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Effect of a Labile Methyl Donor on the Transformation of 5-Demethyltangeretin and the Related Implication on Bioactivity

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S Supporting Information

ABSTRACT: Polymethoxyflavones (PMFs) belong to a subgroup of flavonoids that particularly exist in the peels of citrus fruits. Despite their many health-beneficial biofunctionalities, the lipophilic nature of PMFs limits their water solubility and oral bioavailability. To investigate the effect of the delivery system on the improvement of PMF bioavailibility, a lecithin-based emulsion was formulated for the delivery of two PMF compounds, tangeretin and 5-demethyltangeretin. While the emulsion system improved the digestion kinetics and the total solubilized PMF concentrations in in vitro lipolysis studies, the concentration of 5-demethyltangeretin decreased due to chemical transformation to its permethoxylated counterpart, tangeretin. The emulsifier lecithin used in this emulsion formulation contained a choline headgroup as a labile methyl group donor. The presence of a methyl donor potentially caused the transformation of 5-demethyltangeretin and reduced its anti-cancer-cell-proliferation activities. Moreover, this is the first report in the literature of the transformation from 5-demethyltangeretin to tangeretin in a lecithin-based emulsion during lipolysis, and the mechanism underlying this phenomenon has also been proposed for the first time.

KEYWORDS: *tangeretin, 5-demethyltangeretin, lecithin-based emulsion, lipolysis, transformation, labile methyl donor, in vitro anti-cancer-cell-proliferation activity*

INTRODUCTION

The benefits of consuming fruits and vegetables beyond the basic nutritional requirements were not recognized until recent decades. Due to advancement in research technologies, scientists can now distinguish tens of thousands of natural compounds that possess health-promoting biofunctionalities. These compounds may potentially serve as advantageous alternatives to synthetic drugs in disease prevention and even treatment at an appropriate dosage. The bioactive phytochemicals vary dramatically owing to differences in the species, growth locations and conditions, and segments of the plants, etc. Polymethoxyflavones (PMFs) are an emerging category of phytochemicals which are mainly extracted from the peels of citrus fruits. By definition, PMFs are compounds that have two or more methoxy groups attached to the 15-carbon benzo-γ-pyrone skeleton structure with a carbonyl group on the C_4 position. PMFs, such as nobiletin, tangeretin, sinensetin, and 3,5,6,7,8,3′,4′-heptamethoxyflavone, are well documented to possess anti-inflammatory,[1] antiatherogenic,[1] and anticarcinogenic[2,3] activity and selective antiproliferative activity to cancer over normal cells.[4−6] In addition to permethoxylated PMFs, another group of PMFs, which include single or multiple hydroxy groups on various positions of the C6−C3−C6 flavonoid skeleton, have also been isolated from an aged extract of citrus peel and proved to exhibit stronger efficacies than their permethoxylated counterparts.[7−12]

Due to the multiple substitutions of methoxy groups on the skeleton backbone, PMFs and hydroxylated PMFs (OH-PMFs), similar to many other lipophilic bioactives, have poor aqueous solubility and, consequently, low bioavailability when ingested orally. To augment the oral dose efficiency, many people have relied on the strategy which assumes that improving a compound's aqueous solubility will enhance its bioavailability and bioefficacy. Among many approaches aiming to increase solubility, emulsion encapsulation is one of the convenient and versatile methods which have often been employed by many investigators. Recently, emulsions of different sizes (from >100 nm to <1 μm) and forms, such as solid lipid nanoparticles, self-emulsifying delivery systems, and surface-modified particles, etc., have been acknowledged to significantly improve bioavailability[13−25] and bioefficacy[26,27] in both in vitro and in vivo models.

Being granted the status of generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (USFDA), biocompatible lecithin, or phosphotidylcholine (PC), is a common emulsifier to use when developing emulsion systems for oral delivery. The structure of lecithin includes a nonpolar tail of two long-chain fatty acids and a polar head with a zwitterion phosphate−choline group. Having choline as part of the structure, PC is recognized as an effective dietary supplement for satisfying the body daily choline requirement (~550 mg/day). Choline is a precursor molecule for an important neurotransmitter compound and serves as an active labile methyl (−CH_3) donor during methyl metabolism. It has been well investigated that the increased consumption of PC will boost the concentration of choline and labile methyl groups as a consequence in biological systems. In the present study, an

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emulsion system using PC as the emulsifier has been developed. Two key PMFs, tangeretin (5,6,7,8,4′-pentamethoxyflavone) and 5-demethyltangeretin (5-hydroxy-6,7,8,4′-tetramethoxyflavone), were incorporated in emulsion-based delivery systems of identical compositions. An emulsion system containing either tangeretin or 5-demethyltangeretin was subjected to in vitro bioaccessibility and bioactivity evaluation. In the assessment of bioaccessibility using the in vitro lipolysis model, transformation of 5-demethyltangeretin to its permethoxylated counterpart was observed and confirmed by high-performance liquid chromatography (HPLC). To the best of our knowledge, this phenomenon has not been reported in previous literature and the mechanism underlying the transformation has not yet been elucidated.

■ MATERIALS AND METHODS

Materials. Tangeretin of 98% purity was purchased from Quality Phytochemical LLC (Edison, NJ). 5-Demethyltangeretin (5-hydroxytangeretin) with a purity of >98% was synthesized in our laboratory using a previously published method.[1] PC75 rapeseed lecithin containing 75% phosphatidylcholine was a gift from the American Lecithin Co. (Oxford, CT). Neobee 1053 medium-chain triacylglycerol (MCT) was a gift from Stepan Co. (Northfield, IL). Pancreatin with 8× USP specification and Tris maleate were obtained from Sigma-Aldrich (St. Louis, MO). Sodium taurodeoxycholate (NaTDC) was purchased from CalBiochem (La Jolla, CA). HPLC-grade acetonitrile (ACN) and water were purchased from J.T. Baker (Phillipsburg, NJ). Sterile-filtered, cell-culture-compatible dimethyl sulfoxide (Sigma-Aldrich) was used as the HPLC sample solvent. Minimum essential medium (MEM) was purchased from HyClone Laboratories, Inc. (Logan, UT). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). Penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA). Other chemicals were of reagent grade and used without further purification. Milli-Q water was used throughout the experiment.

Preparation of PMF Emulsions and MCT Suspensions. Tangeretin and 5-hydroxytangeretin emulsions were prepared using the method described in our recent paper.[28] Briefly, the oil phase containing MCT, lecithin, and either tangeretin or 5-hydroxytangeretin was prepared separately from the aqueous phase. The emulsion oil phase was maintained at 130 °C until all materials were completely dissolved. The temperature of the resulting oil phases was then reduced to 70 °C before the aqueous phase (double-deionized water, 70 °C) was added under magnetic stirring to form a crude emulsion, which was then subjected to high-speed homogenization (ULTRA-TURRAX T-25 basic, IKA Works Inc., Willmington, NC) to reduce the sample viscosity before processing using a high-pressure homogenizer (EmulsiFlex-C3, AVESTIN Inc., Ottawa, Canada). The components of the PMF emulsion system are MCT, lecithin, PMFs (tangeretin or 5-demethyltangeretin), and double-deionized water in concentrations of 50.4%, 1.5%, 2.1%, and 46%, respectively.

The MCT suspension was prepared by adding 0.05 g of either tangeretin or 5-hydroxytangeretin into 1 g of MCT. The PMF−MCT suspension was subjected to ultrasonication for 2 min to break up large clumps of precipitated compound. The suspension samples were vortexed for 5 min before the lipolysis study.

In vitro Lipolysis of PMFs in Emulsion or MCT Suspension. To better mimic the digestion activity in the human small intestine, an in vitro lipolysis study was carried out using the method described in our previously published paper [25] with minor modification. In short, a fed-state lipolysis buffer was prepared with Tris maleate, NaCl, $CaCl_2 \cdot H_2O$, NaTDC, and phosphatidylcholine in concentrations of 50, 150, 5, 20, and 5 mM, respectively. Pancreatin was freshly prepared for each lipolysis study by mixing 1 g of pancreatin powder with 5 mL of lipolysis buffer, centrifuging this mixture at 2000 rpm, and storing the mixture on ice. To begin the lipolysis study, equivalent amounts of the lipid samples (0.25 g of MCT suspension; 0.5 g of emulsion sample) and 1 mL of prepared pancreatin solution were added to 9 mL of lipolysis buffer. During the 2 h lipolysis study, the temperature was kept at 37 ± 1 °C and the pH was maintained at 7.50 ± 0.02 by 0.25 N NaOH titration. The volume of NaOH added at each time point was recorded, and the total amount consumed was calculated for data analysis. Upon completion of the 2 h lipolysis study, the whole lipolysis solutions were subjected to ultracentrifugation (type 60 Ti rotor, Beckman Coulter, Brea, CA) for 1 h at 50 000 rpm. After ultracentrifugation, the top layer is the undigested oil and the bottom layer contains precipitated compounds, which cannot be absorbed by the intestinal lining. Therefore, only the middle layer of supernatant was collected and stored at −80 °C for later HPLC analysis because it represented the compounds that could be incorporated into micelle cores of bile salts, which were the major forms for potential intestinal absorption. For HPLC analysis, 200 μL of lipolysis supernatant sample was filtered through a 0.22 μm filter and mixed well with 400 μL of DMSO.

Bioaccessibility (%) Calculation. The concentration of PMFs (tangeretin or 5-demethyltangeretin) solubilized in the supernatant after the lipolysis study was determined using HPLC. The aqueous lipolysis supernatant simulated the small intestinal lumen where PMFs solubilized in this portion were most likely bioaccessible to intestinal cells. The bioaccessibility (%) of PMFs was calculated according to previously published literature[25] using the following equation:

$$\text{bioaccessibility (\%)} = \frac{\text{total mass of solubilized PMFs}}{\text{total mass of PMFs in original lipid sample}} \times 100 \quad (1)$$

The mass of solubilized PMFs was calculated using the concentration of PMFs per volume of supernatant (g/mL) multiplied by the total volume of the lipolysis aqueous phase. The mass of PMFs in the original lipid sample was calculated from the concentration of PMFs in the MCT suspension or emulsion and mass of lipid added.

Determination of the Extent of Lipolysis. The extent of lipolysis, defined as the percentage of triglycerides digested by lipase, can be calculated using the amount of NaOH consumed during a set period of time. To better compare the digestion kinetics of MCT (unformulated) and the emulsion, the extent of lipolysis was determined at the 30 min time point since both the MCT suspension and emulsion sample were 100% digested at the end of the 2 h lipolysis experiment. The calculation of the extent of lipolysis assumed that two molecules of fatty acid were released from digestion of one triglyceride unit while two molecules of NaOH were consumed. Since the lecithin molecule from the emulsion formulation also contributed to the total amount of triglyceride present, the calculation of NaOH consumption for the emulsion sample included both compositional MCT (0.27 g) and lecithin (0.0075 g). The extent of lipolysis was calculated using the following equation in reference to a previously published paper:[25]

$$\text{extent of lipolysis} = \frac{\text{volume of NaOH} \times \text{concn of NaOH}}{2 \times \text{moles of triglyceride}} \times 100 \quad (2)$$

The volume of NaOH was obtained from calculation of the total NaOH volume added during lipolysis and corrected by subtracting the NaOH volume added to blank lipolysis (no lipid added). The concentration of NaOH was 0.25 N in this experiment. The number of moles of triglyceride was estimated from the average molecular weight of triglyceride with the following equation using the saponification value (SV) of MCT and/or lecithin:

$$\text{MW of triglyceride} = \frac{3 \times 1000 \times \text{MW of KOH}}{\text{SV}} \quad (3)$$

The molecular weight of KOH can be found in the literature as 56.1 g/mol. The saponification values used for MCT and lecithin are 334 and 190, respectively.

HPLC Analysis. The UltiMate 3000 HPLC system (Dionex, Sunnyvale, CA) consisted of a quaternary solvent delivery system, an autosampler, and a variable-wavelength detector connected to Supelco's RP-Amide column, 15 cm × 64.6 mm i.d., 3 μm (Bellefonte,

PA). The detection of PMFs was carried out using gradient elution with of a mobile phase of water (solvent A) and ACN (solvent B). The optimized condition was modified from tha in previous literature.[29] The total of 22 min of elution gradient started from 40% B, linearly increased to 55% B in 10 min, linearly increased to 70% at 15 min, linearly increased to 80% at 20 min, and then finally linearly decreased back to 40% at 21 min, the last step lasting for 1 min until the end of analysis. The flow rate during HPLC analysis was kept constant at 1.0 mL/min, the injection volume was 30 μL, and the detection wavelength was 320 nm.

Cell Culture. Human hepatic cancer cell line HepG2 (HB-8065, American Type Culture Collection, Manassas, VA) was cultured and maintained in MEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. The incubation condition for cell culture was 95% relative humidity and 5% CO_2 at 37 °C.

In Vitro Anti-Cancer-Cell-Proliferation Activity. Methylthiazole tetrazolium bromide (MTT) assay was used to evaluate the in vitro anti-cancer-cell-proliferation activity of PMF emulsions in comparison with DMSO-dissolved pure compounds. In the beginning of the test, HepG2 cells were seeded in a flat-bottom 96-well plate with a cell density of 1×10^4 cells/well and then incubated for 24 h. Subsequently, the seeded cells were treated with serum complete media containing various concentrations of PMFs (tangeretin or 5-demethyltangeretin) in either emulsion or DMSO. A negative control (untreated) and blank emulsion vehicle were also cultured along with PMF-treated cells, which were used as a reference for evaluation of in vitro anti-cancer-cell-proliferation activity. After 24 h of incubation, the cell culture medium was replaced by 100 μL of MTT solution (0.5 mg/mL in RPMI 1640 medium). After incubation for 2 h at 37 °C, the MTT solution was carefully aspired, and 100 μL of DMSO was added to each well to dissolve the purple formazan crystals. The DMSO-added plates were stored in the dark for 10 min and then subjected to light absorbance evaluation at 560 nm using an absorbance microplate reader (Molecular Devices, Sunnyvale, CA). The relative viabilities of cancer cells were calculated relative to the untreated control value.

Statistical Analysis. All experiments and analyses were performed in triplicate. The results are expressed as means ± SDs. Using Sigmaplot 10.0 software, Student's *t* test was performed to examine the mean difference between two groups, where $p < 0.01$ (indicated with a single asterisk in the figures) and $p < 0.001$ (indicated with two asterisks) were considered statistically significant.

RESULTS AND DISCUSSION

Effect of the Emulsion on the Digestion Kinetics. The therapeutic dosages of many health-beneficial bioactives are difficult to reach as a result of low aqueous solubility. It has been stated in many reports that the oral bioavailabilities of lipophilic compounds could be greatly increased when the compounds are consumed with lipids.[30−34] Therefore, wide varieties of lipid-based delivery systems have gained popularity among researchers to boost the bioavailable concentrations and to improve the bioefficacies of such compounds. To efficiently determine the most suitable lipid-based option for each specific bioactive candidate, many choose to conduct in vitro screening of potential vehicle formulations before proceeding to in vivo animal studies, which are more costly and time-consuming. Hence, there is a need for simplified in vitro screening that results in the emergence of an in vitro lipolysis model. The dynamic in vitro lipolysis model offers a good mockup of the in vivo lipid digestion process and achieves good in vitro−in vivo correlation (IVIVC) when predicting compound bioavailability.[31,35−37]

In the present study, PMFs (tangeretin or 5-demethyltangeretin) were integrated into a lecithin-emulsified system. The change of digestion kinetics in an emulsion system was compared with that in the crude MCT oil, which is also the base oil for the emulsion lipid dispersion core. As a result of lipid digestion, the pH of the lipolysis sample was lowered and required constant titration using NaOH to maintain the solution's environment at pH 7.5, at which the enzyme exhibited the best activity. On the basis of the assumption that digestion of one triglyceride unit will consume two molecules of NaOH, the digestion process can be monitored by recording the time and volume of NaOH deposited to the lipolysis solution. Namely, the faster the lipid digestion proceeded, the more frequently NaOH was added. The majority of the lipid digestion in the emulsion sample occurred within the first 5 min of the lipolysis study, while a similar degree of lipid digestion in the unformulated MCT lipid sample was reached only after 60 min (Figure 1A). The reason for the faster

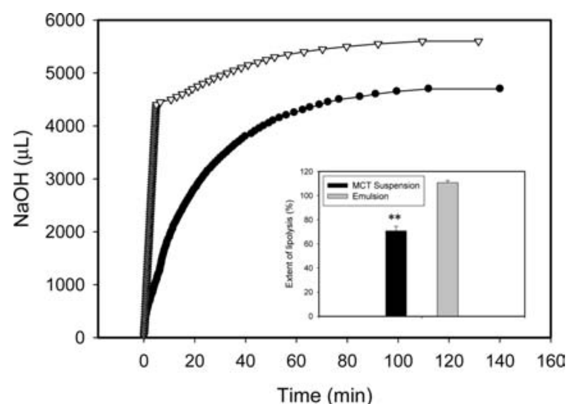

Figure 1. In vitro lipolysis digestion kinetic curve of the blank emulsion (▽) and blank MCT oil (●). Inset: extent of lipolysis at 30 min for the blank emulsion and blank MCT oil. The emulsion preparation is described in section 2.2.

digestion rate observed in the emulsion sample was due to the larger surface area at the lipid−water interface for contact of lipases that are only soluble in an aqueous environment. Since this lipolysis study intends to account for the amount of time required for 100% lipid digestion, the extents of lipolysis for emulsion and unformulated oil samples were compared at the 30 min time point. The extent of lipolysis, defined as the percentage of lipid digested, exhibited a great difference between the emulsion (>100%) and crude MCT (64.5%) samples (Figure 1B). Since digested lipids are constantly adapted into mixed micelles during lipolysis, any nearby lipophilic compounds may as well be incorporated into the micelle available for intestinal absorption. Since intestinal activity is a dynamic process of digestion, absorption, and excretion, lipophilic compounds will have a longer intestinal retention time and, thus, a higher chance to be absorbed if they are rapidly incorporated into the mixed micelles and become soluble in the intestinal lumen.

Effect of the Emulsion on the Total PMF Bioaccessibility. Oral bioavailability is the integration of the compound being bioaccessible to intestinal absorption, the amount that is actually transported across the intestinal lining, and the concentration that remains unchanged by the system's metabolism. Accordingly, increasing bioaccessibility will contribute to the overall enhancement of bioavailability. To be bioaccessible for intestinal uptake, bioactive compounds must be solubilized in the intestinal lumen, which is the medium for active and passive transport. Apart from compounds that naturally have good solubility in the aqueous intestinal lumen,

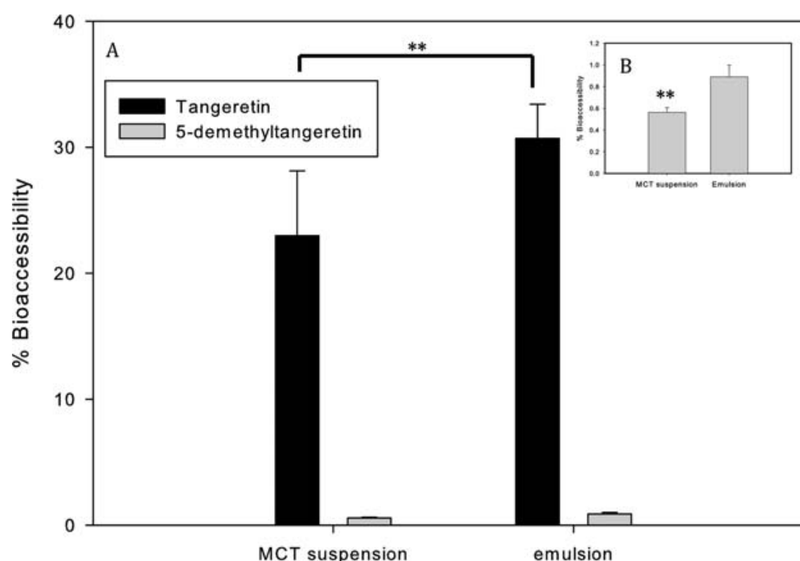

Figure 2. (A) Bioaccessibilities (%) of total PMFs in the MCT oil suspension and emulsion system containing tangeretin or 5-demethyltangeretin. (B) Enlarged picture of total PMFs in the 5-demethyltangeretin MCT suspension and emulsion. Total PMFs is defined as the sum of tangeretin and 5-demethyltangeretin present in the digested lipolysis solutions. The emulsion preparation is described in section 2.2.

Table 1. Solubilized Tangeretin and 5-Demethyltangeretin Concentrations in Digested Lipolysis Solutions

sample digested	tangeretin (mg/mL)	5-demethyltangeretin (mg/mL)	total PMFs (mg/mL)	av NaOH consumption (mL)
tangeretin MCT suspension	1.75 ± 0.39		1.75 ± 0.39	4.67 ± 0.10
tangeretin emulsion	2.27 ± 0.20		2.27 ± 0.20	5.57 ± 0.10
5-demethyltangeretin MCT suspension	$(3.60 \pm 0.33) \times 10^{-3}$	$(4.38 \pm 0.34) \times 10^{-2}$	$(4.74 \pm 0.35) \times 10^{-2}$	4.62 ± 0.41
5-demethyltangeretin emulsion	$(5.80 \pm 0.39) \times 10^{-2}$	$(4.52 \pm 0.86) \times 10^{-2}$	$(10.30 \pm 1.24) \times 10^{-2}$	5.52 ± 0.21

lipophilic compounds become aqueous soluble and bioaccessible after inclusion into mixed micelles. During the in vitro lipolysis study, the lipid is simultaneously digested to form mixed micelles, which imitates the component of the intestinal lumen where the lipophilic compound may be corporately absorbed. The percentage of PMFs from the original emulsion or MCT suspension that becomes bioaccessible was determined after in vitro lipolysis digestion. As total PMFs is defined as the sum of all PMF compounds present in the lipolysis solution, the total PMF bioaccessibility (%) improved for both tangeretin and 5-demethyltangeretin emulsions compared to MCT suspensions (Figure 2). In comparison to the MCT suspension, the tangeretin emulsion increased the total PMF bioaccessibility from 23.0% to 30.7% and the amount of total solubilized PMF from 1.75 ± 0.39 to 2.27 ± 0.20 mg (Table 1). As 5-demethyltangeretin possesses a significant amount of lipophilic structure, the aqueous solubility of this compound is naturally very low and can be improved only slightly by an emulsion delivery vehicle. The bioaccessibility of total PMFs in 5-demethyltangeretin samples increased from 0.6% in the MCT suspension to approximately 0.9% in the emulsion, while the total PMF amount rose from $(4.74 \pm 0.35) \times 10^{-2}$ to $(10.30 \pm 0.12) \times 10^{-2}$ mg (Table 1). The result from the bioaccessibility (%) evaluation was consistent with that previously reported, i.e., that faster lipid digestion kinetics will result in higher bioaccessibility of lipophilic compounds.[16,26]

Effect of the Lecithin-Based Emulsion on 5-Demethyltangeretin Transformations. HPLC standard curves for PMF compounds were prepared using intact tangeretin or 5-demethyltangeretin compounds with average elution times of 10.7 ± 0.1 and 15.3 ± 0.1 min, respectively. Both PMF compounds were of >98% purity (Figure S1A,B, Supporting Information) when intact compounds were sampled by the HPLC system. Furthermore, the undigested emulsion-processed samples were also examined by the HPLC system, and no changes in compound purity or composition were found. However, changes in the PMF composition were observed in samples that contained 5-demethyltangeretin after lipolysis digestion, while the PMFs present in lipolysis solutions after the digestion of tangeretin samples were exclusively tangeretin (Figure S1C,D, Supporting Information). The transformation of 5-demethyltangeretin into its permethoxylated counterpart (Figure S1E,F, Supporting Information) was unprecedented and has not been reported in any previous literature. To further confirm the presence of tangeretin in lipolysis-digested 5-demethyltangeretin samples, a total of six replicates were conducted with both 5-demethyltangeretin emulsions and MCT suspensions on two separate sample batches to avoid any inter- or intrasample variation. Moreover, the digested 5-demethyltangeretin solutions were also subjected to LC−MS analysis, and the chemical structures of both tangeretin and 5-demethyltangeretin were confirmed by comparison with standard compounds.

As discussed above, the total PMF bioaccessibilities were convincingly augmented by incorporation into an emulsion-based delivery system. However, when breaking down the compositional compounds of PMF present in 5-demethyltangeretin lipolysis samples, the amount of 5-demethyltangeretin essentially decreased from $(4.38 \pm 0.36) \times 10^{-2}$ mg in MCT suspension samples to $(4.52 \pm 0.86) \times 10^{-2}$ mg in emulsion samples (Table 1). Namely, the percentage of 5-demethyltangeretin in total suspended PMFs decreased from 93.0% to 40.6% when digested in the form of emulsions (Figure 3). Since

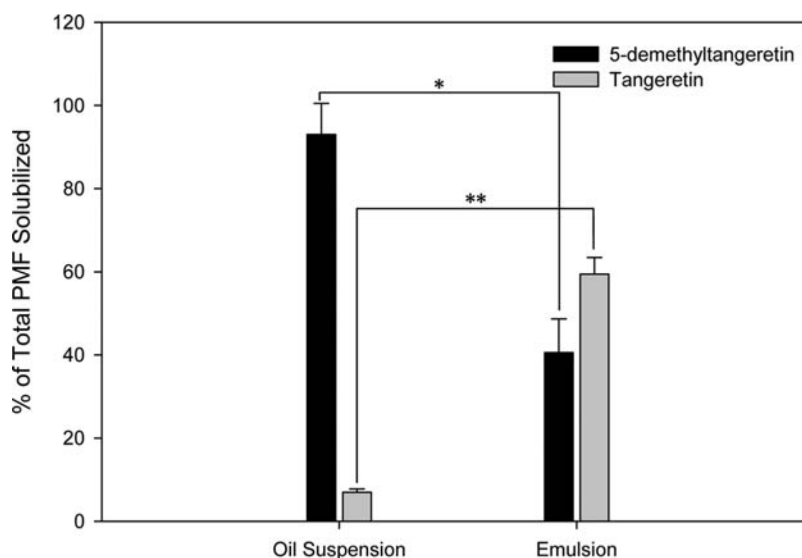

Figure 3. Percentage of tangeretin and 5-demethyltangeretin relative to the total PMFs presented in lipolysis-digested 5-demethyltangeretin MCT suspensions and emulsions (*, $p < 0.01$; **, $p < 0.001$).

the presence of tangeretin in undigested 5-demethyltangeretin MCT suspensions and emulsion samples was not observed, the appearance of tangeretin in digested samples could be conclusively attributed to transformation during the digestion processes. Since the mechanism underlying the transformation is not yet known, one proposed mechanism of such a transformation was suggested as in Figure 4. As the process

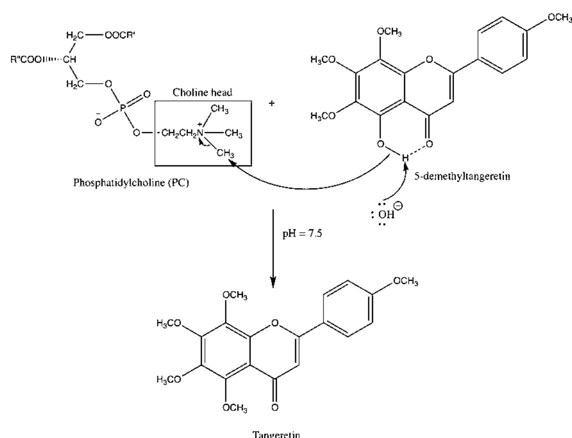

Figure 4. Proposed mechanism by which 5-demethyltangeretin was transformed to tangeretin during in vitro lipolysis study.

of lipolysis is executed in a basic environment (pH 7.5), the constant shock of a free hydroxyl group (OH^-) from the NaOH titration caused the dehydrogenation of the phenolic group on the 5-demethyltangeretin. The negative charge on the resulting oxygen (O^-) at the 5-position then quickly attacked the labile methyl groups ($-CH_3^+$) provided by the choline head of lecithin.

Lecithin, phophatidylcholine, is naturally present in various places within the biological system, such as the digestive track, cell membrane, organs, etc. Due to the inclusion of a choline headgroup as part of its structure, lecithin is commonly regarded as a good dietary source of choline. Choline, as a structural labile methyl donor, is critical for cellular signaling, normal neurofunctions, hormone secretion, protein synthesis, and DNA methylations.[38−45] Therefore, lecithin, as a simple derivative of choline, is a potential reservoir of labile groups. When lecithin is digested as part of an emulsion system, the release of a labile methyl group causes interaction with any compounds that could hypothetically be a methyl group receiver. In the lipolysis experiment, the concentration of labile methyl groups was much higher in samples containing the lecithin-based emulsion than those containing the MCT oil suspension. While tangeretin was not affected by the presence of labile methyl groups, the phenolic group of 5-demethyltangeretin was much more reactive to methylation under a basic environment as proposed in the mechanism. The difference in tangeretin, in terms of the percentage of total PMFs, present in the digested 5-demethyltangeretin solutions was significantly ($p < 0.001$) different between MCT suspensions and emulsions. Since the chemical reaction is dependent on the reactant concentration, a higher concentration of labile methyl groups in the lipolysis solution may cause methyl substitution and proceed at a faster reaction rate. The transformation of 5-demethyltangeretin to tangeretin was also confirmed by a Caco-2 colon carcinoma cell line transport study, which showed a significantly increased tangeretin content in the HPLC chromatogram after 20 min of permeation of 5-demethyltangeretin across the Caco-2 monolayer to the receiver chamber, as shown in Figure S2, Supporting Information.

According to the findings of the lipolysis experiment, one can conclude that the emulsion-based delivery system is an effective means to enhance digestion kinetics, overall PMF bioaccessibility, and, thus, bioavailability. Still, the selection of compositional material may need special consideration to account for possible interaction with the target compound. In our lipolysis experiment, the lecithin-based emulsion system was effective for augmenting the solubilized tangeretin concentration compared to the MCT suspension. However, the sample emulsion formulation did not work well when 5-demethyltangeretin was incorporated. Due to potential compound interaction with labile methyl groups, transformation of 5-demethyltangeretin was observed and resulted in the reduction of solubilized 5-demethyltangeretin in the final lipolysis solution.

Effect of 5-Demethyltangeretin Transformation on the in Vitro Anti-Cancer-Cell-Proliferation Activity. Although the chemical structures of tangeretin and 5-demethyltangeretin differ only in the substituted functional group at the 5-position, their physical properties and bioactivities deviate significantly. Whereas tangeretin was proven to inhibit cancer cell proliferation from G_0/G_1 cell cycle arrest,[46] 5-demethyltangeretin was found to exhibit higher potency in anti-cancer-cell-proliferation activity through G_2/M arrest while inducing cell apoptosis.[10] To investigate the effect of the emulsion formulation on the in vitro anti-cancer-cell-proliferation activities of the two PMF compounds, HepG2 cells were treated with serum complete medium containing PMFs (tangeretin or 5-demethyltangeretin) dissolved in DMSO or dispersed as an emulsion. The potency of the anti-cancer-cell-proliferation activity was evaluated on the basis of the relative cell viability of each treated cell to that of the untreated control. The result from the MTT study was consistent with previously reported literature that DMSO-dissolved tangeretin exhibits lower anti-cancer-cell-proliferation potency than 5-demethyltangeretin at all concentrations. However, the emulsion delivery system utilized in this study showed significant improvement ($p < 0.001$) of the tangeretin activities, with the largest difference of 26.7% at 25 μM (Table 2). With the impressive improvement seen in the case of tangeretin, the emulsion delivery system did not produce similar results when combined with 5-demethyltangeretin. The 5-demethyltangeretin emulsion treated cell groups exhibited a statistically significant higher cell viability than groups treated with DMSO-dissolved 5-demethyltangeretin (Table 2). The lower bioactivity of 5-demethyltangeretin emulsion treated groups may be explained by the findings from the lipolysis study. As discussed in previous sections, the excessive transformation of 5-demethyltangeretin to tangeretin in the presence of a labile methyl donor (lecithin in this case) may be the major reason that the average bioefficacy of the emulsion group was lowered since tangeretin has a lower anti-cancer-cell-proliferation potency than 5-demethyltangeretin. Even though the mechanism underlying the transformation and effects on bioactivities has not yet been confirmed, the finding of such transformation allows us to realize that the effectiveness of a bioactive compound on the inhibition of cancer cell growth may be changed when other biological compounds present.

Table 2. In Vitro Anticancer Activities of PMFs on the HepG2 Cell Line Expressed as Relative Cell Viabilities and IC_{50}[a]

	tangeretin		5-demethyltangeretin	
concn (μM)	DMSO viability (%)	emulsion viability (%)	DMSO viability (%)	emulsion viability (%)
1.56	88.9 ± 15.0	87.2 ± 5.6	65.7 ± 17.5	85.6 ± 14.2
3.125	91.2 ± 11.7	87.1 ± 11.6	48.4 ± 7.8	69.6 ± 21.1
6.25	88.9 ± 9.8	81.0 ± 5.3	47.1 ± 6.7	60.2 ± 21.6[b]
12.5	85.9 ± 7.2	70.1 ± 7.4[b]	48.2 ± 5.4	55.4 ± 17.5[b]
25	78.6 ± 12.0	51.9 ± 11.8[b]	48.8 ± 8.4	51.5 ± 13.4[c]

[a]Data are presented as the mean ± standard deviation. $n = 6$ in each of three separate replications. [b]$p < 0.001$ [c]$p < 0.01$.

In summary, a lipid-based delivery system is regarded as an efficient solution to enhance the bioavailability of lipophilic compounds. In fact, many investigators have formulated their delivery vehicle on the basis of the hypothesis that improved solubility will simultaneously increase the absorption of such compounds. The hypothesis that an emulsion is indeed very effective to increase the concentrations of encapsulated compounds in system circulation has been proven valid in many pharmacokinetic studies. However, according to our results, development of an emulsion delivery vehicle may fail if interactions between formulation materials and the target compound are undesirable. In our study, we examined the effectiveness of lecithin-based emulsions to enhance the digestion kinetics, bioaccessibilities, and bioactivities of two PMF compounds, tangeretin and 5-demethyltangeretin. Emulsions promoted faster lipid digestion kinetics, which result in a higher total solubilized PMF concentration. However, due to the presence of labile methyl donors (choline from lecithin), 5-demethyltangeretin was transformed into its permethoxylated counterpart, tangeretin, thus lowering the solubilized 5-demethyltangeretin concentrations. The anti-cancer-cell-proliferation activities examined using MTT assay again address the decrease in compound bioactivities when affected by labile methyl donors. As a result, the potential interaction between compositional materials and the target compound may be worthy of careful consideration. The finding from our investigation may serve as a reference for future development of delivery vehicles for many other compounds that may sustain similar chemical properties. Since lecithin is a naturally occurring compound in the digestion track, the in vivo transformation of 5-demethyltangeretin or other potential methyl receivers may also occur and requires further investigation. The consequent result from such transformations in a biological system may serve as a link to elucidate the mechanism that underlies important anticancer efficacy of such compounds.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1 showing the HPLC chromatograms of the (A) intact tangeretin standard, (B) intact 5-demethyltangeretin standard, (C) lipolysis-digested tangeretin MCT suspension sample, (D) lipolysis-digested tangeretin emulsion sample, (E) lipolysis-digested 5-demethyltangeretin MCT suspension sample, and (F) lipolysis-digested 5-demethyltangeretin emulsion sample and Figure S2 showing the HPLC analysis of the Caco-2 colon carcinoma cell line transport study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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