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Cancer chemopreventive properties of orally bioavailable flavonoids—Methylated versus unmethylated flavones

Thomas Walle *, *Nga Ta, Toshihiko Kawamori, Xia Wen, Petra A. Tsuji, U. Kristina Walle*

Departments of Cell and Molecular Pharmacology and Experimental Therapeutics and Pathology & Laboratory Medicine (T.K.), Medical University of South Carolina, Charleston, SC 29425, USA

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ABSTRACT

Poor oral bioavailability has been a major limitation for the successful use of dietary flavonoids as cancer chemopreventive agents. In this study, we examined fully methylated flavones as promising improved agents. In the human oral SCC-9 cancer cells, 5,7-dimethoxyflavone and 5,7,4′-trimethoxyflavone were both 10 times more potent inhibitors of cell proliferation (IC_{50} values 5–8 μM) than the corresponding unmethylated analogs chrysin and apigenin. Flow cytometry indicated that both methylated flavones arrested the SCC-9 cells in the G1 phase with a concomitant decrease in the S phase, dramatically different from the unmethylated analogs, which promoted G2/M phase arrest. Both methylated compounds inhibited the proliferation of two other cancer cell lines with very little effect on two immortalized normal cell lines. Examination of additional flavone structures indicated that methylated flavones in general have antiproliferative properties. Finally, we demonstrated that 5,7-dimethoxyflavone, in contrast to its unmethylated analog chrysin, was well absorbed and had high oral bioavailability as well as tissue accumulation *in vivo* in the rat. Thus, fully methylated flavones appear to have great potential as cancer chemopreventive/chemotherapeutic agents, in particular in oral cancer.

1. Introduction

Dietary flavonoids and other polyphenols have over many years been demonstrated to have cancer chemopreventive properties in various biological systems [1–3]. Novel biochemical mechanisms are still being revealed at an accelerating rate in cell culture experiments [4–7]. However, extending such studies to the *in vivo* situation in animals or even more so in humans, using modest clinically tolerable doses, in most cases have been unsuccessful. This clearly can be explained by very poor oral bioavailability of the polyphenols, as has been shown directly in humans for chrysin [8], quercetin [9,10], the tea flavonoids [11,12] and resveratrol [13,14]. Mechanistically, this is related to the free hydroxyl groups of the polyphenols, giving rise to rapid intestinal/hepatic conjugation by glucuronidation and/or sulfation and excretion [15].

In contrast, we have recently described high metabolic stability as well as high intestinal transport of fully methylated flavones [16,17]. Although many of these compounds are present in plants, they have attracted only a modest interest as potential chemopreventive agents. One reason may be their lack of antioxidant properties, which in general has been

* *Corresponding author at*: Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, 173 Ashley Avenue, P.O. Box 250505, Charleston, SC 29425, USA. Tel.: +1 843 792 2507; fax: +1 843 792 2475.
E-mail address: wallet@musc.edu (T. Walle).

Abbreviations: SCC, squamous cell carcinoma; DMF, dimethoxyflavone; TMF, trimethoxyflavone; DHF, dihydroxyflavone; MF, methoxyflavone; HF, hydroxyflavone; PMF, pentamethoxyflavone; BrdU, bromodeoxyuridine; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline

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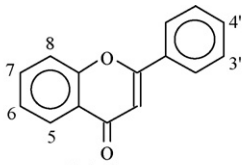

Flavone	Substituent 5	6	7	8	3'	4'
5,7-DMF	OCH_3	H	OCH_3	H	H	H
Chrysin	OH	H	OH	H	H	H
5,7,4'-TMF	OCH_3	H	OCH_3	H	H	OCH_3
Apigenin	OH	H	OH	H	H	OH
7-MF	H	H	OCH_3	H	H	H
7,4'-DMF	H	H	OCH_3	H	H	OCH_3
Tangeretin	OCH_3	OCH_3	OCH_3	OCH_3	H	OCH_3
5,3'-DMF	OCH_3	H	H	H	OCH_3	H
5,4'-DMF	OCH_3	H	H	H	H	OCH_3
7,8-DMF	H	H	OCH_3	OCH_3	H	H
Flavone	H	H	H	H	H	H

Fig. 1 – Structures of flavones used in this study.

associated with free hydroxyl groups [18]. However, some of these methylated flavones show remarkable inhibitory effects on carcinogen activating enzymes, thus, making them potentially useful as inhibitors of carcinogenesis at the initiation stage [19–21]. Although inhibition of cancer cell proliferation has been reported for methylated flavones [22,23], some of which appear highly potent [23], these studies were not pursued further. In contrast, methylation even reduces the biological activities of the isoflavones [24]. Thus, the effect of methylation on the cancer chemopreventive activities of flavonoids is not clearly understood. Our hypothesis was that methylation does not diminish the ability of the flavones to inhibit the proliferation of oral cancer cells while increasing their oral bioavailability.

In this study, we therefore compared the effects of two methylated flavones, i.e. 5,7-dimethoxyflavone (5,7-DMF) and 5,7,4′-trimethoxyflavone (5,7,4′-TMF), with their unmethylated analogs chrysin and apigenin (structures in Fig. 1) on cell proliferation. Both chrysin and in particular apigenin have been extensively studied in the past [5,25–27]. Several other flavones were investigated as well to get a preliminary feel for chemical structure–activity relationships. Our cell models contained cancer as well as noncancer cells and cell proliferation was measured using incorporation of BrdU into newly synthesized DNA as the assay. Effects on cell cycle progression were also investigated. In addition, we determined the cellular uptake as well as the oral bioavailability of 5,7-DMF compared to chrysin in the rat *in vivo*.

2. Materials and methods

2.1. Materials

Chrysin, apigenin, flavone, propidium iodide and Cremophor EL® were obtained from Sigma Chemical Co. (St. Louis, MO). 5,7-Dimethoxyflavone (5,7-DMF), 5,7,4′-trimethoxyflavone (5,7,4′-TMF), 7,4′-dimethoxyflavone (7,4′-DMF), 7-methoxyflavone (7-MF) and 5,4′-dimethoxyflavone (5,4′-DMF) were obtained from Indofine Chemical Co. Inc. (Hillsborough, NJ). 5,6,7,8,4′-Pentamethoxyflavone (tangeretin) was purchased from ChromaDex (Santa Ana, CA). All other chemicals were purchased from Sigma or Fisher.

2.2. Cell culture and treatments

The human oral (tongue) squamous cell carcinoma SCC-9 cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and cultured in DMEM/Ham's F12 medium (Fisher, Pittsburgh, PA), supplemented with 10% fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO), 1% penicillin/streptomycin and 0.4 μg/ml hydrocortisone in a 5% CO_2 atmosphere at 37 °C. Vehicle dimethyl sulfoxide (DMSO, 0.1% final volume) was used as a control in all experiments. The cells were used at passages 18–30. The human pharynx SCC FaDu cells (ATCC) were cultured in minimum essential medium (Eagle) with Earle's salts, nonessential amino acids, sodium pyruvate and 10% FBS. Human mammary MCF-7 cancer cells (ATCC) were grown in minimum essential medium (Eagle) with Earle's salts, 20 mM HEPES, insulin (10 μg/ml), sodium pyruvate and 10% FBS. Virus-transformed normal human esophageal epithelial HET-1A cells [28] were cultured in DMEM with 2% FBS, sodium pyruvate, insulin, transferrin, hydrocortisone and cholera toxin, as previously described [29]. Virus-transformed normal human lung epithelial BEAS-2B cells [30] were cultured in serum-free bronchial epithelial growth medium (Cambrex, Walkersville, MD).

2.3. Cell proliferation assay

The rate of proliferation of cells was determined as the rate of incorporation of bromodeoxyuridine (BrdU) into cellular nucleic acids [31], using a kit from Calbiochem/EMD Biosciences (La Jolla, CA). Briefly, cells seeded in 96-wells at 10^4 cells/well were allowed to attach overnight prior to incubation with flavones (0–100 μM) in serum-free medium with 0.1% bovine serum albumin for 24 h. The cells were then pulsed with BrdU in the presence of the flavone for 4 h. The cells were fixed, denatured and incubated with anti-BrdU primary antibody, secondary antibody and substrate according to the manufacturer's protocol. The absorbance was read at 450 nm with 590 nm background subtraction with appropriate blanks on a FLUOStar Optima Platereader (BMG Labtech Inc., Durham, NC).

2.4. Cell uptake assay

Confluent SCC-9 cells cultured in six-well plates were incubated at 37 °C with 25 μM chrysin or 5,7-DMF for 0.5, 2, 6 or 24 h in complete cell culture medium. The uptake was terminated by rinsing the cell layers twice with ice-cold saline. The flavones were extracted twice from the cells with 1 ml of methanol for 10 min on an orbital shaker. The combined methanol extracts were taken to dryness under nitrogen, reconstituted in 500 μl mobile phase and spun down. An aliquot (100 μl) of the supernatant was analyzed by HPLC as described below. The peak areas were compared to standard curves and the uptake was normalized for cellular protein [32].

2.5. Cell toxicity assay

SCC-9 cells seeded in 96-well plates (2500 cells/well) were allowed to attach overnight. The cells were then treated with 0–100 μM 5,7-DMF or chrysin for 24 h as above. After removal of the medium, the cells were fixed with glutaraldehyde, washed, and stained with 0.1% crystal violet, as previously described [33]. The washed and dried plates were shaken with 10% acetic acid and the absorbance was read at 570 nm.

2.6. Cell cycle analysis

Asynchronized cells were plated in 60 mm^2 plates at 0.1×10^6 cells/ml, grown until 75–80% confluency, then treated with 0, 5, 10, 25 and 50 μM flavonoid for 24, 48 and 72 h [4]. The cells were collected by trypsinization, washed twice with cold phosphate-buffered saline (PBS), and centrifuged. The pellets were resuspended in 100 μl cold PBS and 900 μl cold 70% ethanol and incubated overnight at 4 °C. After centrifugation, the cell pellets were washed with cold PBS and resuspended in PBS containing 1 mg/ml RNase and 100 μg/ml propidium iodide. Following incubation in the dark for 30 min at 4 °C, the cells were analyzed by flow cytometry. Data were acquired on a BD FACSCalibur using CellQuest software and were analyzed using Modfit LT software.

2.7. In vivo bioavailability study

2.7.1. Animals

Male F344 rats, 4 weeks old, were obtained from Charles River Laboratories (Wilmington, MA) and quarantined for 10 days. They were kept under controlled conditions with 12 h light/dark cycle and constant temperature and humidity in the Department of Laboratory Animal Resources and had free access to water and standard rat chow (Harlan Teklad). The protocol was approved by the Institutional Animal Care and Use Committee. The rats were killed by an overdose of gas anesthetic.

2.7.2. Experimental procedure

A suspension of chrysin and 5,7-DMF (2 mg/ml of each) was prepared in Cremophor EL®/ethanol/saline (1/1/8, v/v/v). After 24 h of fasting, the rats were administered 0.2–0.3 ml of the suspension by gavage (for a dose of 5 mg/kg). Control animals received the same volume of vehicle. At 0.5, 1, 2 or 3 h after the drug dosing, the rats were sacrificed. Five animals were used at each time-point. Blood was obtained from the inferior vena cava and centrifuged after the addition of EDTA. The plasma was frozen and stored at −20 °C. Lungs, liver, kidneys and colon were excised and immediately frozen in liquid nitrogen. The tissues were stored at −20 °C until analyzed.

2.7.3. Analysis

Plasma was mixed with an equal volume of methanol, vortex-mixed and centrifuged at 14,000 × *g* for 5 min. Aliquots of the supernatant (100 μl) were analyzed by HPLC, using a Symmetry C18 column (3.9 mm × 150 mm, 5 μm particle size) from Waters Corporation (Milford, MA). The mobile phase was methanol/trifluoroacetic acid/water (55/0.3/45, v/v/v) and detection was with a Model 996 photodiode array detector (Waters) at 268 nm. Quantitation was based on a comparison of peak areas with standard curves (0.05–25 μM) for 5,7-DMF and chrysin. The minimum detectable concentration (two times background noise) was 0.05 μM. Aliquots of lung, liver and kidney tissue (100–400 mg) were homogenized on ice in 1 ml of phosphate-buffered saline, using a Polytron homogenizer. To 0.5 ml of the homogenate was added 0.5 ml of methanol. After 1 min of vortex-mixing, the samples were centrifuged as above and the supernatant analyzed by HPLC. With the colon, the top 2 cm with the first fecal pellet were homogenized separately as above.

2.8. Statistical analysis

Data were expressed as means ± S.E.M. Statistical significance of differences between samples were calculated by Student's two-tailed *t*-test or ANOVA with Dunnett multiple comparison post-test. $P < 0.05$ was considered significant. The IC_{50} values

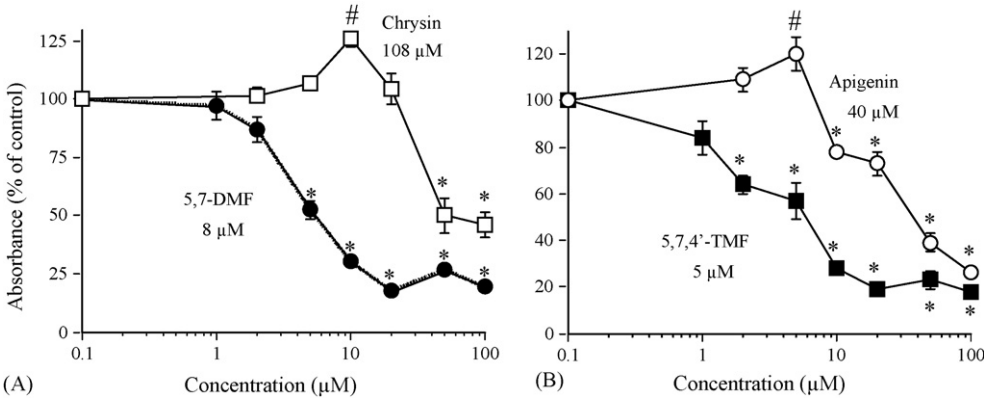

Fig. 2 – Effect of the methylated flavones (A) 5,7-DMF and (B) 5,7,4′-TMF compared to the unmethylated analogs chrysin and apigenin on SCC-9 cell proliferation. Cell proliferation, expressed as percent of control (DMSO-treatment), was measured as BrdU incorporation into cellular DNA after a 24-h exposure of the cells to the flavones. Mean values ± S.E.M. are shown ($n = 10$). The numbers shown in the figure are the calculated IC_{50} values. *Significantly lower than control, $P < 0.05$. #Significantly higher than control, $P < 0.05$.

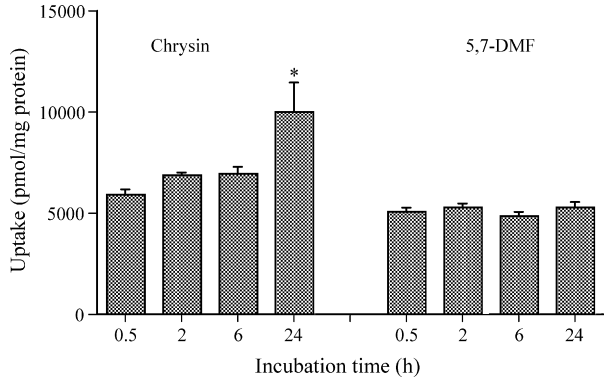

Fig. 3 – SCC-9 cell uptake of chrysin and 5,7-DMF. The cell monolayers were incubated with 25 μM flavones in complete medium for various time. *Significantly higher than 0.5, 2 and 6 h uptake, $P < 0.05$ ($n = 6$).

were calculated using Prism (GraphPad Software, San Diego, CA).

3. Results

3.1. *Effects on cell proliferation and toxicity*

Two pairs of compounds were selected for these studies, i.e. the simple flavones 5,7-dimethoxyflavone (5,7-DMF) and its unmethylated analog chrysin and 5,7,4′-trimethoxyflavone (5,7,4′-TMF) and its unmethylated analog apigenin (structures, see Fig. 1). Chrysin and apigenin are both well-studied flavonoids. Our cancer model was the human oral squamous carcinoma SCC-9 cell, a rather aggressively growing cell line [34], and the effect on cell proliferation was measured as *de novo* DNA synthesis, using the BrdU incorporation assay [31]. 5,7-DMF inhibited cell proliferation with an IC_{50} value of 8 μM (Fig. 2A), which was about 10 times more potent than chrysin. With chrysin, but not with 5,7-DMF, there was a slight activation at the 10 μM concentration. 5,7,4′-TMF had a similar potency as 5,7-DMF (Fig. 2B) with an IC_{50} value of 5 μM, eight times more potent than apigenin. Also with apigenin there was a slight activation at the 5 μM concentration.

The greater potency of the methylated versus the unmethylated flavones in Fig. 2A and B could conceivably be due to greater cell uptake of the methylated flavones. This was examined with 5,7-DMF compared to chrysin. After incubation of the SCC-9 cells for up to 24 h with 25 μM flavones, the uptake was rapid and virtually identical for the two compounds except for the somewhat higher 24 h uptake of chrysin (Fig. 3).

A small number of additional methylated flavones were investigated for antiproliferative effects compared to 5,7,4′-TMF and 5,7-DMF in the SCC-9 cells. The calculated IC_{50} values were 36.5 μM (7-MF), 24.2 μM (7,4′-DMF) and 19.3 μM (tangeretin). In addition, 5,4′-DMF, 5,3′-DMF and 7,8-DMF showed weaker effects. The unsubstituted flavone had an IC_{50} value of 8.3 μM.

As evidence of cell toxicity, we used both crystal violet staining [33] and the MTT assay [35]. Results from the crystal violet assay for 5,7-DMF and chrysin in the SCC-9 cells showed an IC_{50} value of about 50–100 μM for both compounds ($n = 12$). Virtually identical results were obtained with the MTT assay (data not shown).

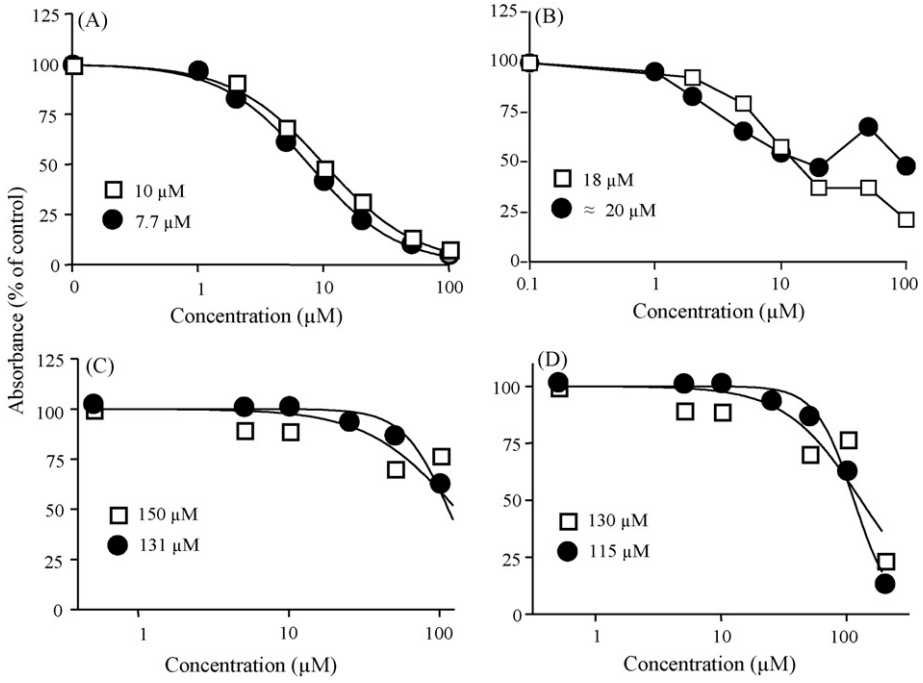

Fig. 4 – Effect of 5,7-DMF (●) compared to chrysin (□) on the proliferation of cancer cells (A and B) and noncancer cells (C and D). (A) FaDu cells; (B) MCF-7 cells; (C) HET-1A cells; (D) BEAS-2B cells. Cell proliferation, expressed as percent of control (DMSO-treatment) was measured as BrdU incorporation into cellular DNA after a 24-h exposure of the cells to the flavones. Mean values ± S.E.M. are shown ($n = 10$). The numbers shown in the figure are the calculated IC_{50} values.

3.2. *Effects on the proliferation of cancer versus noncancer cells*

To determine if the potent antiproliferative effects observed by the methylated flavones on the SCC-9 cells in Fig. 2 were selective for cancer cells versus noncancerous cells, we compared the effects of 5,7-DMF and chrysin in two additional cancer cell lines with those in two noncancer cell lines. In the FaDu human larynx SCC cells (Fig. 4A), both 5,7-DMF and chrysin showed similar potency as 5,7-DMF in the SCC-9 cells (IC_{50}, 8–10 μM). In the MCF-7 human breast cancer cells, both compounds again had similar but slightly lower potency (Fig. 4B) with IC_{50} values of 10–20 μM. 5,7-DMF seemed to exert a bimodal effect with stimulation of cell growth at higher concentrations. In contrast, two normal but transformed human cell lines, i.e. the HET-1A esophageal cells [28] and the BEAS-2B bronchial epithelial cells [30], were much less sensitive to both 5,7-DMF and chrysin. Both of these cell lines retain epithelial morphology and are non-tumorigenic in nude mice [28,30]. Thus, both flavones had estimated IC_{50} values towards cell proliferation of as high as 115–150 μM in these cells (Fig. 4C and D).

3.3. *Effects on cell cycle regulation*

To determine whether the growth inhibitory effect of the two pairs of flavones was accompanied by cell cycle arrest, SCC-9 cells were treated with increasing concentrations, i.e. 0, 5, 10, 25 and 50 μM, of the compounds for 72 h. Asynchronized cells were harvested, fixed in ethanol, stained with propidium iodide and analyzed for cell cycle distribution by flow cytometry. Fig. 5A shows that 5,7-DMF caused a dose-dependent increase in the G1 phase with a concomitant decrease in the S phase and no change in the G2/M population. This was distinctly different from the more modest effects of chrysin on the cell cycle distribution (Fig. 5B) with an increase in the G2/M phase but at the highest concentration (50 μM) only.

Fig. 5C shows the corresponding effects of 5,7,4′-TMF with a dose-dependent increase in the G1 phase, as with 5,7-DMF, and a decrease in the S phase and not much change in the G2/M phase population. Fig. 5D shows the effects of apigenin, which qualitatively is similar to the effects of chrysin with a distinct increase in the G2/M phase population, very different from the effects of 5,7,4′-TMF. When examining the quantitative changes in the G1 phase caused by 5,7-DMF and 5,7,4′-TMF, they appear very similar, with effects already at the lowest concentration investigated (5 μM). This was statistically significant for 5,7,4′-TMF. The results seen by flow cytometry were thus very similar to those seen in the BrdU incorporation assay (Fig. 2).

3.4. *Oral bioavailability in the rat*

In previous *in vitro* studies, we showed that the methylated flavones had increased hepatic metabolic stability as well as intestinal transport in comparison with their unmethylated analogs [16,17]. In the present investigation, these observations were extended to the examination of the oral absorption and bioavailability of one of these compounds, i.e. 5,7-DMF,

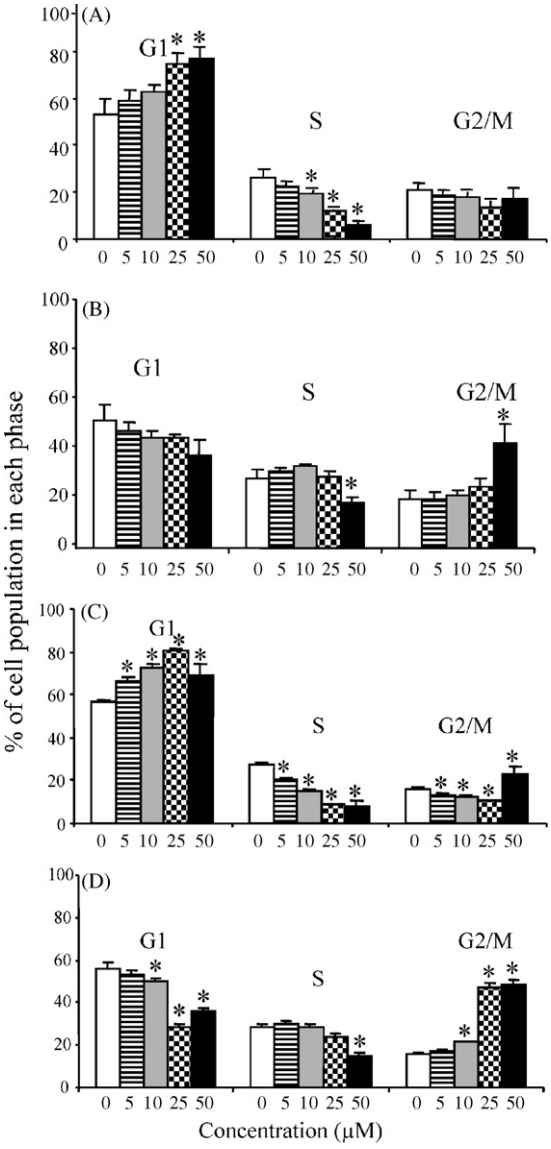

Fig. 5 – Effect of 5,7-DMF (A) compared to chrysin (B) and 5,7,4′-TMF (C) compared to apigenin (D) on SCC-9 cell cycle progression. The cells were exposed to varying concentrations of the flavones for 48 h. The percentage of cells in G1, S and G2/M phase was measured by flow cytometry after propidium iodide staining. Mean values of three experiments with duplicate samples are shown. *Significantly different from control, $P < 0.05$ or better.

compared to chrysin in the rat *in vivo*. This test was considered particularly stringent as the rat is well known to be a species with very high activity of the xenobiotic oxidizing enzymes. Both compounds were administered simultaneously by gavage (5 mg/kg each) and plasma and tissue concentrations were measured by HPLC/UV using a mobile phase that permitted separation of the two compounds. 5,7-DMF was clearly detected in plasma (Fig. 6A) with a peak concentration of 2.5 ± 0.8 μM (mean ± S.E.M.) at 1 h after the dose. The area under the plasma concentration–time curve was 58.8 μg ml^{-1} min. Chrysin was not detectable in plasma at any time-point. 5,7-DMF was also clearly detected in liver, lung

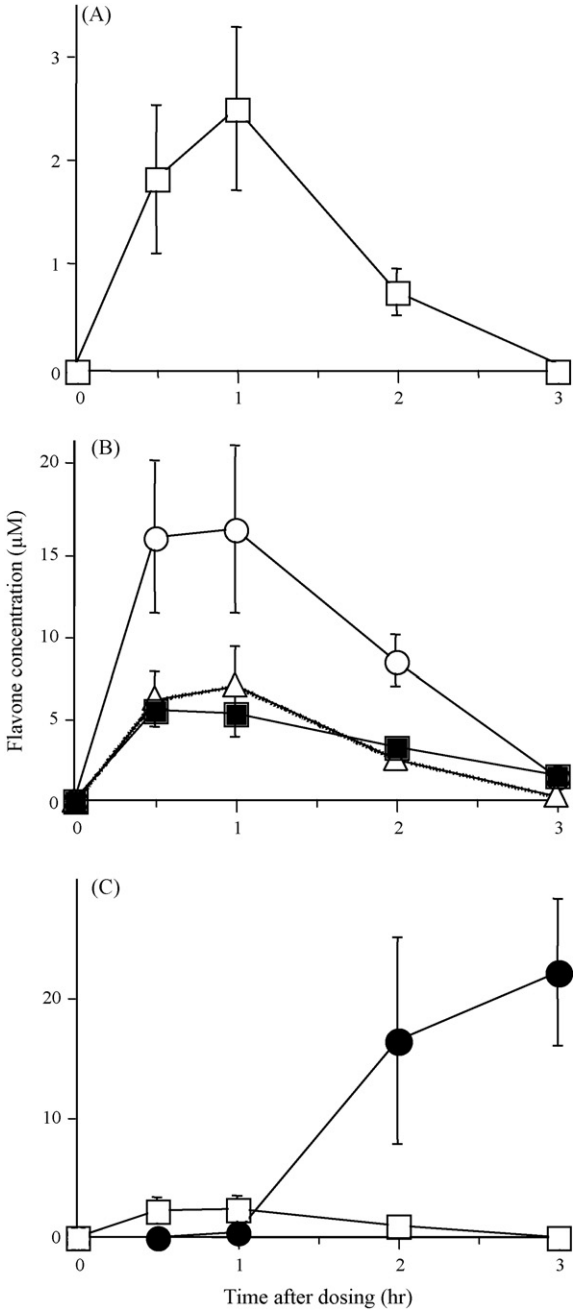

Fig. 6 – Plasma and tissue levels of 5,7-DMF and chrysin after oral administration of 5 mg/kg in rats. (A) Plasma 5,7-DMF (no chrysin could be detected at any time-point); (B) 5,7-DMF in post-absorption tissues, liver (○), lung (■) and kidney (△) (chrysin was barely detectable in a few animals and tissues); (C) 5,7-DMF (□) and chrysin (●) in the proximal 2 cm of the colon with associated fecal pellet. The data represent the mean ± S.E.M. of five animals at each time-point.

and kidney tissues with concentrations in the liver exceeding those in the plasma by as much as seven-fold, i.e. 16.5 ± 5 μM at 1 h after the dose (Fig. 6B). The half-life for 5,7-DMF in plasma was short, i.e. 16 min, most likely reflecting the high oxidative capacity of the rat. Interestingly, the half-life for 5,7-DMF calculated from the tissue measurements was considerably longer, ranging from 29 to 68 min, the latter in the lungs, indicating this tissue as a favorable accumulation site. The liver, which showed the highest accumulation of 5,7-DMF, should be predicted to be a site where 5,7-DMF might exert its greatest activity.

The colon presented a different problem, as the tissue was contaminated with fecal matter. The flavone content in the first 2 cm of the colon and the associated fecal pellet is shown in Fig. 6C. For 5,7-DMF, the low levels were almost exclusively from the colon tissue. These levels were about one-third of those in the kidneys and lungs, but following the same time–course as in plasma and the other tissues. For chrysin, there were no levels detectable in the tissues. However, in the upper colonic fecal matter, there were considerable amounts of chrysin, but only at 2 and 3 h after administration of the flavone mixture (Fig. 6C).

There were no clearly detectable metabolites of the two flavones in either plasma or tissues. Conjugated metabolites of chrysin, as seen in human plasma [8], might have been present but likely were obscured by other highly polar materials present in the samples.

4. Discussion

The fact that the two methylated flavones selected for this study, i.e. 5,7-DMF and 5,7,4′-TMF, both were about 10 times more potent than their unmethylated analogs in inhibiting DNA synthesis in the SCC-9 cells (Fig. 2) was surprising, as many studies in the past have assumed that the free hydroxyl groups of the flavonoids, or polyphenols more generally, are necessary for biological effects. This certainly appears to be the case for antioxidant effects [18], long believed to be the main mechanism of action of these naturally occurring compounds. However, recent findings point to effects on signal transduction pathways as the main sites of action for the flavonoids [36]. Free hydroxyl groups, in particular in the 4′-position of the B-ring, are critically important for the isoflavones [24].

The results of our study suggest a different mechanism of action for the methylated flavones than for the unmethylated analogs. This becomes evident most clearly in their differential effects on cell cycle progression. Apigenin has been studied extensively as a potential dietary chemopreventive agent and several studies [5,25–27], including this one, have shown apigenin to induce cell cycle arrest in the G2/M phase. However, the methylated analog 5,7,4′-TMF as well as 5,7-DMF clearly induced arrest of most cells in the G1 phase (Fig. 4). These differential effects should be worthy of further investigation. It should be pointed out that the 50 μM flavonoid concentration appeared to produce somewhat different effects on the cell cycle progression than the lower concentrations. This may reflect a different, i.e. toxic, response of the cells. This is supported by the data obtained by crystal violet staining and the MTT assay, demonstrating IC_{50} values of 50–100 μM for effects on cell numbers by both 5,7-DMF and chrysin.

Of particular interest was the wide variation in effects on cell proliferation between different cell types. Thus, although

5,7-DMF showed similar potency in the FaDu laryngeal SCC cells as in the tongue SCC-9 cells, chrysin was much more potent in the FaDu cells. These two compounds also had similar effects on the MCF-7 cell proliferation with somewhat diminished potency, in particular for 5,7-DMF, which had a bimodal effect. However, most important, both 5,7-DMF and chrysin had greatly diminished effects on the noncancer transformed esophageal HET-1A cells and lung BEAS-2B cells, emphasizing high selectivity for cancer cells, in particular the tongue cancer SCC-9 cells.

The importance of the chemical structure of the methylated flavones on SCC-9 cell proliferation is not entirely clear from this study. All of the methylated compounds studied so far appear to have some inhibitory effect on SCC-9 cell proliferation, with 5,7-DMF and 5,7,4′-TMF appearing to be two of the most effective agents. These structure–activity relationships will need further investigation with additional methylated flavones. In a survey of various other methoxylated flavones in other types of human cancer cells, using the thymidine incorporation assay, a wide variety of responses were likewise observed [23], some of which indicated high potency.

Although flavonoids commonly are known to inhibit cancer cell proliferation, there are also reports suggesting that some of them may stimulate growth, presumably due to an estrogen-like effect, i.e. to have bimodal effects, such as with quercetin [37]. Following this complex pattern of effects the promising polyphenolic flavonoid mixture silymarin was recently unexpectedly demonstrated to stimulate the growth of MCF-7 breast cancer cells [38]. Whereas both chrysin and apigenin in our study had a tendency toward a biphasic response, with a statistically significant stimulation at low concentrations, this was most certainly not the case with the two methylated analogs (Fig. 2). This may be related to stimulation of the estrogen receptor, such as with quercetin [37].

Although the methylated flavones investigated here were synthetic compounds, some of them have been identified in plants. For example, 5,7,4′-TMF is a citrus flavonoid [39], also present in other plants used in folk medicine [40,41]. 7,4′-DMF has been identified in fruits and leaves from neotropical nutmeg species [42,43], as well as from propolis [44]. Also, 5,7-DMF is a natural product [45,46] highly abundant in pepper vine leaves. In view of the present findings, the search for methylated flavones in plants may be accelerated. The presence of some of these methylated flavones in citrus fruits is of interest in regard to a very recent epidemiological study demonstrating citrus fruits and juices to be protective against oral premalignant lesions [47].

Finally, our previous *in vitro* studies have demonstrated that the methylated flavones have dramatically increased metabolic stability as well as intestinal transport [16,17]. In the present study, we showed *in vivo* in the rat that one of these flavones, i.e. 5,7-DMF, indeed had high oral absorption as well as bioavailability (Fig. 6). In contrast, its unmethylated analog, chrysin, could not be detected except in the stool samples, reflecting negligible systemic availability. Of particular interest was the finding that 5,7-DMF accumulated highly in tissues, in particular in the liver. This is in contrast to observations with unmethylated flavonoids like quercetin, for which tissue accumulation, measured either directly [48] or through volume of distribution measurements [49,50], has been very limited.

In summary, this study demonstrates that fully methylated flavones can be more potent inhibitors of cancer cell proliferation than their corresponding nonmethylated analogs. These effects appear to be selective for cancer versus noncancer cells. In addition, their mode of inhibition appears to be distinctly different. Moreover, we showed that one of these methylated flavones *in vivo* in the rat had high oral absorption, bioavailability and tissue distribution. Together with our previous findings on inhibition of carcinogen bioactivation [19–21], this study suggests that these compounds should be highly attractive as cancer chemopreventive/chemotherapeutic agents deserving further attention. *In vivo* chemopreventive studies are now in progress.

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