNA damage can activate p53 and lead to apoptosis [7]. The upregulation of p53 by 5OH-PMFs observed in this study may be related to the ability of 5OH-PMFs to cause oxidative stress in cancer cells. Further studies are needed to elucidate the relationship between potential oxidative stress caused by 5OH-PMFs and apoptosis in colon cancer cells.

Bax is a Bcl-2 family protein. It promotes apoptosis and is responsible for the mitochondrial damage that leads to caspase activation [8–10]. Bax is largely redundant, but the absence of Bax can render cells highly resistant to apoptosis triggered by a range of cell death stimuli [11, 12]. Herein, we tested the role of Bax in the apoptosis induced by 5OH-PMFs in isogenic HCT116 ($Bax^{+/-}$) and HCT116 ($Bax^{-/-}$) human colon cancer cells. Our Western blotting results have confirmed that HCT116 ($Bax^{+/-}$) cells express ample Bax protein, whereas HCT116 ($Bax^{-/-}$) cells do not express any Bax protein (Fig. 6). According to the Annexin V/PI co-staining assay, all three 5OH-PMFs induced apoptosis in HCT116 ($Bax^{+/-}$) cells, especially 5HHMF and 5HTMF. In contrast, the magnitude of apoptosis induced by the three 5OH-PMFs in HCT116 ($Bax^{-/-}$) cells was much less than in HCT116 ($Bax^{+/-}$) cells. These results demonstrated that cellular apoptosis induced by all three 5OH-PMFs is at least partially through a Bax-dependent mechanism. As Bax plays an important role in mitochondria-mediated apoptosis, our results also suggested the role of mitochondria in the apoptosis caused by OH-PMFs. Consistent with our results, Pan *et al.* [3] have reported that 5HHMF induced apoptosis through reactive oxygen species production that decreased mitochondrial trans-membrane potential in human leukemia cells. Sergeev *et al.* [13] have reported that OH-PMFs could induce an increase in intracellular $Ca^{(2+)}$ and thus activate $Ca^{(2+)}$-dependent apoptotic proteases in human breast cancer cells. It is interesting to further investigate the effects of different OH-PMFs on reactive oxygen species production and intracellular $Ca^{(2+)}$ in human cancer cells. Our results showed that 5HPMF had less potent pro-apoptotic effects in HCT116 ($Bax^{+/-}$) than 5HHMF and 5HTMF. Similar trends were also observed in HCT116 ($p53^{+/+}$) cells. These results suggested that mechanisms other than apoptosis were accounted for the growth inhibitory effects of 5HPMF on HCT116 ($Bax^{+/-}$) and HCT116 ($p53^{+/+}$) cells tested herein. The results from both Annexin V/PI co-staining assay and Western blotting indicated that 5HHMF and 5HTMF also induced apoptosis in HCT116 ($p53^{-/-}$) and HCT116 ($Bax^{-/-}$) cells, although the magnitude was much less than that in HCT116 ($p53^{+/+}$) and HCT116 ($Bax^{+/-}$) cells. This suggested that 5HHMF and 5HTMF are also able to induce p53- and Bax-independent apoptosis in colon cancer cells.

Besides apoptosis, inhibition of cell-cycle progression is an another important strategy to controlling cancer cell growth. Activation of cyclin–CDK complexes regulates progression through the cell cycle [14] CDK inhibitors can inhibit the activities of cyclin–CDK complexes and negatively regulate cell-cycle progression [15] The CDK inhibitor $p21^{WAF1/CIP1}$ is a member of Cip/Kip family and responsible for p53-dependent cell-cycle arrest [16–19]. The $p21^{WAF1/CIP1}$ suppresses cell-cycle progression mainly by inhibiting the cyclin E/CDK2 activity. Utilizing isogenic HCT116 ($p53^{+/+}$), HCT116 ($p53^{-/-}$) and HCT116 ($p21^{-/-}$) cells, we studied the effects of status of p53 and p21 on cell-cycle arrest caused by 5OH-PMFs. HCT116 ($p21^{-/-}$) cells expressed ample amount of p53 but do not express p21. Our results showed that all three 5OH-PMFs caused both G0/G1 arrest and G2/M arrest in HCT116 ($p53^{+/+}$) cells. The treatment with 5HTMF caused the strongest arrest at both G0/G1 and G2/M phases in comparison with 5HPMF and 5HHMF, which suggested that the structure difference on B ring (one methoxyl group *vs* two methoxyl groups) may play a role in inducing cell-cycle arrest. Moreover, the G0/G1 arrest induced by all three 5OH-PMFs was abolished in HCT116 ($p53^{-/-}$) and HCT116 ($p21^{-/-}$) cells, indicating that G0/G1 cell-cycle arrest induced by 5OH-PMFs was through a p53- and p21-dependent mechanism. The data also showed that the 5HTMF-induced G0/G1 arrest was the most responsive to the change of the p21 and p53 status of the colon cancer cells, indicating the importance of the 4′ methoxyl group on B ring of 5HTMF in inducing G0/G1 arrest. Overall, our results are consistent with the fact that $p21^{WAF1/CIP1}$ is one of the major transcription targets of p53 tumor suppressor. As the change of p53 and p21 status did not diminish the G2/M arrest induced by three 5OH-PMFs,

p53- and $p21^{WAF1/CIP1}$-independent mechanisms are likely responsible for G2/M arrest observed in HCT116 cells after treatments with different 5OH-PMFs.

Taken together, our results demonstrated for the first time that (i) 5HHMF and 5HTMF can induce cellular apoptosis in human colon cancer cells by p53- and Bax-dependent mechanisms; (ii) 5HPMF, 5HHMF and 5HTMF induced G0/G1 arrest through p53- and p21-dependent mechanisms, whereas 5HPMF, 5HHMF and 5HTMF induced G2/M arrest through p53- and p21-independent mechanisms.

This work was supported in part by NIH grant CA139174, Healey Endowment Research Grant, CVIP grant and Hatch grant from the University of Massachusetts Amherst and by Pilot Grant from the Center for Excellence in Apoptosis Research at the Pioneer Valley Life Sciences Institute. We thank the Chinese Government for providing funding for Peiju Qiu conducting research at University of Massachusetts, Amherst.

The authors have declared no conflict of interest.

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