DOI 10.1002/mnfr.200800057 Mol. Nutr. Food Res. 2009, *53*, 398–406

Research Article

Monodemethylated polymethoxyflavones from sweet orange (*Citrus sinensis*) peel inhibit growth of human lung cancer cells by apoptosis

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Polymethoxyflavones (PMFs) are almost exclusively found in the Citrus genus, particularly in the peels of sweet orange (*Citrus sinensis* L. Osbeck) and mandarin (*C. reticulate* Blanco). We studied the effects of two major PMFs, namely, nobiletin and 3,5,6,7,8,3′,4′-heptamethoxyflavone (HMF), and two major monodemethylated PMFs, namely 5-hydroxy-3,7,8,3′,4′-pentamethoxyflavone (5HPMF), and 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5HHMF), on the growth of human lung cancer H1299, H441, and H460 cells. Monodemethylated PMFs were much more potent in growth inhibition of lung cancer cells than their permethoxylated counterpart PMFs. In H1299 cells, cell cycle analyses further revealed that monodemethylated PMFs caused significant increase in sub-G0/G1 phase, suggesting possible role of apoptosis in the growth inhibition observed, whereas the permethoxylated counterpart PMFs did not affect cell cycle distribution at same concentrations tested. These results strongly suggested that the phenolic group is essential for the growth inhibitory activity of monodemethylated PMFs. Further studies in H1299 cells demonstrated that monodemethylated PMFs downregulated oncogenic proteins, such as iNOS, COX-2, Mcl-1, and K-ras, as well as induced apoptosis evidenced by activation of caspase-3 and cleavage of PARP. Our results provide rationale to develop orange peel extract enriched with monodemethylated PMFs into value-added nutraceutical products for cancer prevention.

Keywords: Apoptosis / Lung cancer / Monodemethylated polymethoxyflavone

Received: February 8, 2008; revised: April 20, 2008; accepted: June 21, 2008

1 Introduction

Cancer is a major cause of human death in the United States and most other countries. Epidemiological evidence has consistently indicated that diet abundant in fruits and vegetables may reduce the risk of cancer, and this effect has been attributed to bioactive components present in these foods. Flavonoids are universally found in food plants, and an impressive body of evidence has demonstrated their cancer preventive effects [1]. Polymethoxyflavones (PMFs) are a unique class of flavonoids, and almost exclusively exist in the citrus genus, particularly in the peels of sweet oranges (*C. senensis*) and mandarin oranges (*C. reticulata*) [2]. PMFs have shown a wide range of biological activity, including anti-inflammatory, anticarcinogenic, antiviral, antioxidant, antithrombogenic, and anti-artherogenic properties [2–4]. Citrus production worldwide in selected major producing countries between 2003 and 2004 was 73.1 million metric tons while that of the United States was 14.85 million metric tons. Orange juice industry yields about 1.8–2.3 million metric tons of orange peel as byproducts in the United States [2].

Previously, nobiletin and 3,5,6,7,8,3′,4′-heptamethoxyflavone (HMF) were found as major PMFs in an aged

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Abbreviations: COX, cyclooxygenase; **5HHMF,** 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone; **HMF,** 3,5,6,7,8,3′,4′-heptamethoxyflavone; **5HPMF,** 5-hydroxy-3,7,8,3′,4′-pentamethoxyflavone; **iNOS,** inducible nitric oxide; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NSCLC,** nonsmall-cell lung cancer; **PMF,** polymethoxyflavone

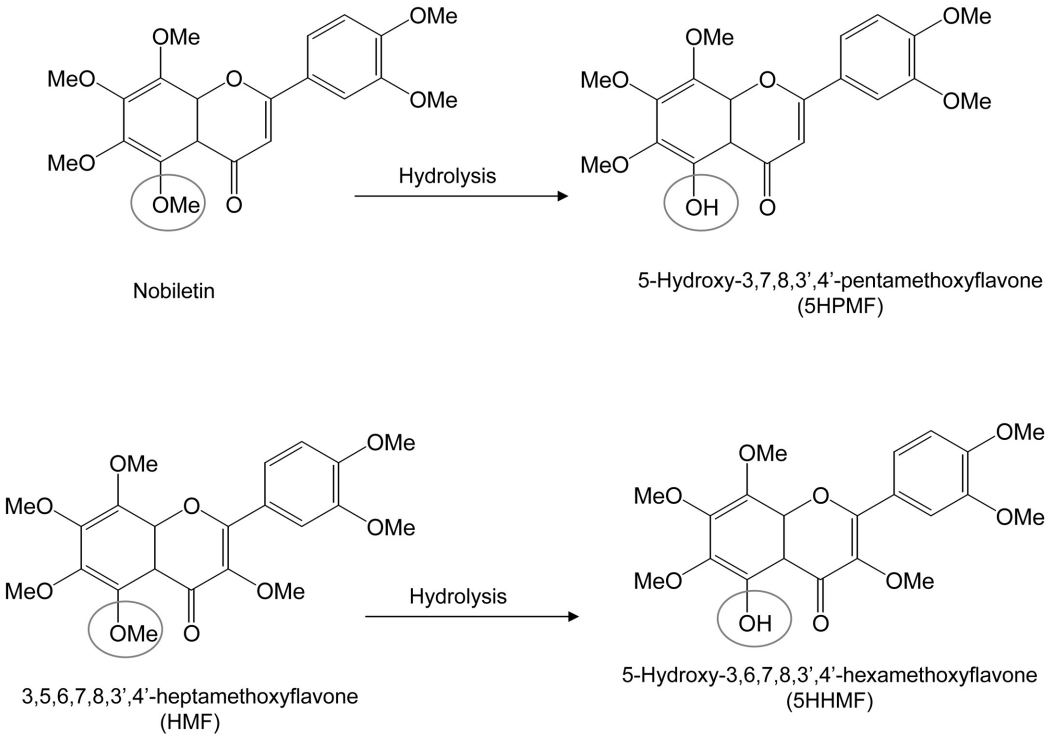

Figure 1. Chemical structures of PMFs.

orange peel extract from cold-pressed sweet orange peel oil [2]. In the same study, besides the permethoxylated PMFs, *i.e.*, nobiletin and HMF, a class of monodemethylated PMFs have also been isolated. Among these monodemethylated PMFs, 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (5HPMF), and 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5HHMF) were two most abundant ones. It was believed that the monodemethylated PMFs could be formed in the orange extract by auto-hydrolysis of their permethoxylated counterparts during long-term storage (Fig. 1). Recently, efforts have been made to compare the biological activities of monodemethylated PMFs and their permethoxylated PMF counterparts in different biological systems. It was found that 4′-monodemethylated nobiletin had much stronger anti-inflammatory activities than nobiletin in LPS-treated RAW264.7 macrophages, *i.e.*, 4′-monodemethylated nobiletin inhibited nitrite production, and decreased the RNA and protein levels of both inducible nitric oxide (iNOS) and cyclooxygenase (COX)-2, while nobiletin did not [5]. In cell culture, 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 [6, 7]. It is intriguing that monodemethylation renders such difference in biological activities of PMFs.

Lung cancer is a leading cause of cancer death in the United States and one of the most common cancer worldwide [8]. Cancer chemoprevention by dietary components may be a practical, economical, and effective approach to reduce the risk of this disease [9]. Herein, we selected the two most abundant monodemethylated PMFs from sweet orange peel extract, *i.e.*, 5HPMF and 5HHMF (Fig. 1), and their permethoxylated counterparts, to investigate their anticancer effects in human lung carcinoma H1299 cells. Our effort was focused on the effects of these PMFs on the cell growth, cell cycle, and apoptosis.

2 Materials and methods

2.1 Isolation and identification of PMFs

PMFs were isolated as previously described [2, 10]. In brief, the sweet orange peel extract from Florida Flavors Company (Lakeland, FL, USA) (10 g) was dissolved in a mixture of methylene chloride (2 mL) and hexanes (2 mL) 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 went to 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 (total run of 50 min). The fractions that had 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 the residue was

dissolved in ACN and water. The dissolved solution was loaded onto a C_{18} RP 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 mixtures were collected. To afford pure compounds, the mixture fractions were subject 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 compound 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) on pure nobiletin, 5HPMF, HMF, and 5HHMF have been reported previously [2].

2.2 Cell culture and treatment

Human lung cancer cell lines H1299, H441, and H460 were obtained from American Type Cell Collection (ATCC, Manassas, VA, USA), and were maintained in RPMI 1640 media (Mediatech, Herndon, VA, USA) supplemented with 10% heat inactivated FBS (Mediatech), 100 U/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 20 passages. DMSO was used as the vehicle to deliver atorvastatin and PPE, and the final concentration of DMSO in all experiments was 0.1%.

2.3 Cell viability assay

H1299 (1500 cells/well), H441 (2000 cells/well), or H460 (2000 cells/well) cells were seeded in 96-well plates. After 24 h, cells were treated with serial concentrations of different PMFs in 200 μL of serum complete media. After suitable treatment periods, 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 2 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 550 nm using a plate reader (TECAN, Phenix Research Products, Candler, NC, USA).

2.4 Cell cycle analyses

H1299 cells (5×10^4 cells/well) were seeded in 6-well plates. After 24 h incubation for attachment, cells were treated with different concentrations of PMFs in 3 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 re-suspended in 0.5 mL of 70% ethanol overnight. After centrifugation ($1500 \times g$, 3 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 Beckman Coulter flow cytometer (FC500), and data were processed using AXP acquisition and analysis software.

2.5 Immunoblotting

H1299 cells were seeded in 10-cm petri dishes. After 24 h, cells were treated with serial concentrations of 5HPMF or 5HHMF. After another 24 h of incubation, cells were washed with ice-cold PBS, collected with cell-scrapers. The cells were combined with floating cells, if any, and incubated on ice for 10 min in lysis buffer (cell signaling, Beverly, MA, USA) supplemented with cocktails of protease inhibitor (1:100), phosphotase inhibitor 1 (1:100), and phosphotase inhibitor 2 (1:100) (Sigma – Aldrich). Cell suspensions were then subject to sonication (5 s, three times). After further incubation of 20 min on ice, followed by centrifugation at $10\,000 \times g$ for 10 min at 4°C, supernatants were collected. Proteins were quantified by BCA™ protein assay kit (Pierce Biotechnology,Rockford, IL, USA), and 20 – 50 μg of proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane. After blocking, proteins of interest were probed using different antibodies at manufacturer's recommended concentrations, and then visualized using Odyssey system (LI-COR, NE, USA) after incubation with suitable IR-antibodies. Antibodies for hyperphospho-Rb, full length PARP, cleaved PARP, and cleaved caspase-3 (Asp175) were from Cell Signaling (Beverly). Antibodies for K-ras (F234), COX-2, and iNOS were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for $p21^{Cip1/Waf1}$ and β-actin were from Upstate (Lake Placid, NY, USA) and Sigma – Aldrich, respectively.

2.6 Statistical analysis

All data were presented as mean ± SD Student's *t*-test was used to test the mean difference between two groups. Analyses 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 Monodemethylated PMFs are more effective than their permethoxylated counterparts in inhibiting the growth of H1299 cells

We studied effect of four pure PMFs isolated from sweet orange peel on growth of human lung cancer cells including

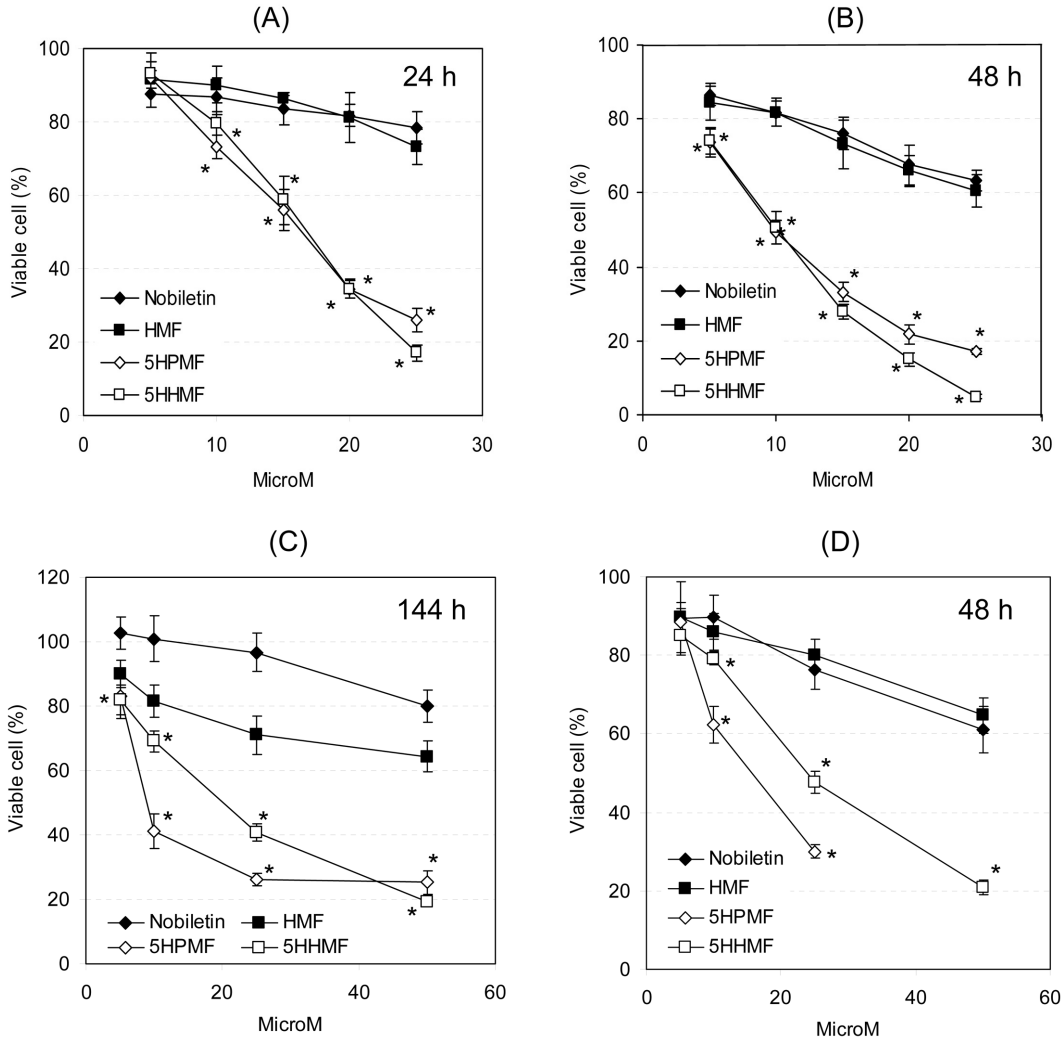

Figure 2. Growth inhibitory effect of PMFs on H1299, H441, and H460 human lung cancer cells. Lung cancer cells H1299 (A and B), H441 (C), and H460 (D) were seeded in 96-well plates, and after 24 h, cells were treated with serial concentrations of nobiletin, HMF, 5HPMF, and 5HHMF. After 24, 48, or 144 h of treatments, growth inhibition was measured by MTT assay as described in Section 2. Data represent mean ± SD, and the asterisks indicate the statistical significance between monodemethylated PMFs and corresponding PMFs at the same concentration according to Student's *t*-test ($p<0.01$, $n = 6$).

H1299, H441, and H460. The chemical structures of the PMFs are shown in Fig. 1. In H1299 cells, after a treatment for 24 h, nobiletin and HMF, up to 25 μM, slightly inhibited cell growth (Figs. 2A and B). However, the monodemethylated compounds, 5HPMF and 5HHMF, showed much stronger inhibitory effect as compared to nobiletin and HMF. Both 5HPMF and 5HHMF showed IC_{50} values of 16.5 μM at 24 h, while nobiletin and HMF had IC_{50} greater than 50 μM in H1299 cells. At 48 h, all four PMFs showed stronger inhibitory effect than that observed at 24 h, and the monodemethylated 5HPMF and 5HHMF showed more extensive inhibition than nobiletin and HMF in H1299 cells. In comparison to H1299 cells, H441, and H460 cells responded to the four test compounds in a similar pattern, which was indicative of that monodemethylated PMFs had much stronger inhibitory effects on cancer cell growth than their permethoxylated counterparts (Figs. 2C and D). To further demonstrate the difference between the effects of monodemethylated and permethoxylated PMFs on the growth of lung cancer cells, we treated H1299 and H460 cells with the four PMFs at 10 and 25 μM, respectively. After 48 h, photos of cells were taken (Fig. 3). Treatments with 5HPMF and 5HHMF clearly caused much stronger growth inhibitory effects on both H1299 and H460 cells than with nobiletin and HMF.

3.2 Monodemethylated PMFs cause sub G0/G1 accumulation of H1299 cells

To further characterize the growth inhibitory effects observed above, cell cycle analyses were performed on H1299 cells after treatment with 10 μM of nobiletin, HMF,

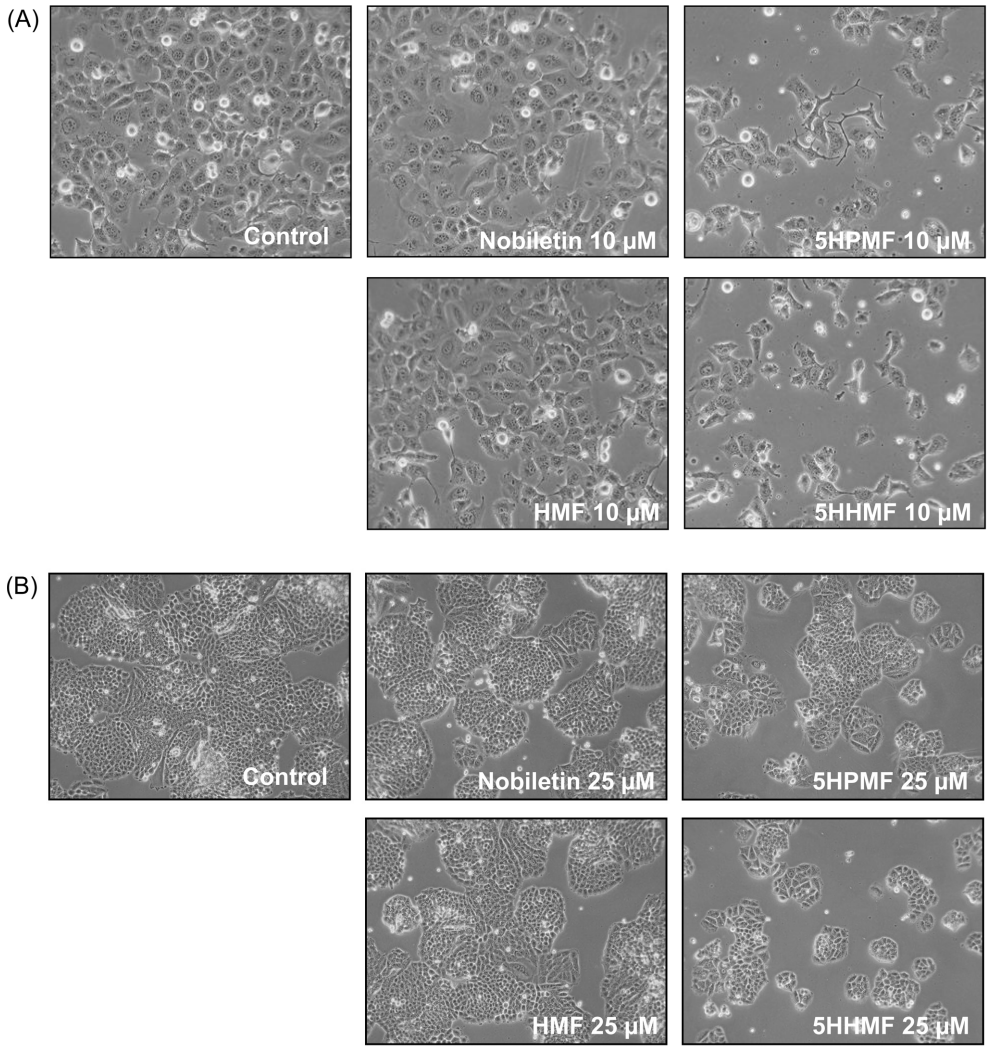

Figure 3. Photos of H1299 and H460 human lung cancer cells after treatments with nobiletin, HMF, 5HPMF, or 5HHMF. H1299 and H460 cells were seeded in 10-cm petri dishes, and after 24 h, cells were treated with 10 and 25 μM, respectively, of nobiletin, HMF, 5HPMF, or 5HHMF. After 48 h of treatments, photos of cells were taken under phase contrast invert microscope. Representative photos are shown ($n = 3$).

5HPMF, or 5HHMF. As shown in Fig. 4A, at 24 h, nobiletin or HMF did not cause any changes in cell cycle distribution compared to the control. However, the monodemethylated PMFs, 5HPMF, and 5HHMF resulted in significant accumulation of sub G0/G1 phase cell population. Sub G0/G1 cell population was increased by 4.1- and 3.8-fold by 5HPMF and 5HHMF, respectively. Similarly, at 48 h, 5HPMF and 5HHMF significantly increased sub-G0/G1 cell population by 5.1- and 4.7-fold, respectively, while nobiletin or HMF did not (Fig. 4B).

3.3 Monodemethylated PMFs cause changes in cell proliferation, apoptosis, and inflammation related proteins

In order to elucidate molecular mechanisms by which monodemethylated PMFs inhibit cancer cell growth, we studied the effects of 5HPMF and 5HHMF on key proteins related with cell proliferation, apoptosis, and inflammation in H1299 human lung carcinoma cells. Figure 5 shows the Western immunoblotting of these proteins after treatments with 5HPMF and 5HHMF at different concentrations for 24 h. Our results demonstrated that 5HPMF and 5HHMF decreased K-ras levels in a dose-dependent manner. Treatments with 5HPMF and 5HHMF also dose-dependently increased levels of $p21^{Cip1/Waf1}$, and decreased levels of hyper-phospho-Rb. As described above, both 5HPMF and 5HHMF caused significant accumulation of sub G0/G1 cell population in H1299 cells, which suggests possible DNA degradation caused by apoptosis. Herein, we tested effects of hydroxylated PMFs on apoptosis-related proteins. Expression levels of Mcl-1, an anti-apoptotic protein overexpressed in H1299 cells, were decreased by both 5HPMF and 5HHMF after 24 h treatment. Cleavage of caspase-3, a hallmark of apoptosis, was observed in the cells treated with both 5HPMF and 5HHMF, and the effects were more appreciable as concentrations of 5HPMF and 5HHMF increased. Moreover, activation of caspase-3 was companied by decrease in full-length PARP protein levels and increase in cleaved PARP protein levels caused by treat-

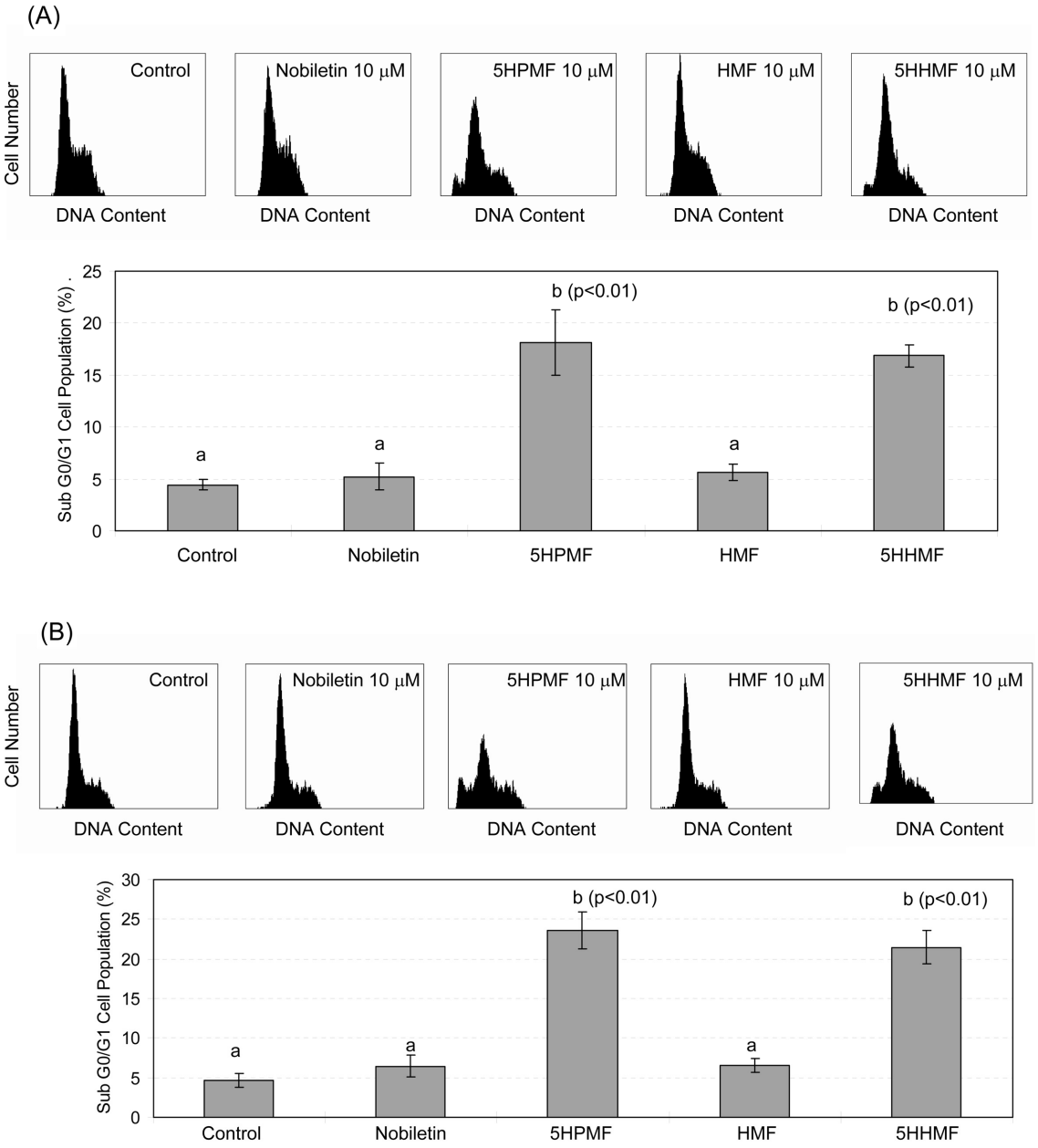

Figure 4. Cell cycle distribution of H1299 cells after treatments with PMFs. H1299 cells were seeded in 6-well plates, and after 24 h, cells were treated with 10 μM of nobiletin, HMF, 5HPMF, or 5HHMF. After 24 h (A) or 48 h (B) of treatments, cells were harvested and subject to cell cycle analyses as described in Section 2. All data represent mean ± SD, and different notations in the bar charts indicate statistical significance ($p < 0.01$, $n = 3$).

ments with 5HPMF and 5HHMF. We also investigated the treatment effect of 5HPMF and 5HHMF on iNOS and COX-2, two well-established inflammation markers associated with carcinogenesis. It was found that 5HPMF and 5HHMF significantly decreased iNOS levels in a dose-dependent manner, while only moderate decrease was observed in COX-2 levels after treatments with 5HPMF and 5HHMF (Fig. 5).

4 Discussion

Nobiletin and HMF are two abundant PMFs found in sweet orange peel, and their monodemethylated compounds are 5HPMF and 5HHMF, respectively. In this study, we investigated effects of these four PMFs on H1299 human lung carcinoma cells. Our results demonstrated that hydroxylated PMFs, *i. e.*, 5HPMF and 5HHMF, have much stronger inhib-

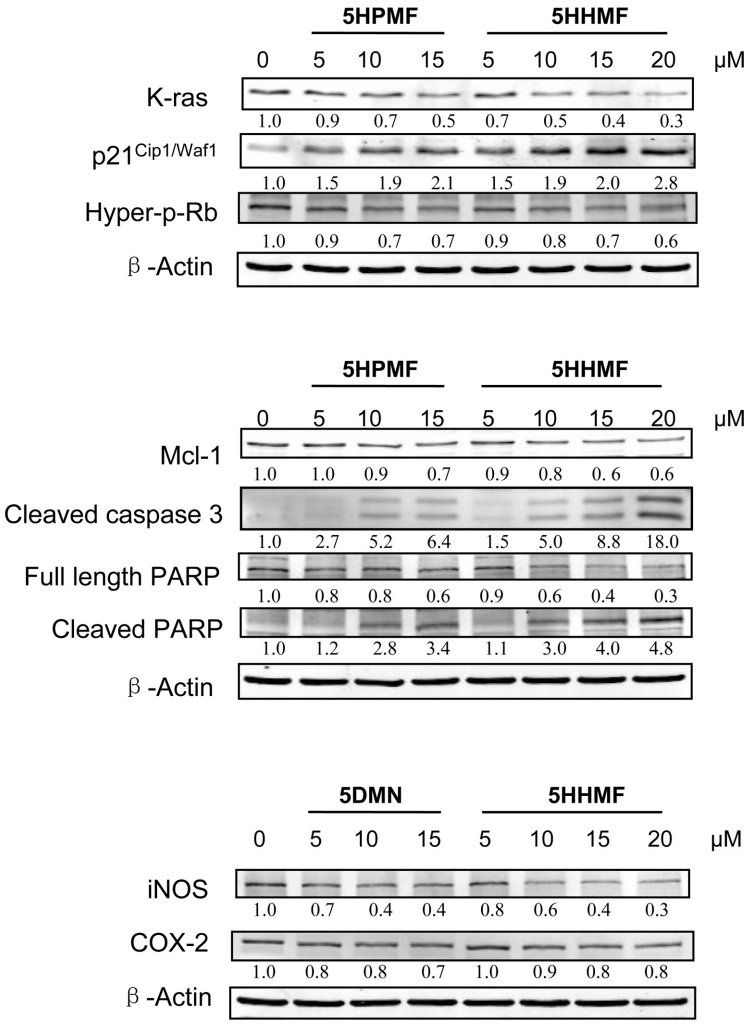

Figure 5. Effects of 5HPMF and 5HHMF on cell proliferation, apoptosis, and inflammation related proteins in H1299 human lung carcinoma cells. H1299 cells were seeded in 10-cm petri dishes. After 24 h, cells were treated with serial concentrations of 5HPMF or 5HHMF. After another 24 h of incubation, cells were harvested for Western immunoblotting as described in Section 2. The numbers underneath of the blots represent mean band intensity (normalized to β-actin) measured by densitometer ($n = 3$), and the range of the SD of the band intensity was within 10% of the mean value. β-Actin was served as an equal loading control. The experiments were repeated at least once.

itory effect on H1299 cell growth than their permethoxylated counterparts, *i.e.*, nobiletin and HMF, respectively. Our results are in agreement with previous reports that showed potent anticancer effects of 5-hydroxylated PMFs in leukemia and breast cancer cell lines [6, 7, 11, 12]. The only chemical structural difference between 5HPMF and nobiletin, or between 5HHMF and HMF, is the substitution of the methoxy group with hydroxyl in 5-position. This suggested the pivotal role of hydroxylation at 5-position in rendering 5HPMF and 5HHMF potent anticancer activity in H1299 cells. How does exactly the hydroxylation influent biological activity of PMFs in cancer cells is unknown, and it is an intriguing topic that warrants further investigation.

Apoptosis, a programmed cell death, is a naturally occurring process that eliminates damaged or unwanted cells such as those with harmful mutations. Deregulation of apoptosis can disrupt the balance between cell proliferation and cell death, and can lead to cancer. As a matter of fact, the evasion of apoptosis is one of the essential alterations in cell physiology that dictate malignant growth and is a hallmark of most types of cancer [13]. Restoration of apoptosis in cancer cells is an effective strategy for cancer prevention and treatment, and is under intensive investigation. In our efforts to elucidate the mechanisms by which monodemethylated PMFs, namely 5HPMF and 5HHMF, inhibit cancer cell growth, it was found that both compounds caused significant accumulation of cell population in sub G0/G1 phase. Increased sub G0/G1 cell population indicated DNA fragmentation caused by 5HPMF and 5HHMF. DNA fragmentation is an important characteristic of apoptosis [14, 15]. We further demonstrated that caspase-3 was activated by treatments with 5HPMF and 5HHMF, and this activation was companied by the cleavage of PARP, a substrate of activated caspase-3. PARP is involved in DNA repair and important to maintain cell viability [16 – 18]. The cleavage of PARP facilitates cellular disassembly and ultimately apoptosis [19]. Mcl-1 (myeloid cell leukemia-1), a member of Bcl-2 anti-apoptotic family, is expressed in approximately 60% of human nonsmall-cell lung cancer (NSCLC) [20]. Multiple NSCLC cell lines, including H1299, express

abundant Mcl-1 proteins. Depletion of Mcl-1 induces apoptosis, and reduction of this protein can sensitize lung cancer cells to apoptosis induced by cytotoxic agents as well as ionizing radiation. Overexpression of Mcl-1 renders H1299 cells resistance to apoptosis induced by chemotherapeutic agents [21]. Treatments with 5HPMF and 5HHMF decreased expression levels of Mcl-1 in H1299 cells, which may be important to their proapoptotic effects.

Our results showed that monodemethylated PMFs inhibited cancer cell growth possibly through modulation on several important proteins associated with lung carcinogenesis. Ras oncogenes play important role in regulating cell growth, differentiation and survival, and dysregulation of ras proteins, especially, K-ras, is common in human cancers, including lung cancer [22]. Both 5HPMF and 5HHMF dose-dependently decreased the level of K-ras in H1299 cells. Loss of cell cycle control and cell proliferation is a hallmark of cancer. Cyclin dependent kinase (CDK) inhibitors, such as p21Cip1/Waf1, are negative regulators of cell cycle progression. They inhibit CDK activities, which causes hypo-phosphorylation of Rb, and in turn, the inhibition leads to decreased cell proliferation [23]. After treatments with 5HPMF and 5HHMF, p21Cip1/Waf1 protein level was significantly elevated, which was companied by the decrease in the level of hyper-phosphorylated Rb protein. Inflammation has been associated with lung carcinogenesis. As a proinflammatory factor, COX-2 has a critical involvement in carcinogenesis due to its role in apoptosis resistance, cell proliferation, and angiogenesis [24]. Several studies have shown the high-level constitutive expression of COX-2 in human NSCLC [25 – 27]. Our results demonstrated that treatments with 5HPMF and 5HHMF moderately decreased the expression level of COX-2. Due to the potential importance of COX-2 in lung carcinogenesis, future efforts should be made to explore the effects of these compounds on the *in vivo* expression level and activity of COX-2. iNOS is a enzyme responsible to produce nitric oxide (NO), and elevated levels of iNOS and NO have been reported in lung cancer [28 – 30]. Chen *et al*. [31] showed that the level of iNOS and NO was significantly higher in lung cancer tissues of smokers than that of nonsmokers. Their results also demonstrated that the increased level of iNOS/NO was concomitant with decreased level of apoptosis, which may be caused by cigarette smoking and lead to the proliferation and growth of lung cancer cells [31]. Our results, that monodemethylated PMFs dose-dependently decreased iNOS protein levels in H1299 cells, support the rationale to study the *in vivo* efficacy of monodemethylated PMFs in the inhibition of lung carcinogenesis in A/J mice treated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco smoke derived carcinogen.

Bioavailability is one of important factors dictating cancer preventive efficacy of dietary components in humans. Poor absorption and extensive conjugative metabolisms in the intestine and liver greatly limit bioavailability of dietary flavonoids and other polyphenols. However, methoxylation of PMFs dramatically increases their metabolic stability and membrane transport in the intestine/liver, which can improve their oral bioavailability [32]. There are only a few studies on the oral bioavailability of PMFs. Murakami *et al*. [33] demonstrated that nobiletin at 27 mg/kg oral dose resulted in liver and kidney levels of 2 – 6 μM between 1 and 4 h after oral administration in rats [33]. There is no report on *in vivo* bioavailability of monodemethylated PMFs, but based on the aforementioned nobiletin data, our results that 5HPMF and 5HHMF inhibited cancer cell growth and caused molecular changes at the concentration of 5 μM provided a strong rationale to further explore cancer preventive efficacy of monodemethylated PMFs in animal models.

Taken together, we demonstrated that monodemethylated PMFs have much stronger inhibitory effect on the growth of lung carcinoma H1299 cells than their permethoxylated counterparts. Monodemethylated PMFs such as 5HPMF and 5HHMF can be formed from their permethoxylated counterparts in orange peel extract by hydrolysis. This process could be accelerated by addition of acids as a catalyst. If indeed hydroxylated PMFs are proved to be effective cancer preventive agents in humans, the orange peel extract enriched with hydroxylated PMFs would be a promising and novel ingredient for cancer preventive functional foods.

The authors have declared no conflict of interest.

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