DOI 10.1002/mnfr.200900605 *Mol. Nutr. Food Res.* 2010, *54*, S244–S252

RESEARCH ARTICLE

Inhibitory effects of 5-hydroxy polymethoxyflavones on colon cancer cells

Peiju Qiu[1,2], Ping Dong[2], Huashi Guan[1], Shiming Li[3], Chi-Tang Ho[4], Min-Hsiung Pan[5], David Julian McClements[2] and Hang Xiao[2]

[1] Marine Drug and Food Institute, Ocean University of China, Qingdao Shandong, P. R. China
[2] Department of Food Science, University of Massachusetts, Amherst, MA, USA
[3] WellGen, Inc., North Brunswick, NJ, USA
[4] Department of Food Science, Rutgers, The State University of New Jersey, New Brunswick, NJ, USA
[5] Department of Seafood Science, National Kaohsiung Marine University, Kaohsiung, Taiwan

Received: December 18, 2009
Revised: January 29, 2010
Accepted: February 2, 2010

Hydroxylated polymethoxyflavones (PMFs) are a class of novel flavonoid compounds mainly found in citrus plants. We studied the effects of three major 5-hydroxy PMFs, namely: 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone, 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone, and 5-hydroxy-6,7,8,4′-tetramethoxyflavone, on human colon cancer HCT116 and HT29 cells. Their effects were compared with those produced by their permethoxylated counterparts, namely: nobiletin, 3,5,6,7,8,3′,4′-heptamethoxylflavone, and tangeretin. 5-Hydroxy PMFs showed much stronger inhibitory effects on the growth of the colon cancer cells in comparison with their permethoxylated counterparts, suggesting the pivotal role of hydroxyl group at 5-position in the enhanced inhibitory activity by 5-hydroxy PMFs. Flow cytometry analysis demonstrated that three 5-hydroxy PMFs produced different effects on the cell cycle and apoptosis, which may suggest that three 5-hydroxy PMFs act through different mechanisms. For example, 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone caused cell cycle arrest at G2/M phase in HT29 cells, while 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone led to significant G0/G1 phase arrest. In contrast, 5-hydroxy-6,7,8,4′-tetramethoxyflavone increased sub-G0/G1 cell population, which has been confirmed to be due to enhanced apoptosis. Our results further demonstrated that the inhibitory effects of 5-hydroxy PMFs were associated with their ability in modulating key signaling proteins related to cell proliferation and apoptosis, such as $p21^{Cip1/Waf1}$, CDK-2, CDK-4, phosphor-Rb, Mcl-1, caspases 3 and 8, and poly ADP ribose polymerase (PARP).

Keywords:
5-hydroxy polymethoxyflavones / Apoptosis / Cell cycle arrest / Cell growth inhibition / Colon cancer

1 Introduction

Polymethoxyflavones (PMFs) refer to flavonoid compounds that bear two or more methoxy groups on their basic benzo-γ-pyrone skeleton with a carbonyl group at the C4 position (Fig. 1). Naturally, PMFs were mainly found in citrus plants, particularly in the peels of sweet oranges (*Citrus sinensis*) and mandarin oranges (*Citrus reticulata*) [1]. Currently, more than 20 PMFs have been isolated and identified from different parts of citrus plants. They exhibited a broad spectrum of biological activity, such as anti-inflammatory, anti-carcinogenic, anti-atherogenic, anti-viral, and anti-oxidative ones [1–3]. The most studied PMFs are permethoxylated PMFs, such as nobiletin, 3,5,6,7,8,3′,4′-heptamethoxylflavone (HMF) and tangeretin (Fig. 1). These PMFs are also most abundant ones found in orange peel extract from cold-pressed sweet orange peel oil [1].

Recently, a class of hydroxylated PMFs have been isolated from aged orange peel extracts, and they could be formed by auto-hydrolysis of their permethoxylated counterparts

Correspondence: Dr. Hang Xiao, Department of Food Science, University of Massachusetts, 100 Holdsworth Way, Amherst, MA 01003, USA
E-mail: hangxiao@foodsci.umass.edu
Fax: +1-413-545-1262

Abbreviation: **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; **HMF,** 3,5,6,7,8,3′,4′-heptamethoxyflavone; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PARP,** poly ADP ribose polymerase; **PI,** propidium iodide; **PMF,** polymethoxyflavone

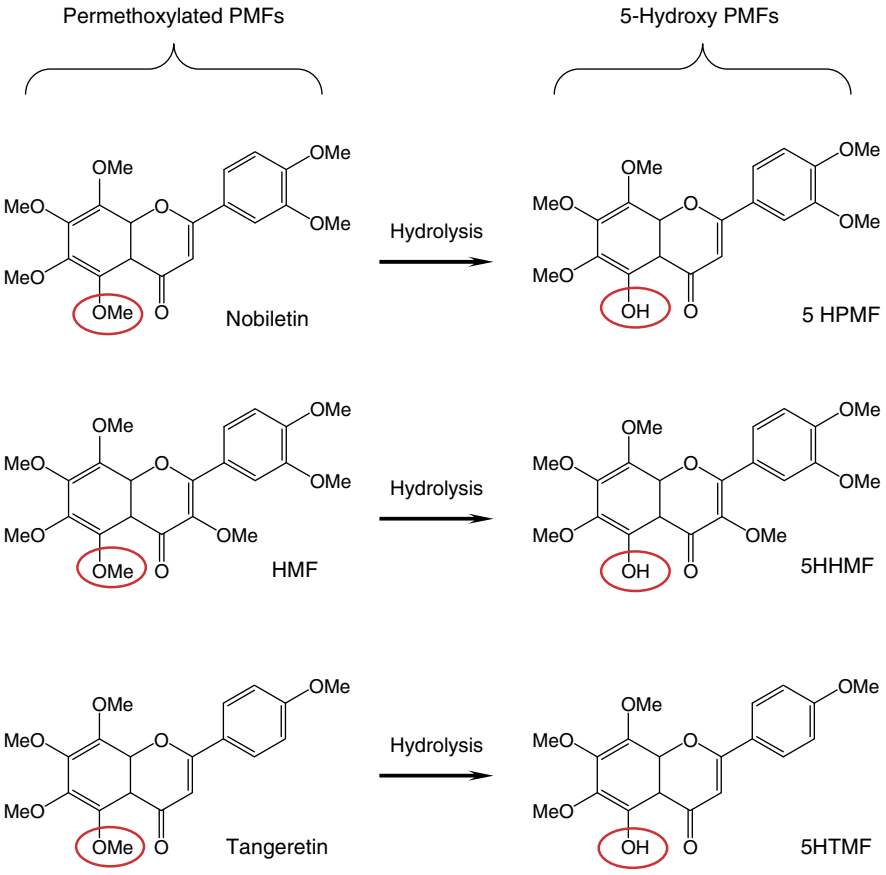

Figure 1. Chemical structures of permethoxylated PMFs and 5-hydroxy PMFs.

during long-term storage (Fig. 1) [1, 4, 5]. We have previously showed that hydroxylated PMFs, *i.e.* 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (5HPMF) and 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5HHMF), had much stronger inhibitory effect on the growth of human lung cancer cells than their permethoxylated counterparts, *i.e.* nobiletin and HMF, respectively [6]. It has also been demonstrated that 5HHMF and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone showed much more potent inhibitory effects on the growth of human leukemia cells and breast cancer cells than their permethoxylated counterparts, HMF and 5,6,7,3′,4′-pentamethoxyflavone, respectively [7, 8]. While aforementioned studies demonstrated promising cancer-preventive effects of hydroxylated PMFs, much more research efforts are needed to understand the mechanisms by which hydroxylated PMFs act against cancer cell growth.

Colon cancer is one of leading causes of cancer death in the developed Western countries such as the United States [9]. The incidence of colon cancer is increasing globally as the dietary patterns and life styles of the industrialized Western countries are adopted worldwide [10, 11]. As part of our efforts to identify novel dietary agents for cancer prevention, we studied effects of three major 5-hydroxy PMFs, namely: 5HPMF, 5HHMF, and 5-hydroxy-6,7,8,4′-tetramethoxyflavone (5HTMF) on two human colon cancer cell lines. We also compared their activities with those of their permethoxylated counterparts, namely, nobiletin, HMF, and tangeretin. It is noteworthy that this is the first report on the colon cancer chemopreventive effects of 5HTMF.

2 Materials and methods

2.1 Isolation of PMFs and cell culture treatments with PMFs

PMFs were isolated and identified as previously described [1, 6, 12]. Human colorectal cancer cell lines HCT116 and HT29 were obtained from American Type Cell Collection (ATCC, Manassas, VA, USA), and were maintained in McCoy's 5A media (ATCC) 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% CO2 and 95% air. Cells were kept sub-confluent and media were changed every other day. DMSO was used as the vehicle to deliver PMFs, and the final concentration of DMSO in all experiments was 0.1%.

2.2 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for cellular viability

HCT116 (1500 cells/well), HT29 (2000 cells/well) cells were seeded in 96-well plates. After 24 h, cells were treated with serial concentrations of PMFs in 200 μL of serum complete media. After 48 h treatments, cells were subject to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Media were replaced by 100 μL fresh media containing 0.5 mg/mL of MTT (Sigma-Aldrich). 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, VT, USA)

2.3 Cell cycle analyses

HCT116 (6×10^4 cells/well) and HT29 (7×10^4 cells/well) cells were seeded in 6-well plates. After 24-h incubation for attachment, cells were treated with different concentrations of hydroxylated PMFs in 2 mL of serum complete media. After another 24 or 48 h, media 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 and then resuspended in 1 mL of 70% ethanol in −20°C overnight. After centrifugation (1600g, 1 min), the supernatant was removed and cells were incubated with 0.5 mL of PBS containing 50 μg 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 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 and HT29 cells were treated exactly same as in cell cycle analyses described above. Apoptotic cells were quantified by Annexin V/propidium iodide (PI) double staining assay. Annexin V/PI staining was done using apoptotic detection kit (BioVision, Mountain View, CA, USA) following the 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 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.

2.5 Immunoblot analysis

Whole-cell lysates were prepared as previously described [6]. For immunoblot analysis, equal amount of proteins (20–50 μg, depending on the proteins of interest) 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 Bioproducts, Ashland, MA, USA). Antibodies for hyperphospho-Rb (Ser780), CDK-2, CDK-4, cleaved caspase 3 (Asp175), caspase 8, poly ADP ribose polymerase (PARP), $p21^{Cip1/Waf1}$, and Mcl-1 were from Cell Signaling Technology (Beverly, MA, USA). Antibody for β-actin was from Sigma-Aldrich.

2.6 Statistical analyses

All data were presented as mean ± SD. Student's *t*-test was used to test the mean difference between two groups. Analyses of variance 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 5-hydroxyl PMFs were more effective than their permethoxylated counterparts in inhibiting colon cancer cell growth

The effects of six PMFs (Fig. 1) were studied on the growth of HCT-116 and HT-29 human colon cancer cells. Three 5-hydroxy PMFs were 5HPMF, 5HHMF, and HTMF, and their corresponding permethoxylated PMFs were nobiletin, HMF, and tangeretin, respectively. In both HCT116 and HT29 cells, all six PMFs showed dose-dependant inhibition on the cell growth (Fig. 2). Interestingly, 5-hydroxy PMFs showed much stronger growth inhibitory effects than their permethoxylated counterparts. For example, in HCT116 cells, 5-hydroxy PMFs, namely, 5HPMF, 5HHMF, and 5HTMF had IC_{50} values of 8.7, 3.5, and 1.6 μM, respectively, while permethoxylated PMFs, namely, nobiletin, HMF, and tangeretin had IC_{50} values of 37, 36, and 22 μM, respectively. Similar trend was also observed in HT29 cells. For example, IC_{50} values of

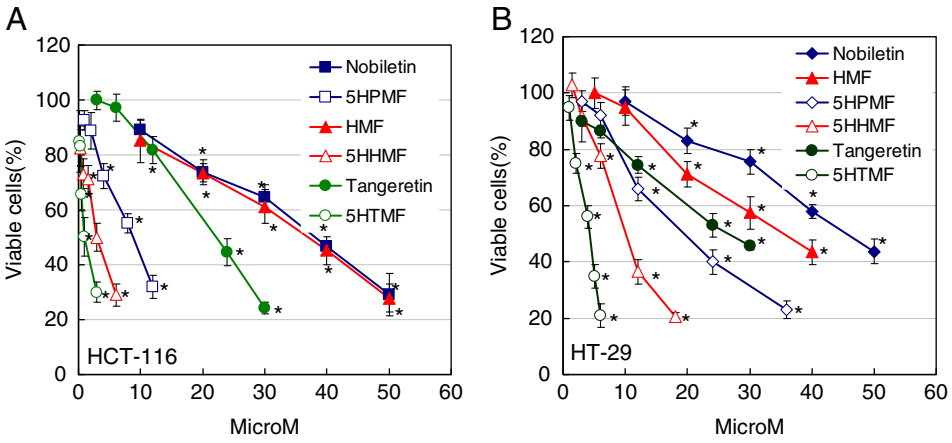

Figure 2. Growth inhibitory effect of 5-hydroxy PMFs and permethoxylated PMFs on HCT116 and HT29 human colon cancer cells. HCT116 (A) and HT29 (B) cells were seeded in 96-well plates, and after 24 h, cells were treated with serial concentrations of permethoxylated PMFS (*i.e.* nobiletin, HMF, and tangeretin) and 5-hydroxy PMFs (*i.e.* 5HPMF, 5HHMF, and 5HTMF). After 48 h of treatments, growth inhibition was measured by MTT assay as described in Section 2. Data represent mean ± SD ($n = 6$). * indicates the statistical significance in comparison with the control cells ($p < 0.01$).

5HPMF, 5HHMF, and 5HTMF were 22.0, 11.2, and 4.04 μM, respectively, and they were 2.1-, 3.1-, and 6.6-fold lower than the IC_{50} values of nobiletin, HMF, and tangeretin, respectively.

3.2 Effect of 5-hydroxy PMFs on cell cycle distribution of colon cancer cells

To further elucidate the mechanisms by which 5-hydroxy PMFs inhibit the growth of colon cancer cells, we studied their effects on cell cycle distribution of cancer cells. According to the relative potency of 5-hydroxy PMFs in growth inhibition of cancer cells (Fig. 2), we selected concentrations for each 5-hydroxy PMFs that produced similar degree of growth inhibition on colon cancer cells to test their effects on cell cycle. In HCT116 cells, treatment with 5HHMF for 24 h increased cell population in both G1/G0 and G2/M phases, and decreased cell population in S phase. In contrast, treatments with 5HPMF and 5HTMF only caused increase of cell population in G2/M phase (Fig. 3A). At the highest concentrations tested, 5HPMF (8 μM), 5HHMF (4 μM), and 5HTMF (3 μM) increased G2/M cell population to 3.7-, 3.4-, and 5.4-fold of that of the control cells, respectively. In HT29 cells, treatment with 5HPMF (36 μM) for 24 h modestly increased G2/M cell population, while much more increase in G2/M cell population was caused by 5HTMF at much lower concentration (6 μM) (Fig. 3B). Moreover, treatment with 5HHMF (18 μM) caused substantial cell accumulation in G0/G1 phase. It is noteworthy that treatments with 5HTMF led to significant increase in sub-G0/G1 cell population in both HCT116 and HT29 cells, which suggested DNA degradation potentially caused by cell death. These results showed that three 5-hydroxy PMFs showed different effects on the cell cycle distribution of two human colon cancer cells.

3.3 5-hydroxy PMFs induced apoptosis in HCT-116 and HT-29 cells

In order to determine the extent to which apoptosis contribute to the growth inhibition caused by 5-hydroxy PMFs, we studied the effects of three 5-hydroxy PMFs on cellular apoptosis in colon cancer cells after 48 h treatment. The apoptotic cells were quantified by Annexin-V/PI double staining assay. Viable cells (FITC-negative) and early apoptotic cells (FITC-positive) were PI-negative, whereas late apoptotic and necrotic cells were PI-positive and FITC-positive. In HCT116 cells, treatments with 5HPMF (8 μM) increased early apoptotic cell population to 2.2-fold of that of control cells (Fig. 4A), while 5HHMF showed similar effects at much lower concentration of 4 μM. Treatment with 5HTMF at even lower concentration of 3 μM increased early apoptotic cell population to 4.4-fold of that of the control cells. Similar effects were observed in late apoptosis/necrosis cell population in HCT116 cells after treatments with three 5-hydroxy PMFs. Similar to HCT116 cells, three 5-hydroxy PMFs induced apoptosis in HT29 cells with descending potency order of 5HTMF > 5HHMF > 5HPMF (Fig. 4B). In contrast, at higher concentrations, treatments with permethoxylated PMFs, namely, nobiletin, HMF, and tangeretin did not increase apoptotic cell population in either HCT116 or HT29 cells after 48 h treatment (Figs. 4A and B).

3.4 5-hydroxy PMFs profoundly modified cell cycle and apoptosis-related signaling proteins

In order to obtain further insights into the molecular mechanisms of 5-hydroxy PMFs, we examined their effects on key proteins related with cell cycle progression and

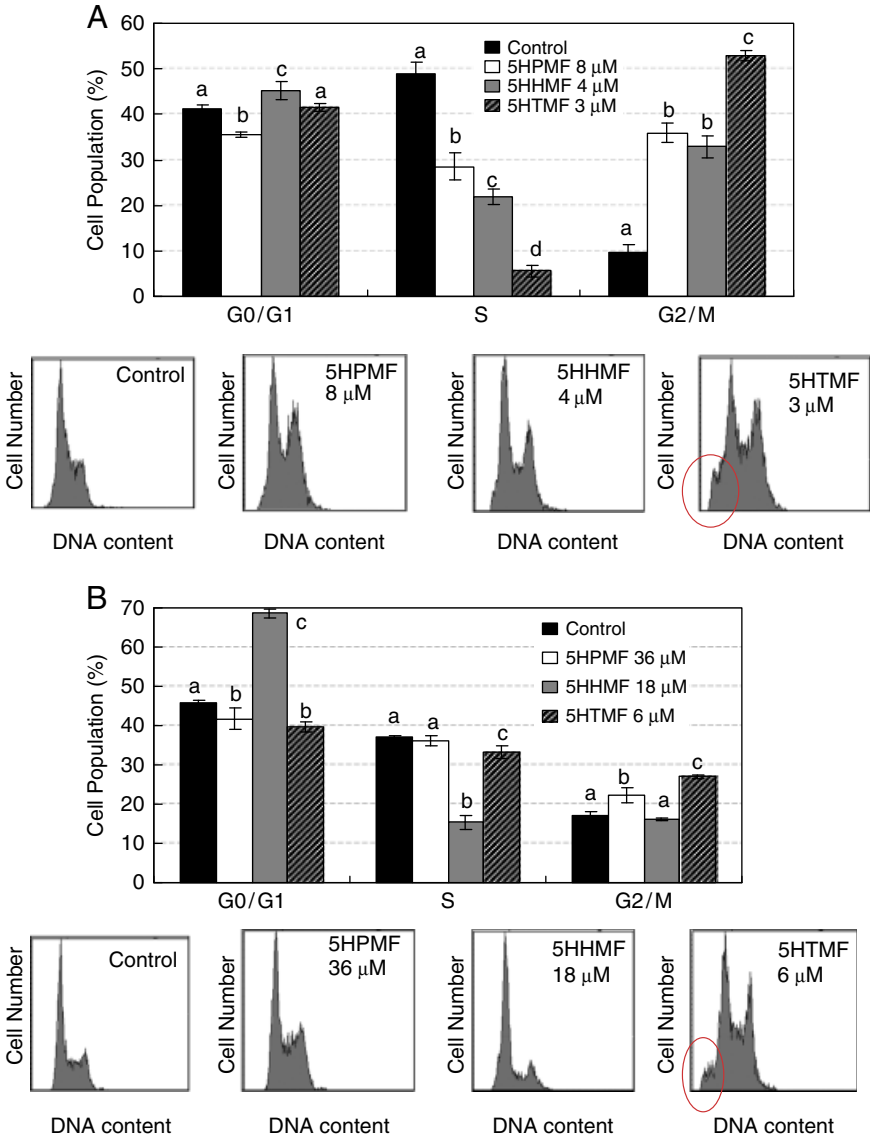

Figure 3. Cell cycle distribution of HCT116 (A) and HT29 (B) cells after treatments with 5-hydroxy PMFs. The HCT116 and HT29 cells were seeded in 6-well plates for 24 h, and then the cells were treated with 5HPMF, 5HHMF, or 5HTMF. After 24 or 48 h of treatments, cells were harvested and subject to cell cycle analyses as described in Section 2. According to the relative potency of 5-hydroxy PMFs in growth inhibition of cancer cells (Fig. 2), we selected concentrations for each 5-hydroxy PMFs that produced similar degree of growth inhibition on colon cancer cells to test their effects on cell cycle. All data represent mean ± SD. Statistical analysis 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 ($p < 0.01$, $n = 3$).

apoptosis in HCT116 cells. Western blotting was performed on the whole-cell lysates after the treatments with 5-hydroxy PMFs. Three permethoxylated PMFs at 16 μM were used as comparisons. The results showed that after 24 h treatment, 5-hydroxy PMFs dose-dependently increased the levels of $p21^{Cip1/Waf1}$ and decreased the levels of CDK-2 (Fig. 5A). Treatments with 5HPMF or 5HHMF did not change the levels of CDK-4, but treatment with 5HTMF (3 μM) significantly decreased CDK-4 levels. All three 5-hydroxy PMFs dose-dependently increased total Rb and decreased p-Rb, especially 5HHMF and 5HTMF. Three permethoxylated PMFs did not cause significant changes on $p21^{Cip1/Waf1}$ or CDK-4, but modestly decreased the levels of CDK-2 and Rb.

Activation of caspase cascade plays a crucial role in the process of apoptosis. First we examined the effects of 5-hydroxy PMFs on Mcl-1, a member of Bcl-2 anti-apoptotic family after 48 h treatments. The results showed that 5HPMF did not cause noticeable change on Mcl-1, while 5HHMF slightly decreased the Mcl-1 level. In contrast, 5HTMF caused dose-dependent decrease in the Mcl-1 levels (Fig. 5B). Caspase 8 was activated by the treatments with all three 5-hydroxy PMFs in a dose-dependent manner. The relative potency in the caspase 8-activation was in the order of 5HPMF < 5HHMF < 5HTMF. Consistent with these results, caspase 3, the downstream effector of caspase 8, was significantly activated by all three 5-hydroxy PMFs dose-dependently. Furthermore, the results confirmed that treatments with three 5-hydroxy PMFs increased the levels of PARP dose-dependently. In contrast to the profound pro-apoptotic effects of 5-hydroxy PMFs, the permethoxylated PMFs at the high concentration (16 μM) did not cause any noticeable change on Mcl-1, caspase 8, caspase 3, or PARP.

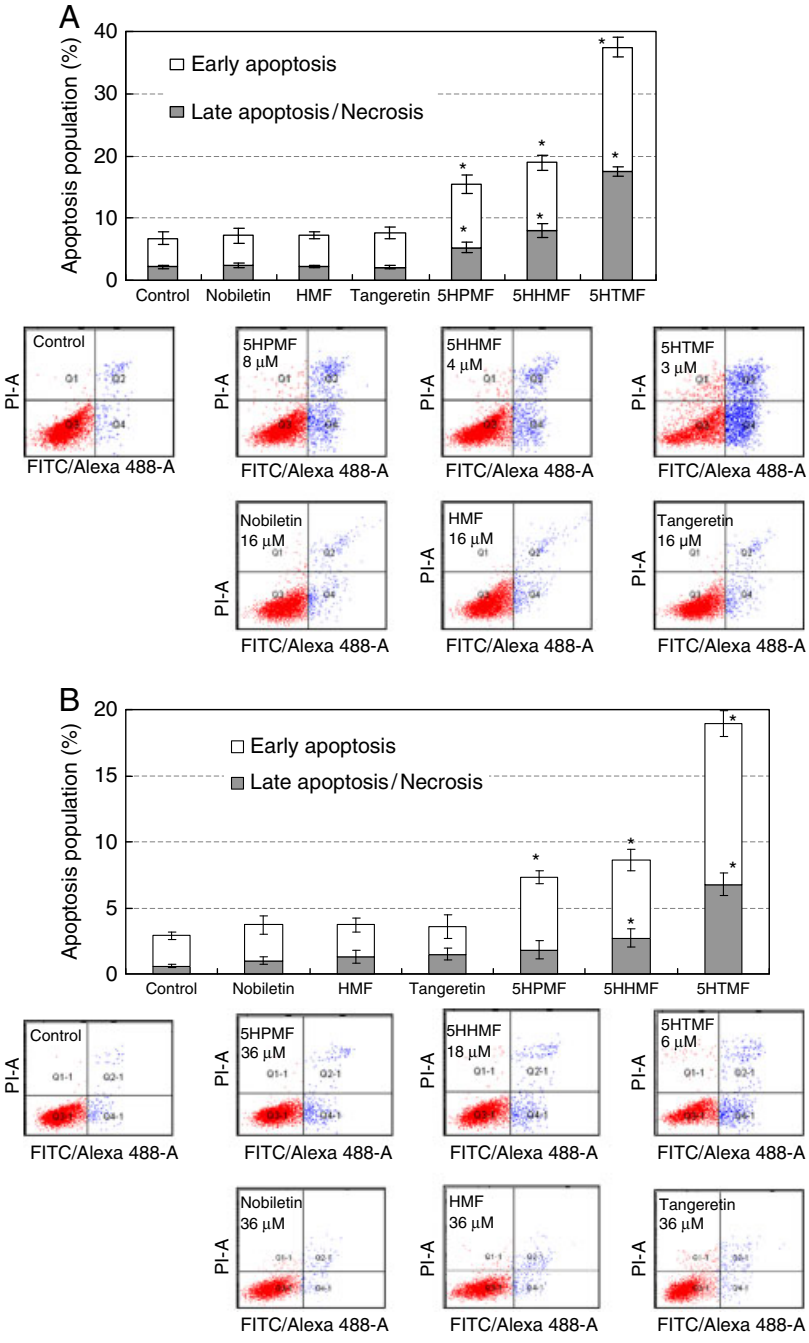

Figure 4. Effect of 5-hydroxy PMFs and permethoxylated PMFs on cell death of HCT-116 (A) and HT-29 (B) cells. The HCT116 and HT29 cells were seeded in 6-well plates for 24 h, and then the cells were treated with 5HPMF, 5HHMF, or 5HTMF. After 24 or 48 h of treatments, cells were harvested and subject to apoptosis analyses as described in Section 2. All data represent mean ± SD, and the asterisk in the bar charts indicate statistical significance in comparison with the control ($p < 0.01$, $n = 3$).

4 Discussion

In this study, we investigated the inhibitory effects of three 5-hydroxy PMFs and their permethoxylated counterparts on two human colon cancer cells. Our results demonstrated that 5-hydroxy PMFs (*i.e.* 5HPMF, 5HHMF, and 5HTMF) had much stronger inhibitory effects on the growth of the cancer cells in comparison with their permethoxylated counterparts (*i.e.* nobiletin, HMF, and tangeretin, respectively). This suggests that the substitution of the methoxy group with hydroxyl group at 5-position of PMFs plays a pivotal role in enhancing inhibitory activity of the PMFs against the growth of colon cancer cells. Previously, we have reported that 5HPMF and 5HHMF were more potent in inhibiting the growth of human lung cancer cells than nobiletin and HMF [6]. Others also demonstrated that 5-hydroxy PMFs were potent agents in inhibiting leukemia and breast cancer cell growth [4, 7, 8, 13]. Moreover,

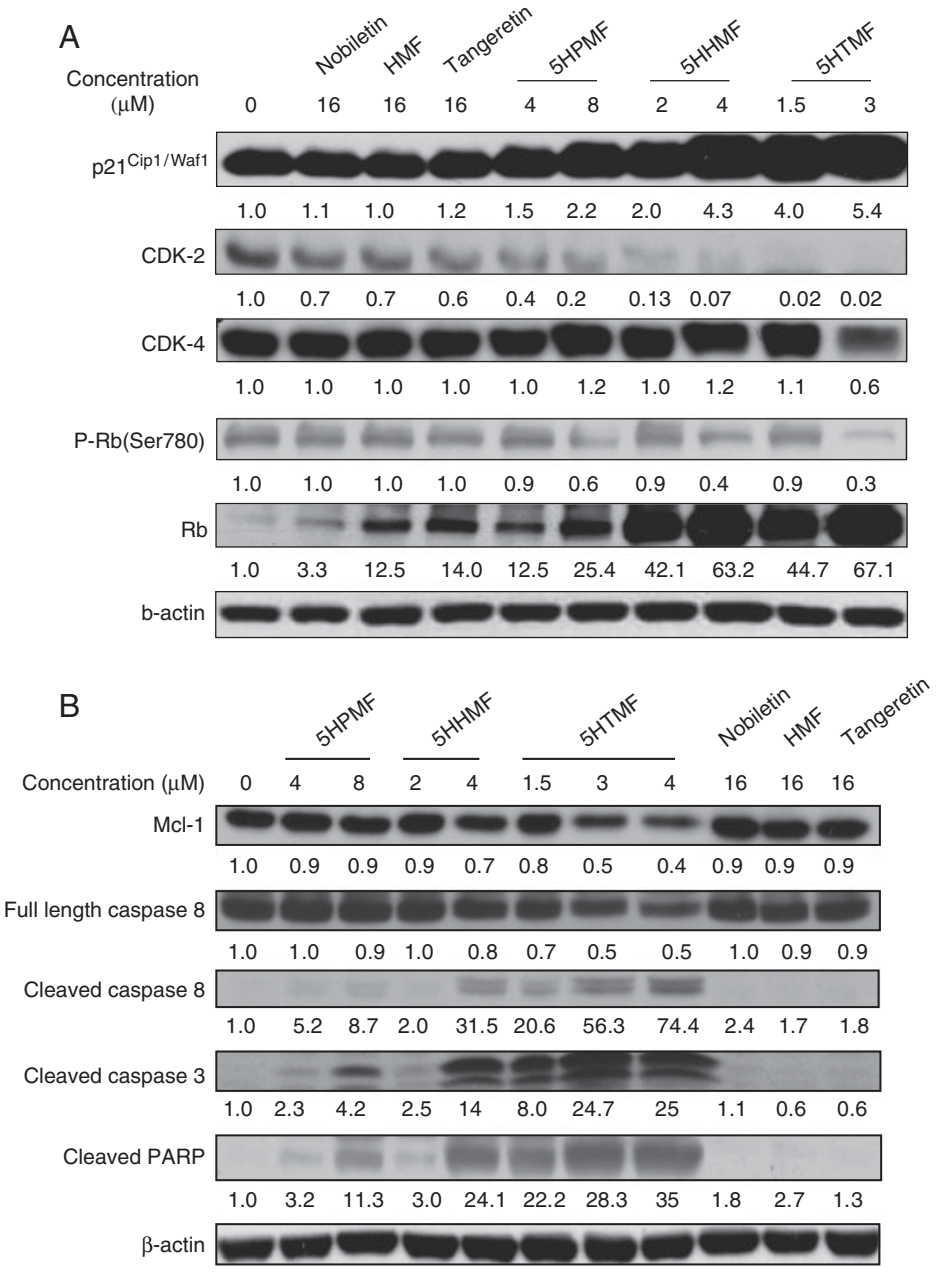

Figure 5. Effects of 5-hydroxy PMFs and permethoxylated PMFs on cell cycle and apoptosis related proteins in HCT116 human colon cancer cells. HCT-116 cells were seeded in 10-cm dishes for 24 h, and then cells were treated with serial concentrations of 5HPMF, 5HHMF 5HTMF, nobiletin, HMF, and tangeretin. After 48 h of incubation, cells were harvested for Western immunoblotting as described in Section 2. The numbers underneath of 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 was served as an equal loading control. The experiments were repeated three times.

hydroxylation of other methoxy groups in the PMFs can also enhance their bioactivities, *e.g.* hydroxylated urinary metabolites of nobiletin were shown to exert much stronger inhibitory effects on LPS-induced inflammation response in murine macrophage cells [14]. It is noteworthy that our results for the first time demonstrated potent inhibitory effects of 5HTMF against colon cancer cells.

Our results demonstrated that three 5-hydroxy PMFs had different potency in inhibiting colon cancer cell growth with relative potency of 5HPMF < 5HHMF < 5HTMF. This may be associated with relative lipophilicity of the 5-hydroxy PMFs. Based on our experience in analyzing and quantifying these PMFs by reverse phase HPLC, the difference on the position and number of methoxy group can change lipophilicity of the PMFs significantly [15]. Among the three 5-hydroxy PMFs, 5HTMF showed strongest lipophilicity, while 5HPMF had weakest lipophilicity. Increased lipophilicity may promote binding of the compound to the plasma membrane, which in turn may increase the uptake of the compound into the cytosol of the cell. Thus increased lipophilicity may enhance bioavailability, and this may subsequently increase the bioactivities of the PMFs. It is also possible that colon cancer cells have different preference in uptaking 5-hydroxy PMFs. The exact mechanism by which

the methoxylation may modulate the bioavailability of PMFs is an attractive topic for future investigation.

Our results demonstrated that the three 5-hydroxy PMFs produced different effects on the cell cycle of colon cancer cells. At the concentrations causing similar degree of growth inhibition, 5HHMF treatment resulted in G0/G1 arrest in HT29 cells, while 5HPMF and 5HTMF led to G2/M arrest. In addition, 5HTMF treatment significantly increased sub-G0/G1 cell population of HT29 cells, suggesting DNA degradation caused by cell death. Similar results were also observed in HCT116 cells. These results indicated that three 5-hydroxy PMFs inhibited colon cancer cell growth by different mechanisms. The cell cycle distribution and cell death are downstream manifestation of upstream molecular events, such as binding of small molecules (*e.g.* 5-hydroxy PMFs) with signaling proteins on cell surface and/or in cytosol. These upstream events can trigger a cascade of signaling events that eventually modulate cell cycle progression and/or cell death. Due to the structural difference, three 5-hydroxy PMFs may bind to different proteins and/or bind to same proteins with different affinity. The different interactions between key signaling proteins and individual 5-hydroxy PMFs accumulatively lead to the different effects on cell cycle progression and cell death in cancer cells treated with different 5-hydroxy PMFs.

Deregulation of cell cycle progression is a distinct feature of cancer cells. Our results showed that three 5-hydroxy PMFs dose-dependently increased the levels of $p21^{Cip1/Waf1}$. As a CDK inhibitor, $p21^{Cip1/Waf1}$ is a negative regulator of cell cycle progression. It inhibits CDK activities, which causes hypo-phosphorylation of Rb, and this in turn leads to cell cycle arrest and decreased cell proliferation [16]. Indeed, the levels of phosphorylation of Rb protein were decreased in colon cancer cells after treatments with 5-hydroxy PMFs. Moreover, treatments with 5-hydroxy PMFs significantly increased total Rb levels, which suggested increased levels of hypo-phosphorylated Rb that can lead to cell cycle arrest. Our results demonstrated that all three 5-hydroxy PMFs dose-dependently decreased the levels of CDK-2, which can form complex with cyclin A or E to contribute to the cell cycle progression [17]. Especially, 5HTMF treatment decreased CDK-2 to a non-detectable level. Besides CDK-2/cyclin complexes, CDK-4/Cyclin D complex also plays important roles in both G1 to S and G2 to M phase transition [16, 18]. Although treatments with 5HPMF and 5HHMF did not decrease the levels of CDK-4, the treatment with 5HTMF at 3 μM significantly decreased the level of CDK-4. Overall, our results have demonstrated profound changes caused by the treatments with different 5-hydroxy PMFs, and the cumulative consequences of these changes were manifested as cell cycle arrest at different phases in colon cancer cells.

The evasion of apoptosis is one of the essential abnormalities in cell physiology that lead to malignant growth in most types of cancer [19]. The results from annexinV/PI co-staining assay clearly demonstrated that all three 5-hydroxy PMFs induced apoptosis in colon cancer cells. However, the magnitude of apoptosis caused by different 5-hydroxy PMFs was much different. At the concentrations causing similar degree of growth inhibition, treatments with 5HPMF and 5HHMF induced moderate apoptosis, while 5HTMF induced much stronger apoptosis. These results indicated that apoptosis contributed much more to the growth inhibition caused by 5HTMF than that caused by 5HPMF or 5HTMF. The pro-apoptotic effects of three 5-hydroxy PMFs were confirmed by Western blotting of key proteins related with apoptosis. The Bcl-2 family proteins are critical regulators of mitochondria-mediated apoptosis by functioning as either promoters or inhibitors of apoptosis [20]. Mcl-1 is an anti-apoptotic member of the Bcl-2 family, and it antagonizes pro-apoptotic Bcl-2 family members [21]. Treatment with 5HPMF and 5HHMF did not change Mcl-1 levels, while treatment with 5HTMF significantly decreased Mcl-1 level. This suggested 5HTMF at least partially induced mitochondria-mediated apoptosis. Caspases play crucial roles in the initiation and execution of apoptosis mediated by both death receptor and mitochondria [22, 23]. Activated caspase 8 cleaves and activates downstream effector caspases such as caspases 1, 3, 6, and 7. Caspase 3 is a central player of apoptosis, as it is responsible for the proteolytic cleavage of many key proteins such as PARP [24]. The cleavage of PARP facilitates cellular disassembly and ultimately apoptosis [25]. Our results showed that caspases 3 and 8 were activated by the treatment with 5HPMF, 5HHMF, and 5HTMF. Consequently, the levels of cleaved PARP were significantly elevated by all three 5-hydroxy PMFs.

Taken together, we demonstrated that 5-hydroxy PMFs, namely, 5HPMF, 5HHMF, and 5HTMF have much stronger inhibitory effect on the growth of human colon cancer cells than their permethoxylated counterparts, namely, nobiletin, HMF, and tangeretin, respectively. The enhanced bioactivity was associated with hydroxylation at 5-position on the A ring of PMFs. Further more, the difference on the position and the number of methoxy groups also modifies the bioactivities of different 5-hydroxy PMFs. These structural differences may cause different 5-hydroxy PMFs to bind different signaling proteins and/or to bind same proteins with different affinity. In addition, the structural difference also changes the lipophilicity of the compounds, which may contribute to the difference in bioavailability. In comparison with permethoxylated PMFs, the enhanced inhibitory effects of 5-hydroxy PMFs against cancer cells make them promising cancer-preventive agents that can be used as health-promoting ingredients in functional foods and dietary supplements.

This work was supported in part by 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.

The authors have declared no conflict of interest.

5 References

[1] Li, S., Lo, C. Y., Ho, C. T., Hydroxylated polymethoxyflavones and methylated flavonoids in sweet orange (*Citrus sinensis*) peel. *J. Agric. Food Chem.* 2006, *54*, 4176–4185.

[2] Middleton Jr E., Kandaswami, C., Theoharides, T. C., The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* 2000, *52*, 673–751.

[3] Manthey, J. A., Grohmann, K., Guthrie, N., Biological properties of citrus flavonoids pertaining to cancer and inflammation. *Curr. Med. Chem.* 2001, *8*, 135–153.

[4] Li, S., Pan, M. H., Lai, C. S., Lo, C. Y. *et al.*, Isolation and syntheses of polymethoxyflavones and hydroxylated polymethoxyflavones as inhibitors of HL-60 cell lines. *Bioorg. Med. Chem.* 2007, *15*, 3381–3389.

[5] Li, S., Lambros, T., Wang, Z., Goodnow, R., Ho, C. T., Efficient and scalable method in isolation of polymethoxyflavones from orange peel extract by supercritical fluid chromatography. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2007, *846*, 291–297.

[6] Xiao, H., Yang, C. S., Li, S., Jin, H. *et al.*, Monodemethylated polymethoxyflavones from sweet orange (*Citrus sinensis*) peel inhibit growth of human lung cancer cells by apoptosis. *Mol. Nutr. Food Res.* 2009, *53*, 398–406.

[7] Pan, M. H., Lai, Y. S., Lai, C. S., Wang, Y. J. *et al.*, 5-Hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone induces apoptosis through reactive oxygen species production, growth arrest and DNA damage-inducible gene 153 expression, and caspase activation in human leukemia cells. *J. Agric. Food Chem.* 2007, *55*, 5081–5091.

[8] Sergeev, I. N., Li, S., Colby, J., Ho, C. T., Dushenkov, S., Polymethoxylated flavones induce Ca(2+)-mediated apoptosis in breast cancer cells. *Life Sci.* 2006, *80*, 245–253.

[9] Jemal, A., Siegel, R., Ward, E., Hao, Y. *et al.*, Cancer statistics, 2009. *CA Cancer J. Clin.* 2009, *59*, 225–249.

[10] Ji, B. T., Devesa, S. S., Chow, W. H., Jin, F., Gao, Y. T., Colorectal cancer incidence trends by subsite in urban Shanghai, 1972–1994. *Cancer Epidemiol. Biomarkers Prev.* 1998, *7*, 661–666.

[11] Yiu, H. Y., Whittemore, A. S., Shibata, A., Increasing colorectal cancer incidence rates in Japan. *Int. J. Cancer* 2004, *109*, 777–781.

[12] Wang, Z., Li, S., Jonca, M., Lambros, T. *et al.*, Comparison of supercritical fluid chromatography and liquid chromatography for the separation of urinary metabolites of nobiletin with chiral and non-chiral stationary phases. *Biomed. Chromatogr.* 2006, *20*, 1206–1215.

[13] Sergeev, I. N., Ho, C. T., Li, S., Colby, J., Dushenkov, S., Apoptosis-inducing activity of hydroxylated polymethoxyflavones and polymethoxyflavones from orange peel in human breast cancer cells. *Mol. Nutr. Food Res.* 2007, *51*, 1478–1484.

[14] Li, S., Sang, S., Pan, M. H., Lai, C. S. *et al.*, Anti-inflammatory property of the urinary metabolites of nobiletin in mouse. *Bioorg. Med. Chem. Lett.* 2007, *17*, 5177–5181.

[15] Dong, P., Qiu, P., Zhu, Y., Li, S. *et al.*, Simultaneous determination of Four 5-Hydroxy polymethoxyflavones by reversed-phase high performance liquid chromatograph with electrochemical detection. *J. Chromatogr. A* 2009, *1217*, 642–647.

[16] Sherr, C. J., Cancer cell cycles. *Science* 1996, *274*, 1672–1677.

[17] Morgan, D. O., Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annu. Rev. Cell Dev. Biol.* 1997, *13*, 261–291.

[18] Gabrielli, B. G., Sarcevic, B., Sinnamon, J., Walker, G. *et al.*, A cyclin D-Cdk4 activity required for G2 phase cell cycle progression is inhibited in ultraviolet radiation-induced G2 phase delay. *J. Biol. Chem.* 1999, *274*, 13961–13969.

[19] Fesik, S. W., Promoting apoptosis as a strategy for cancer drug discovery. *Nat. Rev. Cancer* 2005, *5*, 876–885.

[20] Reed, J. C., Bcl-2 family proteins: regulators of apoptosis and chemoresistance in hematologic malignancies. *Semin. Hematol.* 1997, *34*, 9–19.

[21] Sato, T., Hanada, M., Bodrug, S., Irie, S. *et al.*, Interactions among members of the Bcl-2 protein family analyzed with a yeast two-hybrid system. *Proc. Natl. Acad. Sci. USA* 1994, *91*, 9238–9242.

[22] Kaufmann, S. H., Earnshaw, W. C., Induction of apoptosis by cancer chemotherapy. *Exp. Cell Res.* 2000, *256*, 42–49.

[23] Nunez, G., Benedict, M. A., Hu, Y., Inohara, N., Caspases: the proteases of the apoptotic pathway. *Oncogene* 1998, *17*, 3237–3245.

[24] Fernandes-Alnemri, T., Litwack, G., Alnemri, E. S., CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. *J. Biol. Chem.* 1994, *269*, 30761–30764.

[25] Oliver, F. J., de la Rubia, G., Rolli, V., Ruiz-Ruiz, M. C. *et al.*, Importance of poly(ADP-ribose) polymerase and its cleavage in apoptosis. Lesson from an uncleavable mutant. *J. Biol. Chem.* 1998, *273*, 33533–33539.

