Published in final edited form as:

Mol Nutr Food Res. 2013 December ; 57(12): 2103–2111. doi:10.1002/mnfr.201300136.

5-demethyltangeretin inhibits human non-small cell lung cancer cell growth by inducing G2/M cell cycle arrest and apoptosis

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Abstract

Scope—Tangeretin and 5-demethyltangeretin (5DT) are two closely related polymethoxyflavones found in citrus fruits. We investigated growth inhibitory effects on three human non-small cell lung cancer (NSCLC) cells.

Methods and results—Cell viability assay demonstrated that 5DT inhibited NSCLC cell growth in a time- and dose-dependent manner, and IC₅₀s of 5DT were 79-fold, 57-fold and 56-fold lower than those of tangeretin in A549, H460, and H1299 cells, respectively. Flow cytometry analysis showed that 5DT induced extensive G2/M cell cycle arrest and apoptosis in NSCLC cells, while tangeretin at 10-fold higher concentrations did not. The apoptosis induced by 5DT was further confirmed by activation of caspase-3 and cleavage of PARP. Moreover, 5DT dose-dependently upregulated p53 and p21^{Cip1/Waf1}, and downregulated Cdc-2 (Cdk-1) and cyclin B1. HPLC analysis revealed that the intracellular levels of 5DT in NSCLC cells were 2.7 - 4.9-fold higher than those of tangeretin after the cells were treated with 5DT or tangeretin at the same concentration.

Conclusions—our results demonstrated that 5DT inhibited NSCLC cell growth by inducing G2/M cell cycle arrest and apoptosis. These effects were much stronger than those produced by tangeretin, which is partially due to the higher intracellular uptake of 5DT than tangeretin.

Keywords

5-demethyltangeretin; 5-hydroxy-6,7,8,4'-tetramethoxyflavone; apoptosis; lung cancer; tangeretin

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The authors have declared no conflict of interest.

1. Introduction

Epidemiological studies have suggested that certain phytochemicals derived from edible plants may prevent disease and promote human health. Polymethoxyflavones (PMFs) are a class of flavonoid compounds that are almost exclusively found in the peels of citrus fruits such as sweet oranges (*Citrus sinensis*) and mandarin oranges (*Citrus reticulata*) [1]. Accumulating evidence has demonstrated that PMFs had broad spectrum of biological activities including anti-carcinogenic, anti-inflammatory, anti-atherogenic, anti-viral, and anti-oxidative activities [2]. More than twenty PMFs have been identified, and among them, tangeretin and nobiletin are the most studied in terms of their potential health-promoting effects.

Among different types of PMFs, 5-hydroxylated PMFs are a unique subclass [1]. Although they are relatively less abundant in fresh orange peels in comparison to their permethoxylated counterparts e.g. tangeretin and nobiletin, 5-hydroxylated PMFs can be generated by autohydrolysis from the permethoxylated PMFs [1, 3]. Recently, we and others have demonstrated anti-carcinogenic potential of 5-hydroxylated PMFs against different types of cancers. For example, 5-hydroxylated PMFs have been shown to have strong antiproliferative effects on human lung [4], colon [5-7], breast[8], and leukemia [9] cancer cells. More interestingly, 5-hydroxylated PMFs generally exhibited more potent inhibitory effects on cancer cells than their permethoxylated counterparts. We have demonstrated that 5-hydroxylated 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 (5DT, 5-demethyltangeretin) exhibited stronger growth inhibitory effects than their permethoxylated counterparts, namely nobiletin, 3,5,6,7,8,3',4'-heptamethoxyflavone and tangeretin, respectively, in human colon cancer cells [6].

Lung carcinoma is the most common cause of cancer mortality among all types of cancers in both men and women [10]. Non-small cell lung cancer (NSCLC) accounts for more than 80 % of lung cancer cases including adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma. Generally, the survival rate for lung cancer patients is very low (5 years of less than 15%), and this is due to the fact that NSCLC tends to become resistant to chemotherapeutic treatments and radiation therapy, and NSCLC is frequently diagnosed when the disease progressed beyond surgical intervention [11-12]. Chemoprevention is considered a promising strategy to controlling cancer mortality because it potentially reduces the incidence of malignant cancer by reversing and/or delaying carcinogenesis. However, no effective chemoprevention strategy has been successfully developed for lung cancer, especially NSCLC. Herein, for the first time, we investigated the inhibitory effects of a unique 5-hydroxylated PMF, 5DT (Fig. 1) in three different human NSCLC cells, and compared these effects with those produced by the permethoxylated counterpart of 5DT, tangeretin.

2. Materials and methods

2.1 Cell cultures and treatments

H460, H1299, and A549 human NSCLC cell lines were obtained from American Type Cell Collection (ATCC, Manassas, VA, USA), and maintained in RPMI 1640 media (Mediatech, Herndon, VA, USA) supplemented with 5% heat-inactivated FBS and 100U/ml of penicillin and 0.1mg/ml of streptomycin at 37°C with 5% CO₂ and 95% air. Cells were kept sub-confluent and media were changed every 3-4 days. All cells used in experiments were between 4 and 25 passages. Tangeretin and 5DT were isolated and identified as we previously described [3-4, 13]. DMSO was used as a vehicle to deliver tangeretin and 5DT to the cells. The final concentration of DMSO in all experiments was 0.1% v/v in cell culture media.

2.2 Cell viability assay

H460, H1299, or A549 (2,000 cells/well) cells were seeded in 96-well plates. At 24 hrs, cells were treated with serial concentrations of tangeretin and 5DT in 200 µL of complete media. After desired treatment times, the media were replaced with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich)-containing medium for viability test as we previously reported [4].

2.3 Colony formation assay

H460 (750 cells/well) cells were seeded in 6-well plates. At 24 hrs, cells were treated with serial concentrations of tangeretin and 5DT in 2 mL of serum complete media. The media were refreshed every 4 days. After 12 days of incubation, media were discarded. Colonies were washed with PBS, and then stained with 0.2% crystal violet for 10 minutes. After being washed with double distilled water to remove any residual dye, plates were scanned with a high resolution scanner (HP Inc.) and colonies formed were counted.

2.4 Flow cytometric analysis of cell cycle distribution and apoptosis

H460, H1299, or A549 (5×10^4 cells/well) were seeded in 6-well plates. After 24 hrs of incubation for cell attachment, cells were treated with serial concentrations of tangeretin and 5DT in 2 mL of serum complete media. Media containing any floating cells were harvested and combined with adherent cells that were detached by brief trypsinization (0.25% trypsin-EDTA; Mediatech). Cell pellets were washed with 1 mL of ice-cold PBS then subject to cell cycle and apoptosis analysis as we described previously [4].

2.5 Immunoblotting

Whole Cell lysate were prepared as previously described [4]. Cells were seeded in 150 mm culture dishes. After 24 hrs of incubation, cells were treated with serial concentrations of tangeretin and 5DT. After another 24 or 48 hrs of incubation, cells were washed with ice-cold PBS, and collected with cell-scrapers. Cells were combined with floating cells, if any, and subject to western blotting analysis as we previously described [4]. Antibodies for Bax, p21^{Cip1/Waf1}, p53, cdc-2(cdk-1), cyclin B1, cleaved caspase-3 (Asp175), and poly ADP ribose polymerase (PARP) were obtained from Cell Signaling Technology (Beverly, MA,

USA). β -Actin was used as a loading control, and antibody for β -actin was purchased from Sigma-Aldrich.

2.6 Determination of intracellular levels of tangeretin and 5DT

H460, H1299, and A549 (10^6 cells) were suspended in 1 mL of complete culture media containing serial concentrations of tangeretin and 5DT and then incubated at 37°C for 30, 90, and 180 minutes. The cell suspensions were then centrifuged at $2,147 \times g$ for 1 min at 4°C. The cell pellets were washed twice with 1 mL of ice-cold PBS, re-suspended in 1 mL of PBS, and then sonicated with a probe sonicator to disrupt the cells. After another centrifugation ($2,147 \times g$, 15 min at 4°C), the supernatant was collected and mixed with equal volume of methanol. The mixtures were then subject to HPLC analysis to determine the intracellular levels of tangeretin and 5DT using the method we developed previously [14].

2.7 Statistical analysis

All data were presented as mean $\pm$ SD. Analyses of variance (ANOVA) model was used to compare the differences among more than two groups followed by a post-hoc test for multiple comparisons (Dunnett's test or The Tukey's Range Honesty Significant Difference test as described in figure legends). A 1% significant level was used for all tests.

3. Results

3.1 5DT was stronger than tangeretin in inhibiting human NSCLC cell proliferation

H460, H1299 and A549 cells were treated with 5DT (0.6 – 3 μ M) or tangeretin (8 – 40 μ M) for 24, 48, or 72 hrs (Figure 2). Treatments with tangeretin showed marginal inhibitory effects on all three NSCLC cells. At the highest concentration tested (40 μ M), tangeretin only inhibited cell growth by 36.0%, 27.5%, and 18.4% on H460, H1299, and A549 cells, respectively, after 72 hrs of treatments. In contrast, 5DT showed much more potent inhibition on all three NSCLC cells in a dose-dependent and time-dependent fashion in comparison with tangeretin. At 3 μ M, treatments with 5DT for 72 hrs inhibited cell growth by 93.4%, 93.0%, and 94.2% on H460, H1299, and A549 cells, respectively. The IC_{50} values of 5DT after 72 hrs of treatment were 1.27 μ M, 1.02 μ M, and 0.99 μ M in H460, H1299, and A549 cells, respectively. On the other hand, the projected IC_{50} values of tangeretin after 72 hrs of treatment were 72.6 μ M, 54.5 μ M, and 78.2 μ M in H460, H1299, and A549 cells, respectively. This means that IC_{50} values of 5DT were 57.2-fold, 56.3-fold, and 78.9-fold lower than those of tangeretin in H460, H1299 and A549 cells, respectively.

3.2 5DT inhibited the colony formation of NSCLC cells

To further determine the difference between the effects of 5DT and tangeretin on the growth of human NSCLC cells, we conducted colony formation assay on H460 cells. H460 cells were subjected to the treatments with tangeretin (10 μ M or 20 μ M) or 5DT (1 μ M, 1.5 μ M, or 2 μ M). Tangeretin at 10 μ M or 20 μ M did not significantly decrease the capability of cancer cells to form colonies (Figure 3A). However, 5DT showed potent and dose-dependent inhibition on the formation of colonies, i.e., 5DT at 1 μ M, 1.5 μ M, and 2 μ M inhibited the colony formation by 20%, 75%, and 99%, respectively. The treatment with

5DT also caused distinct changes on the morphology of H460 cells. As shown in Figure 3B, 5DT-treated cells appeared smaller in size, had much less defined nucleus region, and had poor attachment in comparison with the control cells. Treatments with tangeretin did not cause any significant change on cell morphology in comparison to the control (images not shown).

3.3 5DT caused G2/M cell cycle arrest in NSCLC cells

To establish the mode of action of 5DT and tangeretin in inhibiting cell growth, cell cycle analysis was conducted on the NSCLC cells treated with 5DT or tangeretin. As shown in figure 4, tangeretin at 10 μ M, 20 μ M, or 30 μ M did not cause significant changes in cell cycle distribution of three NSCLC cells in comparison to the control cells. In contrast, 5DT caused significant and dose-dependent increase in the percentage of cells in G2/M phase in all three NSCLC cells (Figure 4). Treatments with 5DT at 3 μ M increased the percentage of G2/M cell population to 3.1-fold, 3.5-fold, and 3.7-fold of the control cells in H460, H1299, and A549 cells, respectively.

3.4 5DT induced extensive apoptosis in NSCLC cells

To determine the extent to which apoptosis contributed to the growth inhibition caused by 5DT, we investigated the effects of 5DT on cellular apoptosis of NSCLC cells, and compared them with those produced by tangeretin. After 48 hrs of treatments with 5DT and tangeretin at different concentrations, early and late apoptotic cells were quantified by flow cytometry method with Annexin V/Propidium Iodine (PI) double staining assay. As shown in figure 5, 5DT induced extensive apoptosis in all three NSCLC cells in a dose-dependent manner. In H460 cells, 5DT at 2 μ M and 3 μ M increased early apoptotic cell population by 5.2-fold (from 2.1% to 10.9%) and 16.4-fold (from 2.1% to 34.4%) compared to the control, respectively. Moreover, 5DT at 2 μ M and 3 μ M also increased late apoptotic cell population by 5.3-fold (from 0.8% to 4.2%) and 13.8-fold (from 0.8% to 11.0%) compared to the control, respectively (Fig. 5A). Similar trends were also observed in the H1299 and A549 cells after 5DT treatments (Fig. 5B and C). In contrast, tangeretin at 10 μ M, 20 μ M, and 30 μ M did not cause any significant change in apoptotic cell population in H460, H1299 and A549 NSCLC cells.

3.5 5DT profoundly modulated key proteins related to cell proliferation and apoptosis

In order to further elucidate the molecular mechanisms underlying the inhibitory effects of 5DT on human NSCLC cells, we compared the effects of 5DT and tangeretin on key protein markers related to cell proliferation and apoptosis. Immunoblotting analysis demonstrated that 5DT caused extensive changes in these signaling proteins (Figure 6). In H460 and A549 cells, 5DT at different concentrations, especially 2 and 3 μ M, significantly increased expression levels of p53 and Bax, and decreased expression levels of cdc-2(cdk1) and cyclin B1. In all three NSCLC (H460, H1299 and A549) cells, 5DT at different concentrations, especially 2 and 3 μ M, significantly increased expression levels of p21^{Cip1/Waf1}, cleaved caspase-3 and cleaved PARP. In contrast, tangeretin at much higher concentrations (10, 20 or 30 μ M) did not cause any changes in most of these proteins, except for moderate effects in increasing p21^{Cip1/Waf1} levels in H460, H1299 and A549 cells; increasing Bax levels in

A549 cells; and decreasing cdc-2(cdk1) levels in A549 cells. All of these effects of tangeretin were much weaker than those produced by 5DT at much lower concentrations.

3.6 Intracellular uptake of 5DT was much higher than that of tangeretin in NSCLC cells

Intracellular uptake is an important factor influencing the bioactivities of chemopreventive compounds. Three NSCLC cells were incubated with different concentrations of tangeretin (3, 20, or 30 μ M) or 5DT (1, 2, or 3 μ M) for 30, 90, and 180 min. Then, the intracellular levels (bioavailable fraction to the NSCLC cells) of tangeretin and 5DT in NSCLC cells were determined by the HPLC method developed by us previously [15]. The results showed that the intracellular levels of 5DT and tangeretin increased with increased concentrations of 5DT and tangeretin at 30, 90 and 180 min (Fig. 7, upper panels). Using the data from the upper panels of Fig. 7, the area under curve (AUC) was calculated for tangeretin and 5DT from 0 to 180 min to represent the cumulative amount of tangeretin and 5DT that have been exposed to intracellular compartment of the cancer cells over time. As shown in the lower panels of Fig. 7, intracellular accumulation of 5DT were 4.9-fold, 2.8-fold, and 2.7-fold higher than those of tangeretin in H460, H1299, and A549 cells, respectively, when cells were treated with 5DT or tangeretin at the same concentration of 3 μ M. Actually, the intracellular accumulation of tangeretin at 3 μ M was similar to that of 5DT at 1 μ M, but lower than that of 5DT at 2 μ M. It was also observed that tangeretin at 20 and 30 μ M resulted in higher intracellular accumulation than that produced by 5DT at 2 and 3 μ M. However, the differences were between 2.0 – 4.4-fold (much less than 10-fold). Our results demonstrated superior intracellular uptake of 5DT to tangeretin in all three lung cancer cells.

4. Discussion

Phytochemicals from edible plants have been shown to provide potential protection against carcinogenesis. Tangeretin is one of the most abundant PMFs found in the peels of sweet oranges (*C. senensis*) and mandarin orange (*C. reticulana*), while 5DT exists in these peels in less abundance [1]. Aged orange peels are important ingredients for Traditional Chinese Medicine. The repeated heating and drying process used to produce aged orange peels can convert tangeretin to 5DT by demethylation at 5-position in the A-ring of the tangeretin structure (Fig. 1) [1, 3]. Herein, we reported, for the first time, the inhibitory effects of 5DT on human NSCLC cells.

Flavones, including PMFs, have been showed to have anti-proliferative effects on many cancerous cells [4-5, 8-9, 16], and their chemical structures dictates their bioactivities. It was suggested that the presence of C₂-C₃ double bond in the C-ring core structure of flavones played a pivotal role for their anticancer activities [17-18]. Moreover, the number and position of substituents in the flavone core structures can also influence the anti-proliferative activities of flavones on cancer cells [19]. Previously, we compared the effects of two 5-hydroxylated PMFs, namely 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone and 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone, with those produced by their permethoxylated counterparts, namely nobletin and 3,5,6,7,8,3',4'-heptamethoxyflavone, on human NSCLC cells [4]. It was found that 5-hydroxylated PMFs had much stronger inhibitory effects on cancer cells in comparison with their permethoxylated counterparts. In this study, we further

investigated the effects of 5DT and its permethoxylated counterpart, tangeretin on human NSCLC cells. The results from cell viability assay indicated that 5DT was 55-80 fold more potent than tangeretin in inhibiting NSCLC cell growth (Fig. 2). The colony formation assay showed that 5DT was able to eliminate 99% of colonies formed by NSCLC cells, while tangeretin at 10-fold higher concentration did not have any inhibitory effects on the colonies (Fig. 3). These results demonstrated that 5DT had much stronger inhibitory effects on NSCLC cells than tangeretin. Since the only structural difference between 5DT and tangeretin is the demethylation at 5-position in their A-ring core structure (Fig. 1), our results reinforced the notion that 5-demethylation can significantly enhance the inhibitory effects of PMFs against cancer cell growth [4-5, 8-9].

One of the hallmarks of carcinogenesis is uncontrolled cell proliferation, which is associated with deregulation of cell cycle progression and apoptosis [20-21]. Therefore, inducing cell cycle arrest and apoptosis in cancer cells is potentially an effective strategy for cancer chemoprevention and treatment. Our results from flow cytometry analysis clearly demonstrated that 5DT treatments not only caused significant cell cycle arrest at G₂/M phase, but also led to extensive apoptosis in all NSCLC cells tested (Fig. 4 and 5). However, tangeretin at 10-fold higher concentrations did not cause any change on cell cycle distribution or apoptosis of cancer cells. These findings suggested that 5DT was able to inhibit cancer cell growth by impede cell cycle progression and increase programmed cell death.

Cyclins, cyclin dependent kinases (CDKs), and CDK inhibitors act in concert to regulate cell cycle progression, i.e. cyclin/CDK complexes promote cell cycle progression whereas CDK inhibitors drive cell cycle arrest [22-23]. In H460 and A549 cells, 5DT significantly decreased the expression levels of both cyclin B1 and Cdc2, which at least in part, led to the G₂/M arrest (Fig. 6). This is because that the complex of cyclin B1/Cdc2 is required for entry into mitosis in G₂/M transition [24]. Furthermore, 5DT caused drastic increases in the levels of p21^{Cip1/Waf1} in the NSCLC cells, which also contributed to the cell cycle arrest observed. p21^{Cip1/Waf1} is a small 165 amino acid Cdk inhibitor that inhibits activity of cyclin B1/Cdc2 complex by directly bound to the complex, which blocks cells in G₂/M phase [25-26]. In addition, p21^{Cip1/Waf1} could decrease Cdc2 protein levels by decreasing Cdc2 mRNA levels and its promoter activity [27]. Both H460 and A549 cells contain wild-type p53, a tumor suppressor. Our results showed that 5DT treatments dose-dependently increased expression levels of p53 in these cells. p53 has a broad range of cellular functions and plays key roles in inhibiting carcinogenesis [28] by inducing cell cycle arrest [25], cellular apoptosis [29], and DNA repair [30]. Increased levels of p53 can result in G₂/M cell cycle arrest by downregulating transcription of both cyclin B1 and Cdc2 [25, 31-32]. Moreover, p21^{Cip1/Waf1} is one of the transcriptional targets of p53, and increased levels of p53 can result in increased levels of p21^{Cip1/Waf1}, which in turn contributes to cell cycle arrest [25, 33].

The immunoblotting results illustrated that 5DT-mediated G₂/M cell cycle arrest in H460 and A549 (p53 wild-type) cells was associated with dose-dependent increases in the levels of p53. However, we also observed the G₂/M cell cycle arrest in 5DT-treated H1299 cells that are p53-null NSCLC cells. In the mean time, 5DT also caused increased levels of

p21^{Cip1/Waf1} and decreased levels of Cdc2 in H1299 cells. These findings suggested that 5DT-mediated cell cycle arrest at G2/M phase was at least partially independent of p53 status of the NSCLC cells. Moreover, our results showed that 5DT dose-dependently activated caspase 3 and PARP in all H460, A549, and H1299 cells, suggesting that similarly to the 5DT-mediated cell cycle arrest, 5DT-mediated apoptosis was, at least partially independent of p53 status of the NSCLC cells. These findings in NSCLC cells were not consistent with our previous report showing that 5DT caused p53-dependent cell cycle arrest and apoptosis in human colorectal cancer cells [7]. This suggests that the effects of 5DT may be cancer-type and/or cell-type specific.

Intracellular accumulation of chemopreventive compounds is one of key factors that dictate the efficacy of these compounds in inhibiting cancer cell growth. Cancer cells, including NSCLC cells can acquire abilities to decrease intracellular accumulation of cytotoxic agents by various mechanisms, such as overexpression of multi-drug resistant efflux proteins that can pump the cytotoxic agents out of cancer cells [34]. Our results showed that NSCLC cells had much higher (2.7-4.9 fold) intracellular accumulation of 5DT than tangeretin after incubation with 5DT or tangeretin at the same concentration (Fig. 7). This suggested that NSCLC cells may have better uptake and/or less efflux of 5DT in comparison with tangeretin. HPLC analysis showed that 5DT had higher lipophilicity than tangeretin [14]. High lipophilicity could enhance binding of 5DT to the plasma membrane, which in turn may promote the uptake of 5DT into cytosol of the cancer cells. It is of importance to elucidate the exact mechanism by which 5DT accumulated more intracellularly than tangeretin in cancer cells in future investigation. Our results from HPLC analysis further revealed that tangeretin at 20-30 μ M led to its higher (2-4 fold) intracellular accumulation than 5DT at 2-3 μ M. However, tangeretin at 20-30 μ M still failed to significantly inhibit NSCLC cells, while 5DT at 2-3 μ M showed very strong inhibition on these cells. We demonstrated that 5DT had profound effects in modulating cell cycle progression and apoptosis. These effects are likely the results of interactions between 5DT and its direct protein targets in cancer cells. The binding of 5DT to these protein targets could initiate a cascade of signaling events that result in downstream phenotype outcomes such as cell cycle arrest and apoptosis. Due to the difference in the chemical structure, 5DT and tangeretin may bind to different proteins and/or bind to same proteins but with different affinity, which leads to different downstream phenotype outcomes. The higher intracellular accumulation of 5DT in comparison to tangeretin at the same concentration provides 5DT higher potential to interact with its protein targets, which in turn potentially lead to stronger inhibitory effects on cancer cells.

In conclusion, we demonstrated, for the first time the potent inhibitory effects of 5DT on human NSCLC cells. The inhibition on cancer cells was associated with extensive cell cycle arrest and apoptosis caused by 5DT as the result of modulation of oncogenic signaling proteins. Moreover, 5DT showed high intracellular accumulation in cancer cells, which may enhance its anti-cancer potential.

Acknowledgements

This work was partially supported by a NIH grant (CA139174), an AICR grant, and a USDA Special Grant on bioactive food components.

Abbreviations

5DT	5-demethyltangeretin
AUC	area under curve
FBS	fetal bovine serum
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NSCLC	non-small cell lung cancer
PARP	poly (ADP-ribose) polymerase
PI	propidium iodine
PMFs	polymethoxyflavones
TAN	tangeretin

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Figure 1. Chemical Structures of tangeretin and 5-demethyltangeretin (5DT, 5-hydroxy-6,7,8,4'-tetramethoxyflavone).

Figure 2. 5DT significantly inhibits the proliferation of H460, H1299, and A549 human NSCLC cells. H460 (A), H1299 (B), and A549 (C) were seeded in 96-well plates, and after 24 hrs of incubation, cells were treated with serial concentrations of tangeretin and 5DT. After another 24, 48, or 72h of treatment, cell viability was measured by MTT assay as described in method Section. Data represent mean $\pm$ SD ($n = 6$), and the asterisks (* for 24h, ** for 48h, and *** for 72h) indicate the statistical significance between 5DT at different concentrations and tangeretin at the highest concentration (40 μ M) at the same incubation time based on ANOVA test followed by Dunnett's post-hoc test ($p < 0.01$, $n = 6$).

Figure 3.

Inhibitory effects of 5DT and tangeretin on colony formation of NSCLC cells. A. 5DT potently inhibited colony formation of human H460 NSCLC cells. Cells were seeded into 6-well plates. After 24 hrs of incubation, cells were subjected to treatments with serial concentrations of tangeretin and 5DT as shown in the figure. After 12 days of incubation, the numbers of colonies formed was photographed and counted as described in the methods. Data represent mean $\pm$ SD ($n = 3$), and all the 5DT-treated groups showed significant difference in comparison with the control and tangeretin-treated group based on ANOVA followed by Tukey's HSD post-hoc test. B. Phase-contrast pictures of control and 5DT-treated colonies of H460 NSCLC cells.

Figure 4.

Effects of tangeretin and 5DT on cell cycle progression of human NSCLC cells. H460 (A), H1299 (B), and A549 (C) were seeded in 6-well plates for 24h, and then treated with serial concentrations of tangeretin and 5DT. After another 24h, cells were collected and subjected to cell cycle analyses as described in methods section. All data represent mean $\pm$ SD, and the asterisk in the bar charts indicated statistical significance of 5DT-treated groups in comparison with the control and tangeretin-treated groups at all concentrations based on ANOVA followed by Tukey's HSD post-hoc test ($p < 0.01$, $n = 3$).

Figure 5.

Effects of tangeretin and 5DT on apoptosis of human NSCLC cells. H460 (A), H1299 (B), and A549 (C) cells were seeded in 6-well plates for 24 hr, and then treated with different concentrations of tangeretin and 5DT. After another 48h, cells were collected and subjected to apoptosis analyses described in methods section. All data represent mean $\pm$ SD, and the asterisk in the bar charts indicated statistical significance of 5DT-treated groups in comparison with the control and tangeretin-treated groups at all concentration based on ANOVA followed by Tukey's HSD post-hoc test ($p < 0.01$, $n = 3$).

Figure 6.

Effects of tangeretin and 5DT on cell cycle and apoptosis related key proteins in human NSCLC cells. H460 (A), H1299 (B), and A549 (C) cells were seeded into 15 cm culture dishes for 24h, and then cells were treated with serial concentrations of tangeretin and 5DT. After another 24h or 48h of incubation, cells were collected for western blotting as described in methods section. 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 $\pm 15\%$ of the means) were not shown. β -Actin served as an equal loading control.

Figure 7.

Intracellular levels of tangeretin and 5DT in human NSCLC cells. H460 (A), H1299 (B), and A549 (C) cells were incubated with different concentrations of tangeretin or 5DT for 30, 90, or 180 min. The intracellular concentrations of tangeretin and 5DT were quantified by HPLC as previously described. Bar graphs in the lower panel were based on the area under the curve calculated from the cellular uptake data in the upper panel. All data represent mean $\pm$ SD ($n = 3$). All results for 5DT at 2–3 μ M were significantly higher than those for tangeretin at 3 μ M as tested by ANOVA followed by Dunnett's post-hoc test (*, $p < 0.01$, $n = 3$).