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Identification of xanthomicrol as a major colonic metabolite of 5-demethyltangeretin in mice and their anti-inflammatory effects in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages

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Abbreviations: 5DT, 5-demethyltengeretin; XAN, xanthomicrol; PMF, polymethoxyflavone; LPS, lipopolysaccharide; NO, nitric oxide; PGE_2, prostaglandin E_2; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; interleukin-1β (IL-1β); HO-1, heme oxygenase-1;

Abstract

Scope: 5-demethyltengeretin (5DT) is a citrus flavonoid with various potential health benefits. To provide physiologically relevant information on the anti-inflammatory properties of 5DT, we identified the major metabolite of 5DT in the mouse colon and established its anti-inflammatory effects in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages.

Methods and results: CD-1 mice were fed with a 5DT-containing diet for four weeks, and colonic mucosa samples were collected and subjected to LC-MS analysis. Xanthomicrol (XAN) was identified as the major metabolite of 5DT in the mouse colon. More importantly, the colonic level of XAN was about 3.1-fold higher than that of 5DT. The anti-inflammatory effects of 5DT and XAN were determined in LPS-stimulated macrophages. XAN produced significant inhibitory effects on the production of nitric oxide and PGE_2. Western blotting and real-time PCR analyses demonstrated that XAN greatly decreased the protein and mRNA levels of iNOS as well as the protein level of COX-2. Furthermore, XAN also reduced the production of pro-inflammatory cytokine IL-1β and induced the expression of anti-oxidative enzyme HO-1.

Conclusion: Our results demonstrated that XAN is a major metabolite of 5DT in the colon of mice fed with 5DT, and XAN may play important roles in the anti-inflammatory effects elicited by orally administered 5DT.

Keywords: 5-hydroxy-6, 7, 8, 4'-tetramethoxyflavone, 5-demethyltangeretin, polymethoxyflavones, xanthomicrol, metabolite

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. Naturally, PMFs are found mainly in the *Citrus* genus, particularly in the peel of sweet oranges (*Citrus sinensis*) and mandarin oranges (*Citrus reticulate*) [1]. PMFs have been of particular research interest due to their broad spectrum of documented biological activities including anti-inflammation [2, 3], anti-carcinogenic [4-6], and anti-atherogenic properties [7]. The most studied PMFs are permethoxylated PMFs, such as nobiletin and tangeretin. Recently, a unique class of hydroxylated PMFs has been isolated from aged orange peel extracts, and they can be formed by auto-hydrolysis of their permethoxylated counterparts during long-term storage [1, 8, 9]. Accumulating evidence has shown that hydroxylated PMFs possess potent anticancer effects against various cancer cells such as human lung [6, 10], colon [4, 5, 11, 12], and breast [13] cancer cells. Animal studies also confirmed anticancer effects of hydroxylated PMFs [3, 14, 15].

It is well recognized that there are intriguing links between inflammation and the initiation and progress of chronic diseases such as colon cancers. Inflammation can be oncogenic in the colon by various mechanisms including induction of genomic instability, promoting angiogenesis, altering the genomic epigenetic status, and enhancing cell proliferation [16]. This study aimed to provide physiologically relevant information on the anti-inflammatory potential of 5-demethyltengeretin (5-hydroxy-6, 7, 8, 4'-tetramethoxyflavone, 5DT) in the colon. 5DT is one of the major hydroxylated PMFs presented in citrus fruits and their byproducts such as citrus oil and molasses [1, 17]. It also has been reported that 5DT had more potent biological activities than its permetylated counterpart, tangeretin [6]. However, as one important PMF, 5DT has not been well studied and no detailed information on its anti-inflammatory effects is available.

Biotransformation of dietary PMFs plays an important role in their biological activities because they can be transformed to different metabolites that could possess distinct bioactivities [4, 18-21]. For example, biotransformation of nobiletin and tangeretin, two major PMFs found in citrus fruits, has been determined in various experimental models [22, 23]. More than 10 different demethylated metabolites of nobiletin have been identified [22]. We recently reported that total colonic concentration of three major demethylated metabolites of nobiletin was about 20-fold higher than that of nobiletin itself [4]. More importantly, nobiletin metabolites exerted much stronger anti-inflammatory and anti-cancer activities than did nobiletin [4, 5, 18, 19]. Therefore, biotransformation plays a critical role in health benefits of orally administered PMFs such as nobiletin observed in animal models. In this study, in order to better understand the anti-inflammatory effects of 5DT as a dietary component, we aimed to identify the major colonic metabolite of orally ingested 5DT in mice, and determine its anti-inflammatory effects in comparison with 5DT in lipopolysaccharide (LPS)-treated RAW 264.7 macrophages.

2. Materials and methods

2.1 Identification of the major metabolite of 5DT in the mouse colon

As reported in previous studies, 5DT and XAN was synthesized by acidolysis from tangeretin and 4'-demethyltangeretin, respectively, with purity greater than 99% (Chemical structures of 5DT and XAN are illustrated in Figure 1A) [8, 11]. Tangeretin was isolated from orange peels and 4'-demethyltangeretin was chemically synthesized [1, 11]. The study complied with all institutional and national guidelines (Guide for the Care and Use of Laboratory Animals 8th Edition, by the Institute of Laboratory Animal Research, National Research Council). The

protocol for the animal experiment was approved by the Institutional Animal Care and Use Committee of the University of Massachusetts (#2011-0066). Ten male CD-1 mice (5 week of age) were obtained from Charles River Laboratories (Wilmington, MA, USA). Upon arrival, the mice were kept in an air-conditioned room with the temperature of 23°C, humidity of 65-70%, 12-h light/night cycle and free access to water and AIN93G diet. After one week of acclimation, mice were randomly divided into two groups (5 mice per group). The mice in the control group were fed AIN93G diet, while the 5DT-treated group was fed with AIN93G diet containing 0.1% (w/w) 5DT. Both groups were allowed to eat and drink *ad libitum* during the entire experiment period. After four weeks of treatment, all mice were sacrificed by CO_2 asphyxiation. Colonic mucosa were collected and stored at -80 °C for LC-MS analysis. Aliquots of colonic mucosa samples (100 mg) were homogenized with 50% methanol (1 ml) using a Bead Ruptor homogenizer (Omni International, Kennesaw, GA, USA). Methanol was the removed by vacuum evaporation, and PBS was used to resuspend the pellet. The homogenates were then extracted with equal volume (500 µl) of ethyl acetate for three times. After centrifugation, supernatants were pooled and dried under vacuum. The residue was dissolved in 50% methanol and subjected to LC-MS analysis as we previously reported [24]. The identities and concentrations of 5DT and XAN in the colonic mucosa were determined by comparing the LC-MS profiles of the colonic samples with those of chemically synthesized standards and their corresponding standard curves.

2.2 Cell viability and nitric oxide assay

RAW 264.7 cells were seeded in 96-well plates. After 24 h, cells were treated with serial concentrations of test compounds in 200µL of serum complete media. Dimethyl sulfoxide (DMSO) was used as the vehicle to deliver test compounds to the culture media, and the final concentration of DMSO in all experiments was 0.1% v/v in cell culture media. After 24 h

treatments, cells were subject to MTT assay[10, 25]. Media were replaced by 100µL of fresh media containing 0.1 mg/mL of MTT (Sigma-Aldrich). After 2 h incubation at 37 °C with 5% CO_2 and 95% air, MTT-containing media were removed and the reduced formazan dye was solubilized by adding 100µL of DMSO to each well. After gentle mixing, the absorbance was monitored at 570nm using a plate reader (Elx800TM absorbance microplate reader, BioTek Instrument, VT, USA). The nitrite concentration in the culture media was measured as an indicator of nitric oxide (NO) production by the Griess reaction [25]. The culture media were mixed with an equal volume of Griess reagent A and B (A: 1% sulfanilamide in 5% phosphoric acid, and B: 0.1% naphthylethylenediamine dihydrochloride in water). Absorbance at 540nm was measured by a plate reader, and concentrations of nitrite were calculated according to a standard curve constructed with sodium nitrite as a standard.

2.3 ELISA for prostaglandin E_2 (PGE_2) and interleukin-1 (IL-1)

RAW 264.7 cells were seeded in 6-well plates. After 24 h, cells were treated with 1µg/mL LPS alone or with serial concentrations of test compounds in 2 mL of serum complete media. After another 24 h incubation, the culture media were collected and analyzed for PGE_2 (Cayman Chemical Company, Ann Arbor, MI, USA) and IL-1 (R&D Systems, Minneapolis, MN, USA) levels by ELISA kits, according to the manufacturer's instructions.

2.4 Immunoblot Analysis

Whole cell lysates were prepared according to the method we reported previously [25]. Equal amount of proteins (50µg) were resolved over 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, proteins of interest were probed using different antibodies at manufacturer's recommended concentrations, and then

visualized using enhanced chemiluminescence (Boston Bioproducts, Ashland, MA). Antibodies for iNOS, COX-2, and HO-1 were purchased from Cell Signaling Technology (Beverly, CA, USA). Anti-β actin antibody was from Sigma-Aldrich (St. Louis, MO).

2.5 qRT-PCR Analysis

Total RNA was isolated by RNeasy Plus Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). RNA concentrations were determined using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). From each sample, total RNA was converted to single-stranded cDNA which was then amplified by Brilliant II SYBR Green QRT-PCR Master Mix Kit, 1-Step (Agilent Technologies, Santa Clara, CA, USA) to quantify the gene expression of iNOS, COX-2, HO-1, IL-1β, and GAPDH (an internal standard) using Mx3000P QPCR System (Stratagene, La Jolla, CA, USA). The primer pairs were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa, USA) and the sequences were as followed: iNOS, forward primer 5'-TCC TAC ACC ACA CCA AAC -3', reverse primer 5'-CTC CAA TCT CTG CCT ATC C -3'; COX-2, forward primer 5'-CCT CTG CGA TGC TCT TCC -3', reverse primer 5'-TCA CAC TTA TAC TGG TCA AAT CC -3'; HO-1, forward primer 5'-AAG AGG CTA AGA CCG CCT TC -3', reverse primer 5'-GTC GTC GTC AGT CAA CAT GG -3'; IL-1β, forward primer 5'-GAG TGT GGA TCC CAA GCA AT -3', reverse primer 5'-CTC AGT GCA GGC TAT GGA CCA -3'; GAPDH, forward primer 5'-TCA ACG GCA CAG TCA AGG -3', reverse primer 5'-ACT CCA CGA CAT ACT CAG C -3'. Minimum of three independent experiments (each with triplicates) were carried out. The copy number of each transcript was calculated relative to the GADPH copy number using the $2^{-\Delta\Delta Ct}$ Method [25].

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. Analysis of variance (ANOVA) model was used for the comparison of differences among three or more groups.

3. Results and discussion

3.1 XAN is the major metabolite of 5DT in the colon after oral administration of 5DT in mice

It is known that many dietary flavonoids undergo extensive biotransformation to produce different metabolites due to the first-pass metabolism in the gastrointestinal tract and liver. These metabolites have attracted increasing attention because they may possess unique and potent biological activities. The biotransformation of 5DT has not been reported in the literature. In this study, we aimed to elucidate the metabolism of 5DT *in vivo*. 5DT was orally administrated to the mice as part of regular diet (0.1% w/w) for four weeks. Body weight and food intake was monitored twice a week as indicators of potential toxicity of dietary 5DT. Compared to the control mice, there was no difference in body weight, liver weight or spleen weight between the 5DT-treated group and control group, and no noticeable behavioral or appearance difference was observed either, suggesting no apparent adverse effects caused by dietary feeding of 5DT to the mice. In order to identify the metabolites of 5DT in the colon, we analyzed the colonic mucosa samples from both groups using LC-MS. First, we identified and quantified the colonic levels of 5DT (SIM: *m/z*359 $[M-H]^+$, retention time of 22.7 min, Figure 1B) using synthesized 5DT as

standard. We found that the colonic level of 5DT was 1.76 ± 0.13 nmol/g of tissue in 5DT-fed mice (Figure 1D).

Moreover, we successfully observed multiple potential metabolites in the colonic mucosa of 5DT-fed mice. Among these metabolites, LC-MS analysis indicated that one metabolite (SIM: *m/z* 345 $[M-H]^+$, retention time of 19.2 min, Figure 1C) showed the highest abundance. It has been reported that PMFs such as nobiletin and 5-demethylnobiletin were transformed to demethylated metabolites in mice. The demethylation primarily occurred at 3′ and 4′-positions on B ring of the PMFs[4, 11]. There is only one methoxyl group at 4′-positioin of 5DT (Figure 1A). Therefore, we hypothesized that the biotransformation of 5DT is similar to that of nobiletin and 5-demethylnobiletin, thus a potential metabolite of 5DT is 4′-demethylated 5DT (XAN, Figure 1A). To test this hypothesis, we chemically synthesized XAN by acidolysis of 4′-demethyltangeretin using a method we reported previously [11], and the purity of XAN was greater than 99%. Using synthesized XAN standard, we confirmed that the major metabolite (SIM: *m/z* 345 $[M-H]^+$, retention time of 19.2 min, Figure 1C) was XAN. We further determined the level of XAN in the colonic mucosa of 5DT-fed mice. As shown in Figure 1D, the concentration of XAN in mouse colonic mucosa was 5.46±1.37 nmol/g of tissue. It is noteworthy that the level of XAN was about 3.1-fold higher than that of 5DT in the colonic mucosa. This is a very important finding because it suggested that due to its high abundance, XAN might play a critical role in eliciting the biological activities of orally administered 5DT, such as anti-inflammatory effects in the colon against colitis and colon carcinogenesis. In the following sections, we determined the anti-inflammatory potential of XAN and 5DT.

3.2 XAN inhibited NO and PGE_2 production

Upon the stimulation by pro-inflammatory agents, epithelial cells express iNOS, and increased iNOS expression was associated with various malignant tumors in human, including brain, breast, colorectal, lung, prostate, pancreatic carcinoma, and melanoma [26-32]. NO is the major product of enzymatic activity of iNOS, and it has been implicated in epithelia carcinogenesis [33-35]. Overproduction of NO can cause mutagenesis, damage DNA structure, and promote the formation of carcinogenic *N*-nitrosoamines [36-38]. To evaluate the anti-inflammatory effects of 5DT and its major metabolite XAN, we determined their inhibitory effects on the LPS-induced NO production in RAW264.7 macrophages. Using MTT assay[10], we first established non-toxic dose ranges of 5DT and XAN in macrophages. As shown in figure 2A, 5DT and XAN did not cause significant inhibition (< 10%) on the viability of macrophages when their concentrations were lower than 2.0 and 30 μM, respectively. It is noteworthy that XAN was much better tolerated by the macrophages than 5DT. Using the non-toxic dose ranges established, we further determined the inhibitory effects of the two compounds on LPS-induced NO production in macrophages. This was to ensure that the inhibitory effects observed were not due to disruption of normal cellular functions. As shown in figure 2B, within the non-toxic dose ranges, 5DT only caused marginal inhibition (<15%) on NO production, whereas, XAN showed much stronger inhibitory effects (49-73%) although the concentrations used were higher than those of 5DT. Besides iNOS, cyclooxygenase-2 (COX-2) is another important inducible enzyme in the pro-inflammatory pathways. Increased COX-2activity leads to the production of a series of prostaglandins including PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 or thromboxane A2 by tissue-specific isomerases [39]. Among these prostaglandins, PGE_2 has been closely associated with carcinogenesis by promoting cell survival, angiogenesis, and metastasis [40-42]. As shown in Figure 2C, LPS treatment resulted in a dramatic increase of PGE_2 level, and this increase was

slightly inhibited by XAN at 10 μM (7% inhibition). However, XAN or 5DT at 2μM showed no effect on LPS-induced PGE_2 production. Overall, our results demonstrated for the first time that XAN, the major metabolite of 5DT, effectively inhibited LPS-induced inflammation in macrophages. Meanwhile, 5DT at non-toxic dose ranges, showed very limited anti-inflammatory efficacy.

The aforementioned findings were physiologically relevant to the *in vivo* situation because that 2 μM of 5DT and 2 and 10 μM of XAN were achievable in mouse colon after oral consumption of 5DT. If we consider that 1 gram of tissue is about 1 mL in volume, the concentrations of 5DT and XAN in the colonic mucosa were 1.76 and 5.46 μM, respectively. In addition, this level of XAN was achieved by *ad libitum* intake of AIN93G diet uniformly containing 5DT throughout the feeding period of the mice (overnight), and it thus reflected sustained level (not the peak level after an acute exposure) of XAN in the colon mucosa after long-term chronic dietary exposure to 5DT. It was reasonable to expect that much higher levels of XAN (10 μM) was achievable by one time single dose oral administration of 5DT, such as via taking dietary supplement in capsules, or by consuming 5DT at higher oral doses than 0.1% (w/w).

3.3 XAN inhibited iNOS and COX-2 expression

To further determine the mechanisms by which XAN inhibited LPS-induced inflammation, the effects of 5DT and XAN on the protein expression levels of iNOS and COX-2 were assayed. LPS-stimulated RAW 264.7 macrophages were treated with a series of concentrations of 5DT or XAN for 24 h, and then the protein levels of iNOS and COX-2 were determined by Western blotting. As shown in figure 3A, XAN significantly decreased iNOS

protein levels by 74% and 92% at 2 and 10 μM, respectively, while 5DT only caused a marginal decrease in the protein levels of iNOS at 2μM in comparison to the LPS-treated positive control. These results were consistent with the extent to which 5DT and XAN inhibited the LPS-induced production of NO (Figure 1B). Furthermore, XAN at 10μM significantly suppressed COX-2 protein expression by 68%, compared to control cells. In contrast, 5DT did not decrease the protein level of COX-2 at 2 μM. Despite the potent suppression of COX-2 signaling produced by XAN at 10μM, it only led to a moderate inhibition on PGE_2 production (Figure 2C). Since both COX-1 and COX-2 are involved in the LPS-induced synthesis of PGE_2 in macrophages and it has been found that COX-2 predominantly catalyzes PGE_2 release in the first 8 h, whereas COX-1 couples the newly expressed microsomal prostaglandin E_2 synthase-1 (mPGES-1) that contributes to the PGE_2 release [43]. And COX-1 activity mostly remains unchanged after LPS stimulation. Thus in this study, COX-1 derived PGE_2 might constitute a great proportion of total level of PGE_2, which was not significantly altered by XAN treatment. Another possible explanation could be that down-regulated protein expression of COX-2 by XAN treatment may result in reduced levels of other COX-2 derived prostaglandins besides PGE_2 [39].

Using qRT-PCR, we determined the effects of 5DT and XAN on the mRNA levels of iNOS and COX-2 after 24 hours of treatments. As shown in figure 3B and 3C, the LPS treatments significantly increased the mRNA levels of both iNOS and COX-2 in comparison to the negative control. 5DT at 2μM did not decrease mRNA levels of iNOS or COX-2, while XAN at 2 and 10 μM dose-dependently decreased the mRNA level of iNOS by 18% and 25%, respectively, compared to the LPS-treated positive control. However, same XAN treatments did not cause any decrease in the mRNA level of COX-2. Together, our results showed that XAN caused dose-dependent inhibition on the protein levels of both iNOS and COX-2, but XAN

treatment only decreased the mRNA level of iNOS not that of COX-2. It is likely that XAN decreased the protein level of iNOS mainly by inhibiting transcription of iNOS gene, whereas XAN might inhibit translation and/or promote degradation of COX-2 protein to cause the decrease of protein level of COX-2 in LPS-stimulated macrophages. Previous studies have shown that COX-2 protein can be degraded *via* two pathways, initiated by either post-translational *N*-glycosylation at Asn-594 or substrate-dependent suicide inactivation in LPS-stimulated RAW 264.7 cells [44, 45]. Other dietary bioactive compounds have also been found to disrupt the translation step of COX-2 mRNA in RAW 264.7 cells [2].

3.4 XAN inhibited IL-1β gene expression

Accumulating studies have demonstrated that pro-inflammatory cytokines such as IL-1β may promote the progression of various diseases including cancer [46]. Using ELISA, we determined the effects of 5DT and XAN on the LPS-induced production of IL-1β in RAW 264.7 macrophages. As shown in figure 4A, LPS treatment resulted in a significant production of IL-1β in the macrophages. XAN at 2 and 10 μM dose-dependently suppressed protein levels of IL-1β by 19% and 63%, respectively, compared to the LPS-treated positive control cells. However, 5DT at 2 μM did not inhibit LPS-induced production of IL-1β. Using qRT-PCR, we further determined the effects of 5DT and XAN on the mRNA levels of IL-1β after 24 hours of treatments. XAN at 2 and 10 μM showed a dose-dependent inhibition on the mRNA level of IL-1β by 18% and 42%, respectively (Figure 4B). In contrast, 5DT had no effects on the mRNA level of IL-1β in LPS-stimulated macrophages. Our results suggested that XAN suppressed the LPS-induced IL-1β production, and this suppression was at least partially due to the inhibition on the transcription of IL-1β gene by XAN. As a pro-inflammatory cytokine, IL-1β can increase transcription of both iNOS and COX-2 by activation of NF-κB [47, 48]. Therefore, the

suppression on IL-1β production may also contribute to the decrease of the mRNA levels of iNOS caused by XAN.

3.5 Induction of anti-oxidative enzyme HO-1 by XAN and 5DT

HO-1 is an important phase II antioxidant enzyme, and it has been reported to suppress inflammatory responses and provide protection against the pro-inflammatory effects of toxic agents [49-55]. Previously, we have reported that HO-1 is an important target of PMFs [5, 19], therefore we further examined the effects of 5DT and XAN on the gene expression level of HO-1 in LPS-treated macrophages. As shown in figure 5A, Western-blotting results demonstrated that 5DT at 2 μM increased protein levels of HO-1 by 2.1-fold compared to the LPS-treated cells. XAN at 10 μM caused an increase on the protein level of HO-1 by 1.9-fold, whereas XAN at 2 μM did not result in a significantly induction. We also determined the effects of 5DT and XAN on mRNA level of HO-1 in LPS-stimulated macrophages. The results from qRT-PCR demonstrated that 5DT at 2 μM increased the mRNA level of HO-1 by 58% in comparison with the LPS-treated control cells (Figure 5B). XAN at 10μM increased the mRNA level of HO-1 by 67%, while XAN at 2μM did not caused any significant change. These results showed that the effects of 5DT and XAN on the protein levels of HO-1 shared a similar trend with their effects on the mRNA levels of HO-1, suggesting that induction of transcription of HO-1 gene contributed to the increases on the protein level of HO-1 by both 5DT and XAN. Increased HO-1 levels may contribute to the anti-inflammatory effects by 5DT and XAN. It was reported that HO-1 can increase cellular anti-oxidant status by generating antioxidant such as bilirubin [56]. Bilirubin inhibits iNOS protein expression and suppresses NO production in LPS-stimulated RAW 264.7 macrophages, but showed no effect on LPS-stimulated COX-2 expression [57]. In addition, Carbon monoxide (CO), a major product of HO-1 activity, was shown to cause

downregulation of pro-inflammatory cytokine production through p38 mitogen-activated protein kinase (MAPK)-dependent pathways [58]. Moreover, CO was shown to inhibit iNOS enzymatic activity thus decreases NO production [59].

In summary, we have identified, for the first time, XAN as the major metabolite of 5DT in the mouse colon after oral administration of 5DT. The difference between the chemical structures of 5DT and XAN is that 5DT has a methoxyl group on the B ring, while XAN has a hydroxyl group at the same position. This structural difference results in very different biological activities of 5DT and XAN in LPS-stimulated RAW 264.7 macrophages. At physiologically achievable concentrations, XAN showed stronger anti-inflammatory effects in LPS-stimulated macrophages in comparison with those of 5DT, which was evidenced by lowered NO and PGE_2 production and decreased protein levels of iNOS and COX-2. These anti-inflammatory effects were associated with suppression of IL-1β gene expression and induction of phase II enzyme HO-1. To our knowledge, this is the first report to show the anti-inflammatory effects of XAN in LPS-treated RAW 264.7 macrophages. Since XAN is a major metabolite of 5DT *in vivo*, it is important to investigate the biological effects of XAN in order to obtain meaningful mechanistic information on the biological effects of orally ingested 5DT. Furthermore, it is also important to investigate the biological effects of 5DT and XAN in combination because they likely co-exist in the target tissues and organs *in vivo*.

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S.G., X.W. and H.X. conceived and designed experiments. S.G., X.W., J.Z., N.C., P.D., P.Q. and M.S. performed experiments. S.G., X.W. and H.X. analyzed the data and wrote the manuscript. All authors read and approved the final manuscript. The authors have declared no conflict of interest.

References

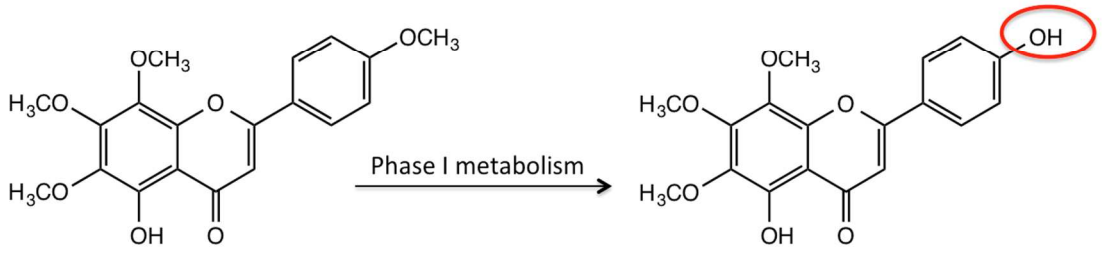
1. Li S, Lo CY, Ho CT: **Hydroxylated polymethoxyflavones and methylated flavonoids in sweet orange (Citrus sinensis) peel.** *J Agric Food Chem* 2006, **54:**4176-4185.
2. Murakami A, Shigemori T, Ohigashi H: **Zingiberaceous and citrus constituents, 1'-acetoxychavicol acetate, zerumbone, auraptene, and nobiletin, suppress lipopolysaccharide-induced cyclooxygenase-2 expression in RAW264.7 murine macrophages through different modes of action.** *J Nutr* 2005, **135:**2987S-2992S.
3. Lai CS, Li S, Chai CY, Lo CY, Ho CT, Wang YJ, Pan MH: **Inhibitory effect of citrus 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone on 12-O-tetradecanoylphorbol 13-acetate-induced skin inflammation and tumor promotion in mice.** *Carcinogenesis* 2007, **28:**2581-2588.
4. Wu X, Song M, Wang M, Zheng J, Gao Z, Xu F, Zhang G, Xiao H: **Chemopreventive effects of nobiletin and its colonic metabolites on colon carcinogenesis.** *Mol Nutr Food Res* 2015, **59:**2383-2394.
5. Wu X, Song M, Gao Z, Sun Y, Wang M, Li F, Zheng J, Xiao H: **Nobiletin and its colonic metabolites suppress colitis-associated colon carcinogenesis by down-regulating iNOS, inducing antioxidative enzymes and arresting cell cycle progression.** *J Nutr Biochem* 2017, **42:**17-25.
6. Charoensinphon N, Qiu P, Dong P, Zheng J, Ngauv P, Cao Y, Li S, Ho CT, Xiao H: **5-demethyltangeretin inhibits human nonsmall cell lung cancer cell growth by inducing G2/M cell cycle arrest and apoptosis.** *Mol Nutr Food Res* 2013, **57:**2103-2111.
7. Kurowska EM, Manthey JA: **Hypolipidemic effects and absorption of citrus polymethoxylated flavones in hamsters with diet-induced hypercholesterolemia.** *J Agric Food Chem* 2004, **52:**2879-2886.
8. Li S, Pan MH, Lai CS, Lo CY, Dushenkov S, Ho CT: **Isolation and syntheses of polymethoxyflavones and hydroxylated polymethoxyflavones as inhibitors of HL-60 cell lines.** *Bioorg Med Chem* 2007, **15:**3381-3389.
9. Li S, Lambros T, Wang Z, Goodnow R, Ho CT: **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.
10. Xiao H, Yang CS, Li S, Jin H, Ho CT, Patel T: **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.
11. Zheng J, Song M, Dong P, Qiu P, Guo S, Zhong Z, Li S, Ho CT, Xiao H: **Identification of novel bioactive metabolites of 5-demethylnobiletin in mice.** *Mol Nutr Food Res* 2013, **57:**1999-2007.
12. Qiu P, Dong P, Guan H, Li S, Ho CT, Pan MH, McClements DJ, Xiao H: **Inhibitory effects of 5-hydroxy polymethoxyflavones on colon cancer cells.** *Mol Nutr Food Res* 2010, **54 Suppl 2:**S244-252.
13. Sergeev IN, Ho CT, 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. Lai CS, Tsai ML, Cheng AC, Li S, Lo CY, Wang Y, Xiao H, Ho CT, Wang YJ, Pan MH: **Chemoprevention of colonic tumorigenesis by dietary hydroxylated polymethoxyflavones in azoxymethane-treated mice.** *Mol Nutr Food Res* 2011, **55:**278-290.
15. Ma N, Lai C-S, Chung C-H, Yang J-M, Hsu K-C, Chen C-Y, Chung T-S, Li S, Ho C-T, Pan M-H: **5-Demethyltangeretin is more potent than tangeretin in inhibiting dimethylbenz (a) anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin tumorigenesis.** *Journal of Functional Foods* 2014, **11:**528-537.

16. Schetter AJ, Heegaard NH, Harris CC: **Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways.** *Carcinogenesis* 2010, **31:**37-49.
17. Manthey JA, Grohmann K: **Phenols in citrus peel byproducts. Concentrations of hydroxycinnamates and polymethoxylated flavones in citrus peel molasses.** *J Agric Food Chem* 2001, **49:**3268-3273.
18. Wu X, Song M, Rakariyatham K, Zheng J, Guo S, Tang Z, Zhou S, Xiao H: **Anti-inflammatory effects of 4'-demethylnobiletin, a major metabolite of nobiletin.** *J Funct Foods* 2015, **19:**278-287.
19. Wu X, Song M, Rakariyatham K, Zheng J, Wang M, Xu F, Gao Z, Xiao H: **Inhibitory Effects of 4'-Demethylnobiletin, a Metabolite of Nobiletin, on 12-O-Tetradecanoylphorbol-13-acetate (TPA)-Induced Inflammation in Mouse Ears.** *J Agric Food Chem* 2015, **63:**10921-10927.
20. Song M, Charoensinphon N, Wu X, Zheng J, Gao Z, Xu F, Wang M, Xiao H: **Inhibitory Effects of Metabolites of 5-Demethylnobiletin on Human Nonsmall Cell Lung Cancer Cells.** *J Agric Food Chem* 2016, **64:**4943-4949.
21. Song M, Wu X, Charoensinphon N, Wang M, Zheng J, Gao Z, Xu F, Li Z, Li F, Zhou J, Xiao H: **Dietary 5-demethylnobiletin inhibits cigarette carcinogen NNK-induced lung tumorigenesis in mice.** *Food Funct* 2017, **8:**954-963.
22. Li SM, Wang H, Guo LM, Zhao H, Ho CT: **Chemistry and bioactivity of nobiletin and its metabolites.** *Journal of Functional Foods* 2014, **6:**2-10.
23. Nielsen SE, Breinholt V, Cornett C, Dragsted LO: **Biotransformation of the citrus flavone tangeretin in rats. Identification of metabolites with intact flavane nucleus.** *Food Chem Toxicol* 2000, **38:**739-746.
24. Sun Y, Wu X, Cai X, Song M, Zheng J, Pan C, Qiu P, Zhang L, Zhou S, Tang Z: **Identification of pinostilbene as a major colonic metabolite of pterostilbene and its inhibitory effects on colon cancer cells.** *Molecular Nutrition & Food Research* 2016.
25. Guo SS, Qiu PJ, Xu G, Wu X, Dong P, Yang GP, Zheng JK, McClements DJ, Xiao H: **Synergistic Anti-inflammatory Effects of Nobiletin and Sulforaphane in Lipopolysaccharide-Stimulated RAW 264.7 Cells.** *Journal of Agricultural and Food Chemistry* 2012, **60:**2157-2164.
26. Lagares-Garcia JA, Moore RA, Collier B, Heggere M, Diaz F, Qian F: **Nitric oxide synthase as a marker in colorectal carcinoma.** *Am Surg* 2001, **67:**709-713.
27. Thomsen LL, Miles DW, Happerfield L, Bobrow LG, Knowles RG, Moncada S: **Nitric oxide synthase activity in human breast cancer.** *Br J Cancer* 1995, **72:**41-44.
28. Cobbs CS, Brenman JE, Aldape KD, Bredt DS, Israel MA: **Expression of nitric oxide synthase in human central nervous system tumors.** *Cancer Res* 1995, **55:**727-730.
29. Masri FA, Comhair SA, Koeck T, Xu W, Janocha A, Ghosh S, Dweik RA, Golish J, Kinter M, Stuehr DJ, et al: **Abnormalities in nitric oxide and its derivatives in lung cancer.** *Am J Respir Crit Care Med* 2005, **172:**597-605.
30. Klotz T, Bloch W, Volberg C, Engelmann U, Addicks K: **Selective expression of inducible nitric oxide synthase in human prostate carcinoma.** *Cancer* 1998, **82:**1897-1903.
31. Hajri A, Metzger E, Vallat F, Coffy S, Flatter E, Evrard S, Marescaux J, Aprahamian M: **Role of nitric oxide in pancreatic tumour growth: in vivo and in vitro studies.** *Br J Cancer* 1998, **78:**841-849.
32. Ekmekcioglu S, Ellerhorst J, Smid CM, Prieto VG, Munsell M, Buzaid AC, Grimm EA: **Inducible nitric oxide synthase and nitrotyrosine in human metastatic melanoma tumors correlate with poor survival.** *Clin Cancer Res* 2000, **6:**4768-4775.
33. Ohshima H, Bartsch H: **Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis.** *Mutat Res* 1994, **305:**253-264.

34. Tsuji S, Kawano S, Tsujii M, Takei Y, Tanaka M, Sawaoka H, Nagano K, Fusamoto H, Kamada T: **Helicobacter pylori extract stimulates inflammatory nitric oxide production.** *Cancer Lett* 1996, **108:**195-200.
35. Xie K, Huang S, Dong Z, Juang SH, Wang Y, Fidler IJ: **Destruction of bystander cells by tumor cells transfected with inducible nitric oxide (NO) synthase gene.** *J Natl Cancer Inst* 1997, **89:**421-427.
36. Arroyo PL, Hatch-Pigott V, Mower HF, Cooney RV: **Mutagenicity of nitric oxide and its inhibition by antioxidants.** *Mutat Res* 1992, **281:**193-202.
37. Miwa M, Stuehr DJ, Marletta MA, Wishnok JS, Tannenbaum SR: **Nitrosation of amines by stimulated macrophages.** *Carcinogenesis* 1987, **8:**955-958.
38. Wink DA, Kasprzak KS, Maragos CM, Elespuru RK, Misra M, Dunams TM, Cebula TA, Koch WH, Andrews AW, Allen JS, et al.: **DNA deaminating ability and genotoxicity of nitric oxide and its progenitors.** *Science* 1991, **254:**1001-1003.
39. O'Banion MK: **Cyclooxygenase-2: molecular biology, pharmacology, and neurobiology.** *Crit Rev Neurobiol* 1999, **13:**45-82.
40. Tsujii M, Kawano S, DuBois RN: **Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential.** *Proc Natl Acad Sci U S A* 1997, **94:**3336-3340.
41. Sheng H, Shao J, Morrow JD, Beauchamp RD, DuBois RN: **Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells.** *Cancer Res* 1998, **58:**362-366.
42. Liu XH, Kirschenbaum A, Yao S, Stearns ME, Holland JF, Claffey K, Levine AC: **Upregulation of vascular endothelial growth factor by cobalt chloride-simulated hypoxia is mediated by persistent induction of cyclooxygenase-2 in a metastatic human prostate cancer cell line.** *Clin Exp Metastasis* 1999, **17:**687-694.
43. Bezugla Y, Kolada A, Kamionka S, Bernard B, Scheibe R, Dieter P: **COX-1 and COX-2 contribute differentially to the LPS-induced release of PGE2 and TxA2 in liver macrophages.** *Prostaglandins Other Lipid Mediat* 2006, **79:**93-100.
44. Mbonye UR, Yuan C, Harris CE, Sidhu RS, Song I, Arakawa T, Smith WL: **Two distinct pathways for cyclooxygenase-2 protein degradation.** *J Biol Chem* 2008, **283:**8611-8623.
45. Riese J, Hoff T, Nordhoff A, DeWitt DL, Resch K, Kaever V: **Transient expression of prostaglandin endoperoxide synthase-2 during mouse macrophage activation.** *J Leukoc Biol* 1994, **55:**476-482.
46. Lin WW, Karin M: **A cytokine-mediated link between innate immunity, inflammation, and cancer.** *J Clin Invest* 2007, **117:**1175-1183.
47. Hibbs JB, Jr.: **Synthesis of nitric oxide from L-arginine: a recently discovered pathway induced by cytokines with antitumour and antimicrobial activity.** *Res Immunol* 1991, **142:**565-569; discussion 596-568.
48. Dinarello CA: **The paradox of pro-inflammatory cytokines in cancer.** *Cancer Metastasis Rev* 2006, **25:**307-313.
49. Chen XL, Kunsch C: **Induction of cytoprotective genes through Nrf2/antioxidant response element pathway: a new therapeutic approach for the treatment of inflammatory diseases.** *Curr Pharm Des* 2004, **10:**879-891.
50. Li N, Alam J, Venkatesan MI, Eiguren-Fernandez A, Schmitz D, Di Stefano E, Slaughter N, Killeen E, Wang X, Huang A, et al: **Nrf2 is a key transcription factor that regulates antioxidant defense in macrophages and epithelial cells: protecting against the proinflammatory and oxidizing effects of diesel exhaust chemicals.** *J Immunol* 2004, **173:**3467-3481.
51. Syapin PJ: **Regulation of haeme oxygenase-1 for treatment of neuroinflammation and brain disorders.** *Br J Pharmacol* 2008, **155:**623-640.
52. Innamorato NG, Lastres-Becker I, Cuadrado A: **Role of microglial redox balance in modulation of neuroinflammation.** *Curr Opin Neurol* 2009, **22:**308-314.

53. Jeon WK, Kim BC: **WITHDRAWN: Heme oxygenase-1 mediates the anti-inflammatory effect of propyl gallate in LPS-stimulated macrophages.** *Biochem Biophys Res Commun* 2007, **361:**645-650.
54. Lee TS, Chau LY: **Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice.** *Nat Med* 2002, **8:**240-246.
55. Lee TS, Tsai HL, Chau LY: **Induction of heme oxygenase-1 expression in murine macrophages is essential for the anti-inflammatory effect of low dose 15-deoxy-Delta 12,14-prostaglandin J2.** *J Biol Chem* 2003, **278:**19325-19330.
56. Lin CC, Liu XM, Peyton K, Wang H, Yang WC, Lin SJ, Durante W: **Far infrared therapy inhibits vascular endothelial inflammation via the induction of heme oxygenase-1.** *Arterioscler Thromb Vasc Biol* 2008, **28:**739-745.
57. Wang WW, Smith DL, Zucker SD: **Bilirubin inhibits iNOS expression and NO production in response to endotoxin in rats.** *Hepatology* 2004, **40:**424-433.
58. Ryter SW, Alam J, Choi AM: **Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications.** *Physiol Rev* 2006, **86:**583-650.
59. Thorup C, Jones CL, Gross SS, Moore LC, Goligorsky MS: **Carbon monoxide induces vasodilation and nitric oxide release but suppresses endothelial NOS.** *Am J Physiol* 1999, **277:**F882-889.

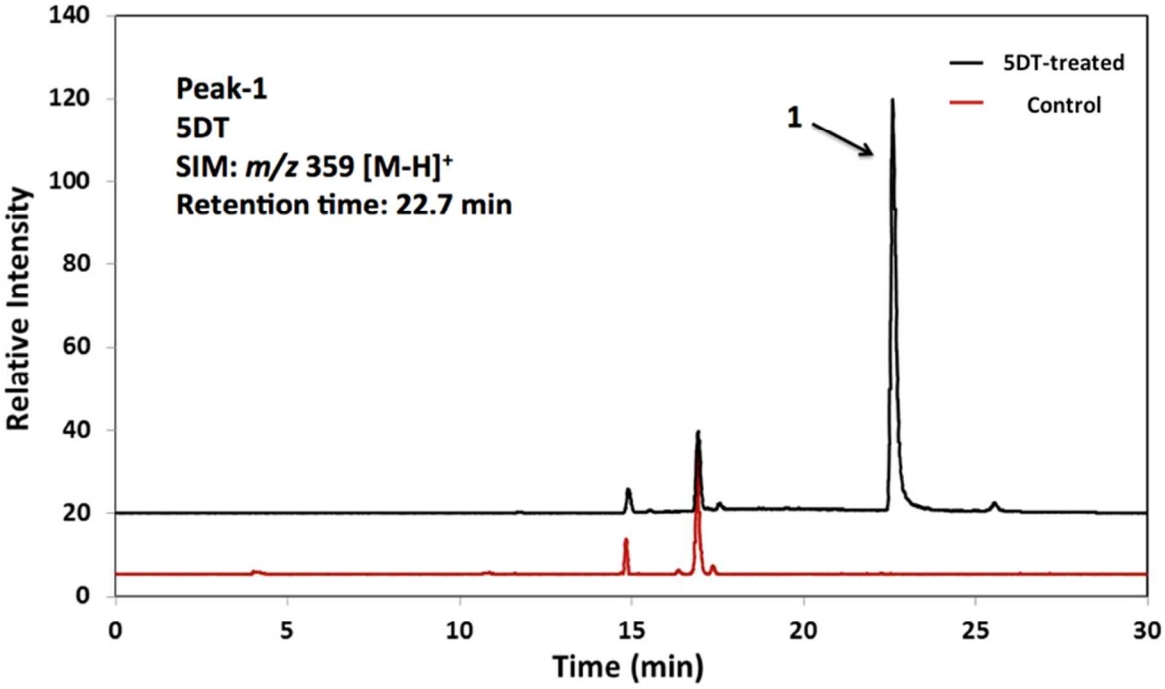
(A)



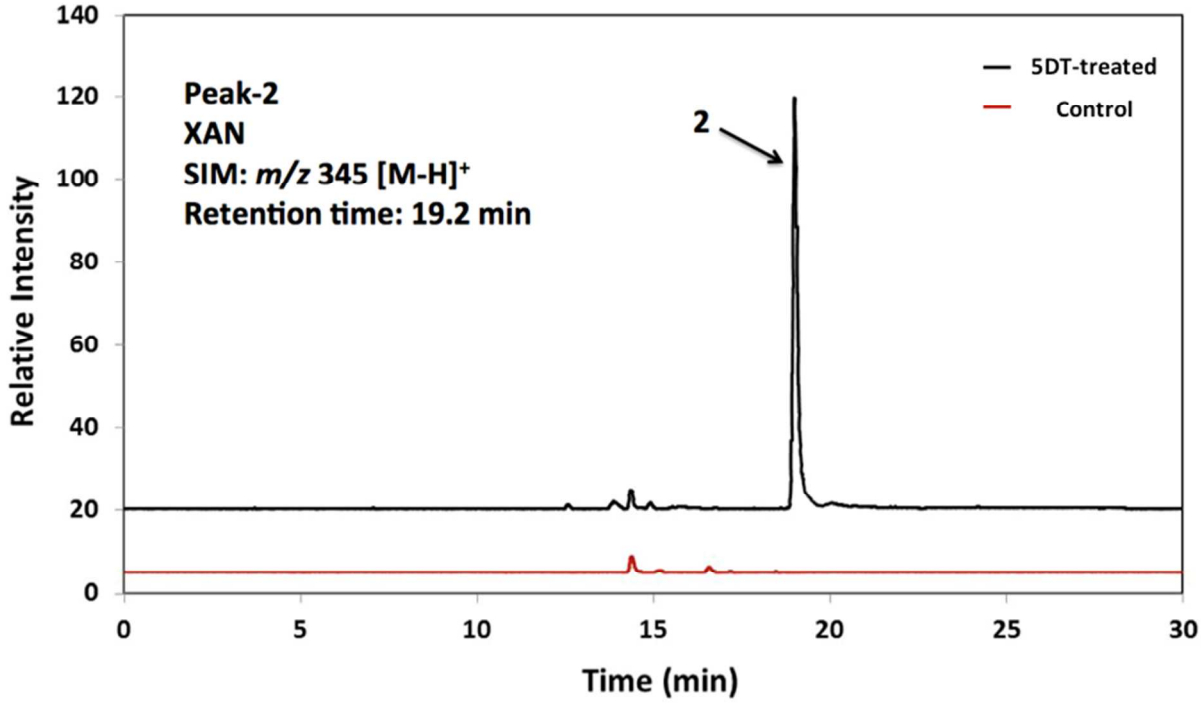
5-Demethyltengeretin (5DT)

Xanthomicrol (XAN)

(B)



(C)


Peak-2
XAN
SIM: m/z 345 [M-H]+
Retention time: 19.2 min
2
5DT-treated
Control
Relative Intensity
Time (min)
0
20
40
60
80
100
120
140
0
5
10
15
20
25
30

(D)

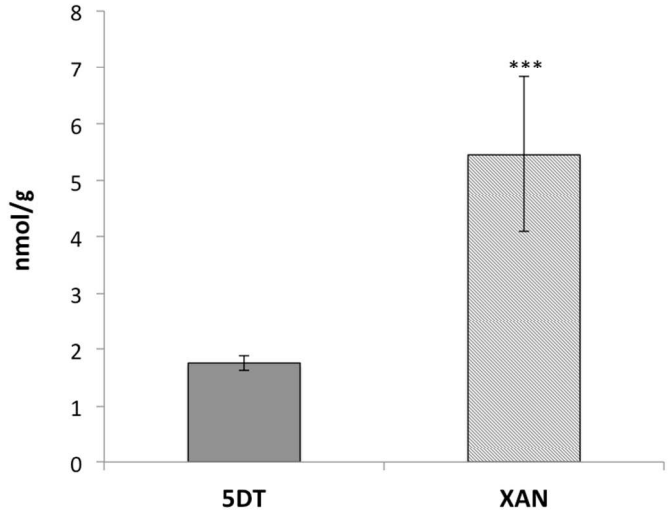
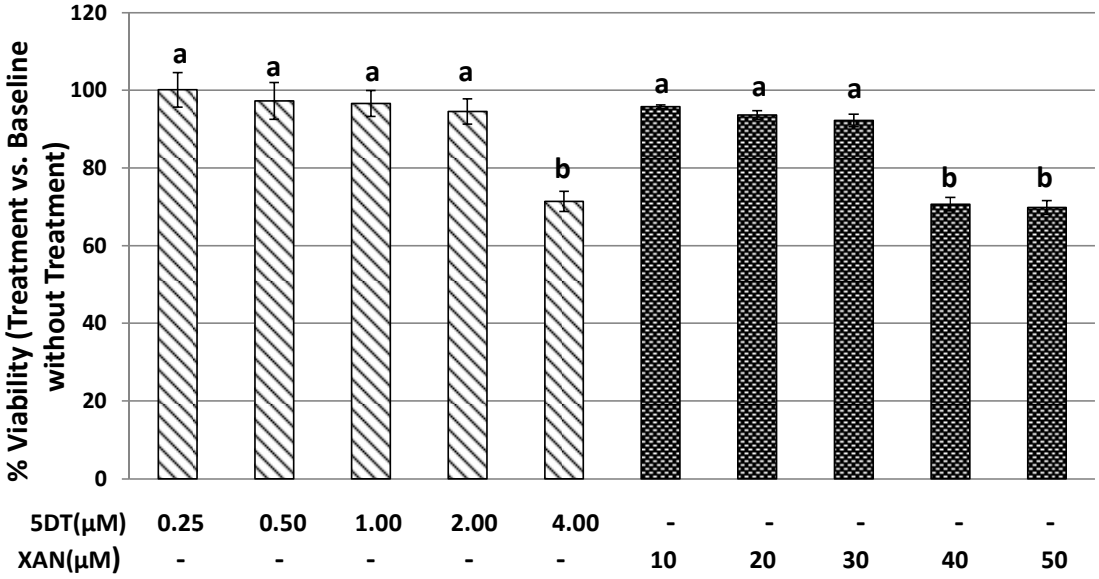
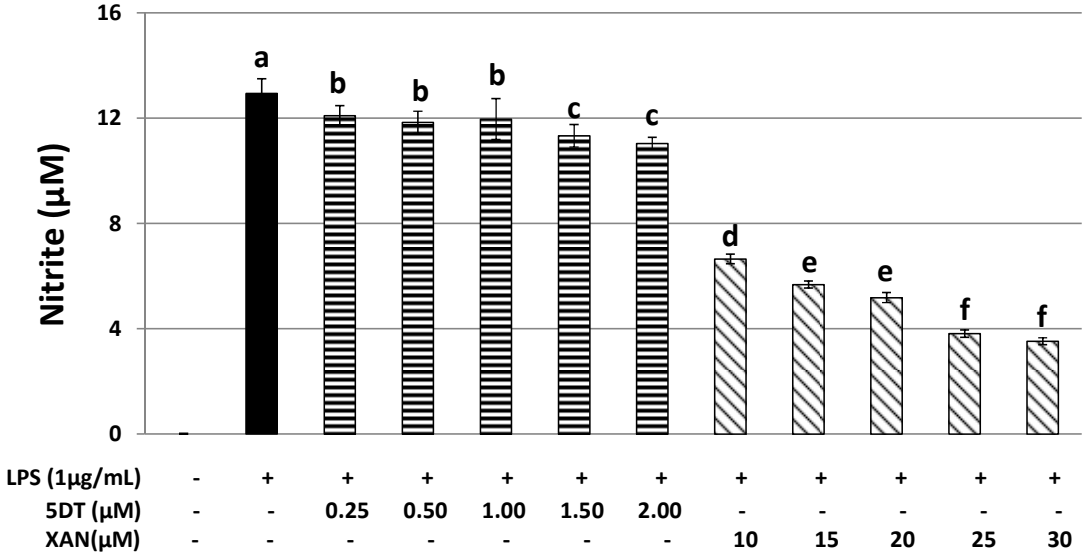

nmol/g
***
0
1
2
3
4
5
6
7
8
5DT
XAN

Figure 1. (A) Chemical structures of 5DT and XAN.LC/ESI-MS (positive ion) chromatograms of 5DT **(B)** and XAN **(C)** in colonic mucosa samples obtained from 5DT-fed mice and control mice. **(D)** The levels of 5DT and XAN in the colonic mucosa of 5DT-fed mice. *** represents $p < 0.001$, compared to 5DT. All data represent mean ± SD ($n = 5$).

(A)


% Viability (Treatment vs. Baseline without Treatment)
120
100
80
60
40
20
0
a a a a b a a a b b
5DT(μM) 0.25 0.50 1.00 2.00 4.00 - - - - -
XAN(μM) - - - - - 10 20 30 40 50

(B)


Nitrite (μM)
16
12
8
4
0
a b b b c c d e e f f
LPS (1μg/mL) - + + + + + + + + + + +
5DT (μM) - - 0.25 0.50 1.00 1.50 2.00 - - - - -
XAN(μM) - - - - - - - 10 15 20 25 30

(C)

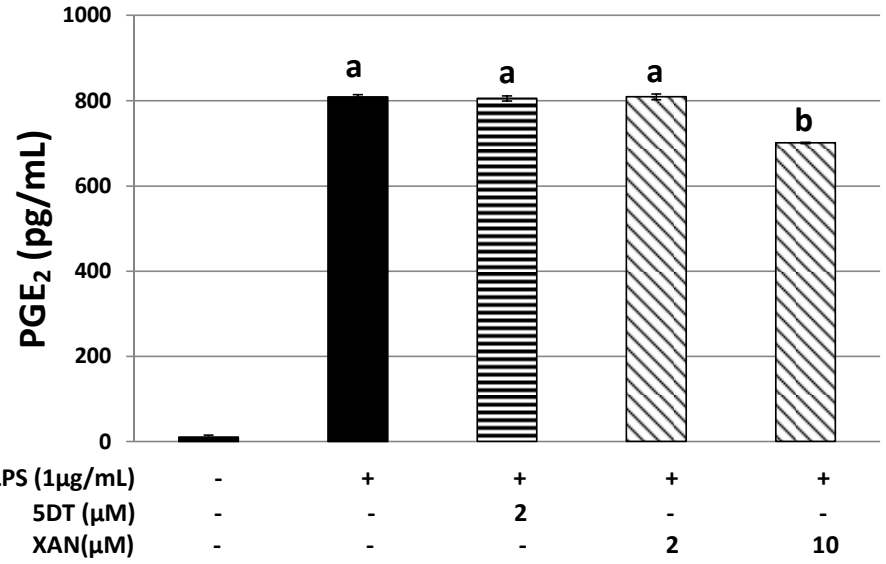
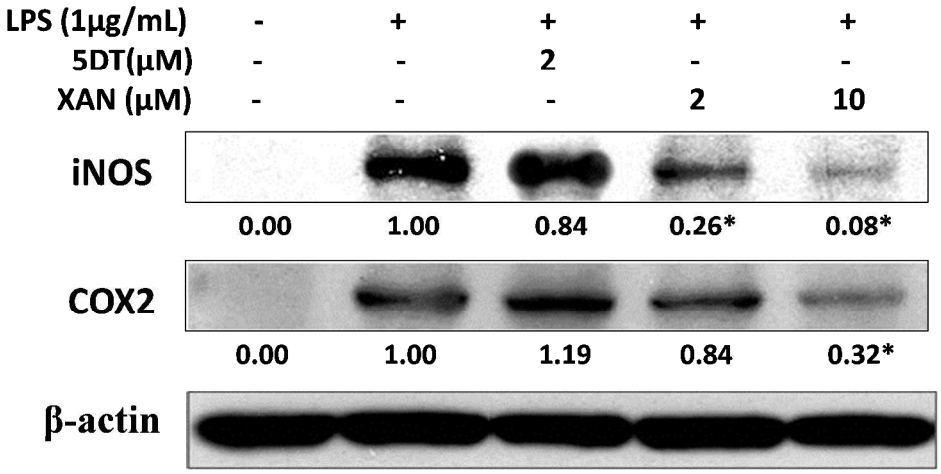
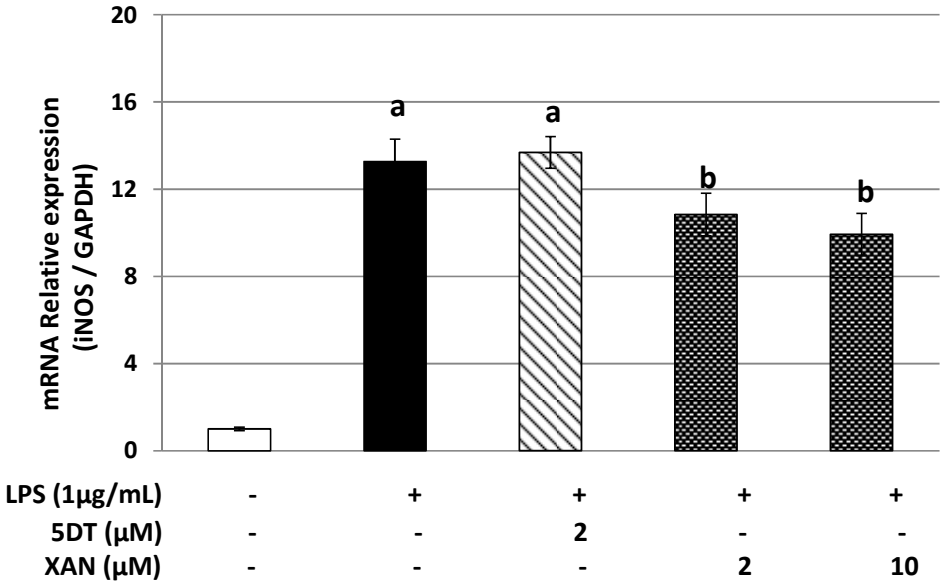


Figure2. (**A**) Cytotoxicity profiles of 5DT and XAN on RAW 264.7 macrophages. Macrophages in 96-well plates were treated with a series of concentrations of 5DT and XAN for 24 hours, and cell viability was determined MTT assay. The viability of control cells was set as the reference with a value of 100%. **(B)** Effects of 5DT and XAN on LPS-induced NO production in RAW 264.7 cells. The LPS-stimulated cells were treated with 5DT and XAN for 24 hours, and the culture media were collected for NO assay as described in the Materials and methods section. **(C)** Effects of 5DT and XAN on LPS-induced PGE_2 production in RAW 264.7 macrophages. The LPS-stimulated cells were treated with 5DT and XAN for 24 hours, and the culture media were collected and analyzed for protein levels of PGE_2 by ELISA. Results are presented as mean ± SD from six replicates. Different annotations indicate statistical significance ($p<0.01$, $n = 6$) by ANOVA.

(A)


LPS (1μg/mL) - + + + +
5DT(μM) - - 2 - -
XAN (μM) - - - 2 10
iNOS
0.00 1.00 0.84 0.26* 0.08*
COX2
0.00 1.00 1.19 0.84 0.32*
β-actin

(B)


mRNA Relative expression (iNOS / GAPDH)
20
16
12
8
4
0
a
a
b
b
LPS (1μg/mL) - + + + +
5DT (μM) - - 2 - -
XAN (μM) - - - 2 10

(C)

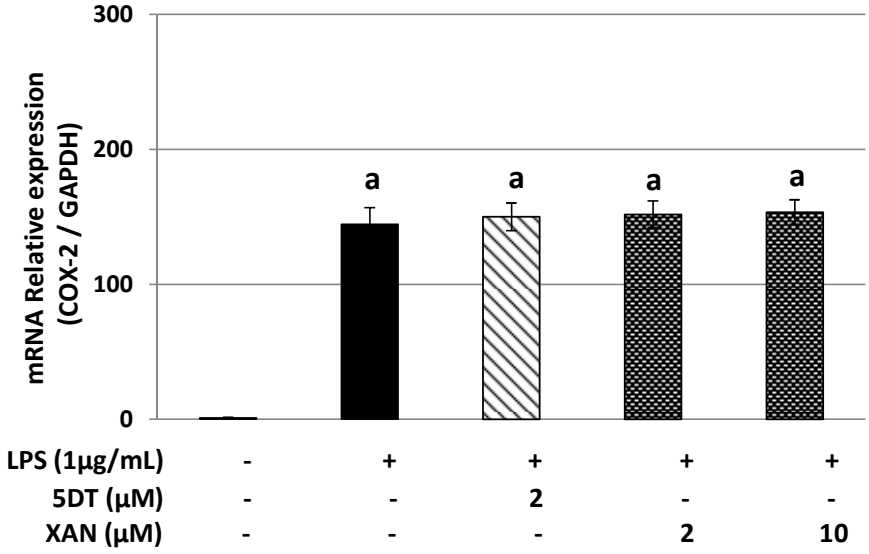
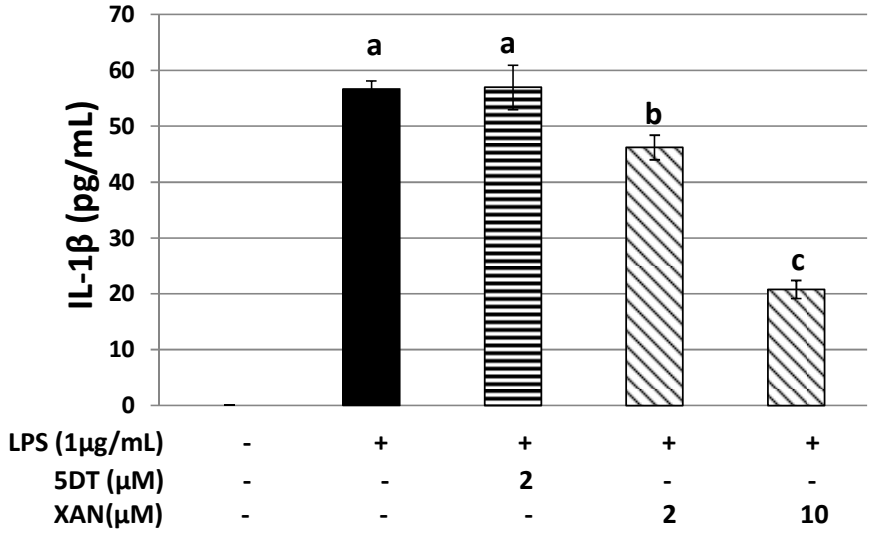


Figure3. The effects of 5DT and XAN on the protein levels of iNOS and COX-2 **(A)**, mRNA levels of iNOS **(B)**, and mRNA levels of COX-2 **(C)** in LPS-stimulated RAW 264.7 macrophages. The LPS-stimulated cells were treated with 5DT and XAN for 24 hours, and then the whole cell lysate was collected and subject to Western-blotting and real-time RT-PCR as described in Materials and methods section. In **(A)**, the numbers underneath of the Western 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. "*" indicates statistical significance ($P < 0.05$, $n = 3$). In **(B)** and **(C)**, each value represents the mean ± SD of three independent experiments. Different annotations indicate statistical significance ($p < 0.05$, $n = 3$) by ANOVA.

(A)


IL-1β (pg/mL)
70
60
50
40
30
20
10
0
a
a
b
c
LPS (1μg/mL) - + + + +
5DT (μM) - - 2 - -
XAN(μM) - - - 2 10

(B)

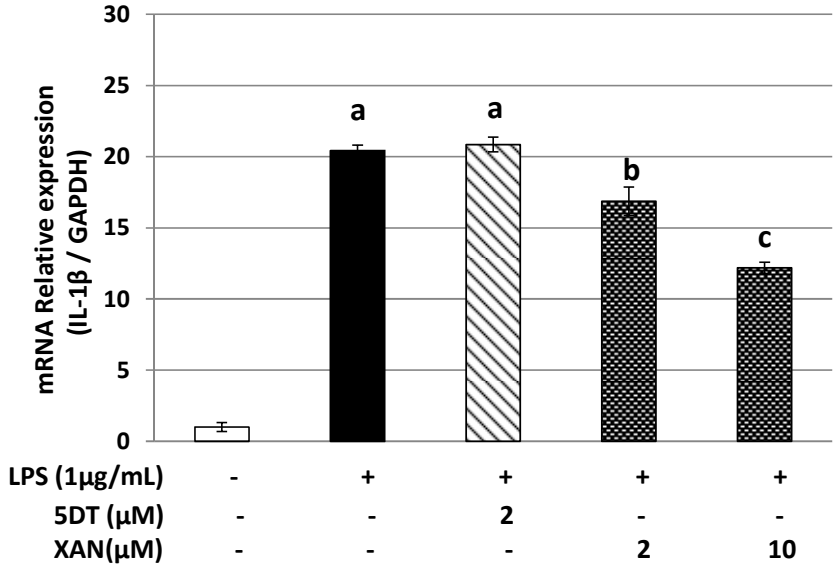
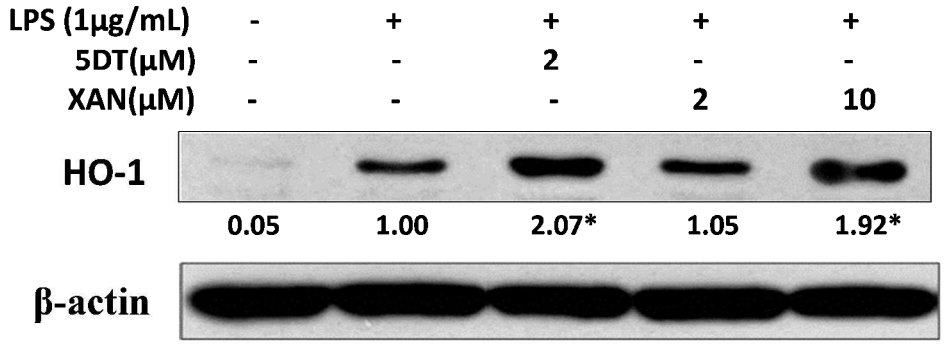

mRNA Relative expression (IL-1β / GAPDH)
30
25
20
15
10
5
0
a
a
b
c
LPS (1μg/mL) - + + + +
5DT (μM) - - 2 - -
XAN(μM) - - - 2 10

Figure4. Inhibitory effects of 5DT and XAN on the protein levels **(A)** and mRNA levels **(B)** of IL-1β in LPS-stimulated RAW 264.7 macrophages. The LPS-stimulated cells were treated with 5DT and XAN for 24 hours, and then the culture media were collected and subject to ELISA and real-time RT-PCR as described in Materials and methods section. Results are presented as mean ± SD. Different annotations indicate statistical significance ($p < 0.05$, $n = 6$ for **A**, and $n = 3$ for **B**) by ANOVA.

(A)



(B)

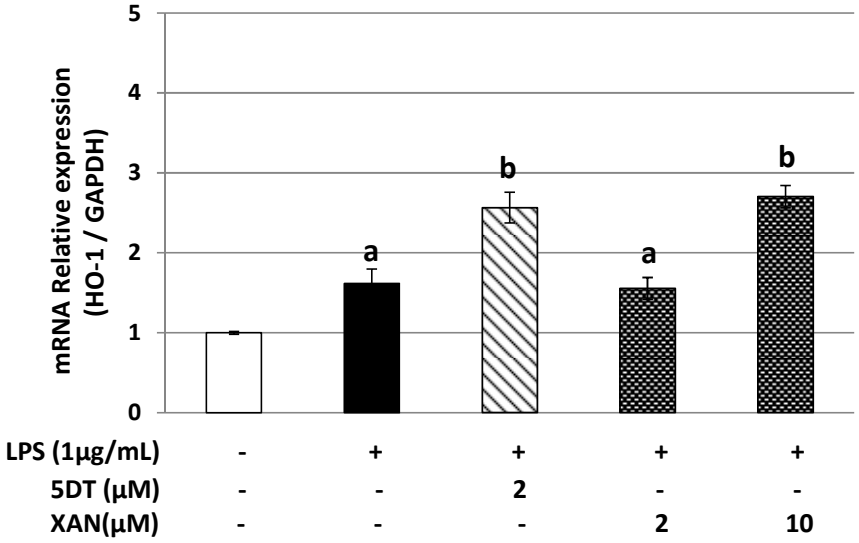
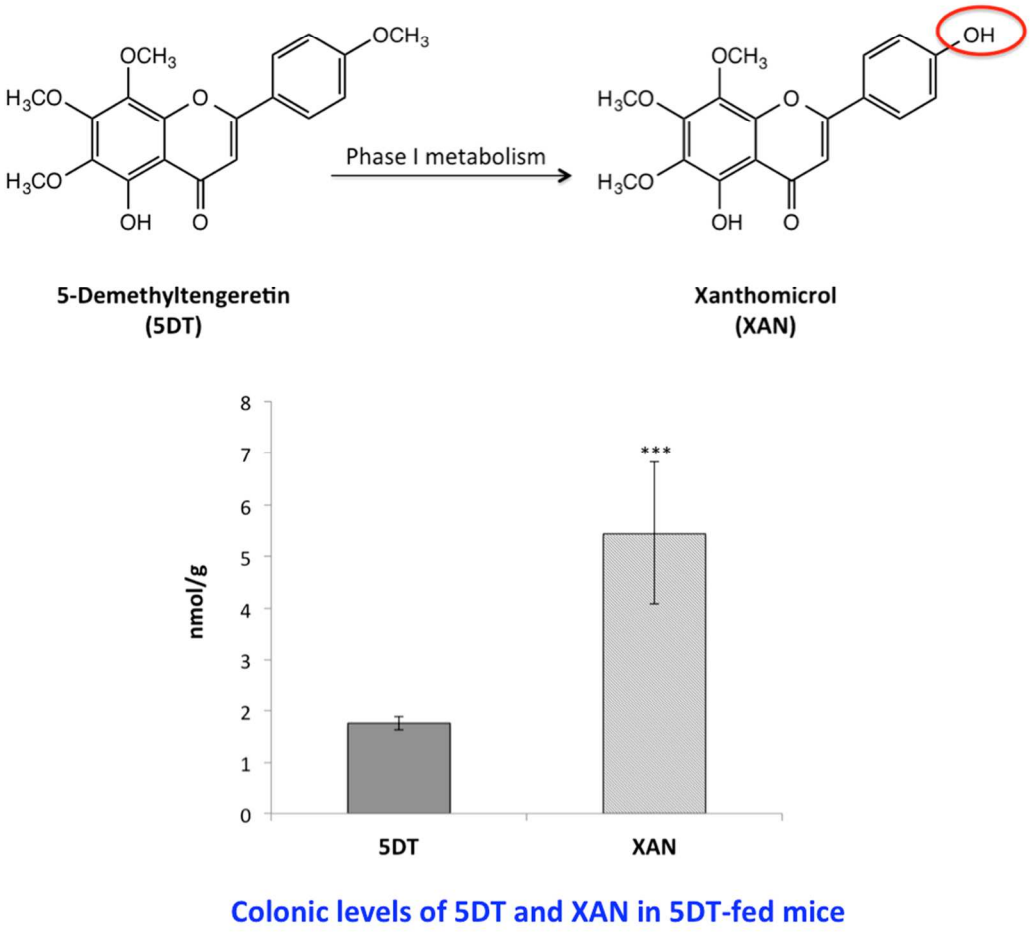


Figure5. Effects of 5DT and XAN on the protein levels **(A)** and mRNA levels **(B)** of HO-1 in LPS-stimulated RAW 264.7 macrophages. The LPS-stimulated cells were treated with 5DT and XAN for 24 hours, and then the whole cell lysate was collected and subject to Western-blotting and real-time RT-PCR as described in Materials and methods section. In **(A)**, the numbers underneath of the Western 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. "*" indicates statistical significance ($P < 0.05$, $n = 3$). In **(B)**, each value represents the mean ± SD of

three independent experiments. Different annotations indicate statistical significance ($p < 0.05$, $n = 3$) by ANOVA.

Graphical Abstract



5-Demethyltengeretin (5DT) is a citrus flavonoid with various health benefits. Our results shown that orally administered 5DT was mainly metabolized to form xanthomicrol (XAN) via demethylation in mice. More importantly, the colonic level of XAN was much higher than that of 5DT. Furthermore, we found that XAN inhibited LPS-stimulated inflammation in RAW 264.7 macrophages via suppressing IL-1β, iNOS and COX-2, as well as inducing HO-1. These results provided insights on the anti-inflammatory effects of 5DT.