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Flavones as Colorectal Cancer Chemopreventive Agents—Phenol-*O*-Methylation Enhances Efficacy

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Abstract Flavonoids occur ubiquitously in plants, and some possess preclinical cancer chemopreventive activity. Little is known about molecular features that mediate chemopreventive efficacy of flavonoids. Here, three related flavones, apigenin (4′,5,7-trihydroxyflavone), tricin (4′,5,7-trihydroxy-3′,5′-dimethoxyflavone), and 3′,4′,5′,5,7-pentamethoxyflavone (PMF), were compared in terms of their effects on (*a*) adenoma development in Apc^{Min} mice, a model of human gastrointestinal malignancies; (*b*) growth of APC10.1 mouse adenoma cells *in vitro*; and (*c*) prostaglandin E-2 generation in HCA-7 human-derived colorectal cancer cells *in vitro*. Life-long consumption of PMF with the diet at 0.2% reduced Apc^{Min} mouse adenoma number and burden by 43% and 61%, respectively, whereas apigenin was inactive. Tricin has previously shown activity in this model. IC_{50} values for murine adenoma cell growth inhibition by PMF, tricin, and apigenin were 6, 13, and 18 μmol/L, respectively. In Apc^{Min} mice that received flavones (0.2%) for 4 weeks, adenoma cell proliferation as reflected by Ki-67 staining was reduced by PMF and tricin, but not by apigenin. On incubation with HCA-7 cells for 6 hours, PMF reduced prostaglandin E-2 generation with an IC_{50} of 0.8 μmol/L, a fraction of the respective values reported for tricin or apigenin. *In silico* PMF docked into the cyclooxygenase active site with greater affinity than tricin or apigenin. The results suggest that the rank order of cancer chemopreventive efficacy in Apc^{Min} mice is PMF > tricin > apigenin, supporting the notion that the presence of *O*-methyl in the flavone molecular scaffold promotes gastrointestinal cancer chemopreventive efficacy.

Flavonoids are polyphenolic phytochemicals that occur ubiquitously in the leaves, seeds, bark, and flowers of plants, many of which are consumed with the diet. Certain flavonoids exemplified by the flavonol epigallocatechin-3-gallate contained in green tea, the isoflavone genistein from soya, and the flavonol quercetin found in apples and onions have been the focus of considerable research interest as they can prevent malignancies in rodent models of prostate, breast, or gastrointestinal carcinogenesis (1–3). More than 4,000 flavonoids have been identified to date, and only a handful of which, including epigallocatechin-3-gallate, genistein, and quercetin, have been investigated in depth. The putative cancer chemopreventive properties of most flavonoids are virtually unknown. Knowledge of molecular features that impart anticarcinogenic activity on flavonoids would allow prioritization of agents for preclinical development, but such information is extremely scarce. The flavone tricin (4′,5,7-trihydroxy-3′,5′-dimethoxyflavone; Fig. 1) contained in rice bran and other grass species has recently been shown to interfere with small intestinal carcinogenesis in the Apc^{Min} mouse (4), a model of the human heritable condition familial adenomatous polyposis coli (5). Tricin inhibited prostaglandin E-2 (PGE-2) generation in colorectal cells *in vitro* and in Apc^{Min} mice *in vivo* (4). PGE-2 is generated from arachidonic acid via the cyclooxygenase (COX) pathway. Inhibition and/or down-regulation of components of this pathway, especially of the isoenzyme COX-2, are considered to be important mechanisms of colorectal cancer chemoprevention (6).

Recently, methoxy moieties have been characterized as pharmacophor imparting anticarcinogenic properties onto flavones. 5,7-Dimethoxyflavone and 5,7,4′-trimethoxyflavone were superior to their hydroxy congeners in terms of systemic availability in rats and ability to inhibit oral cancer cell proliferation *in vitro* (7). The latter finding implies an intrinsic advantage of methoxylated flavones over hydroxylated ones in terms of growth-inhibitory properties. In the light of all this evidence, we wished to explore the role of phenyl methyl ether moieties in the adenoma-preventive efficacy of flavones in the Apc^{Min} mouse. Two analogues of tricin differing in *O*-methylation status were selected, apigenin (4′,5,7-trihydroxyflavone) and

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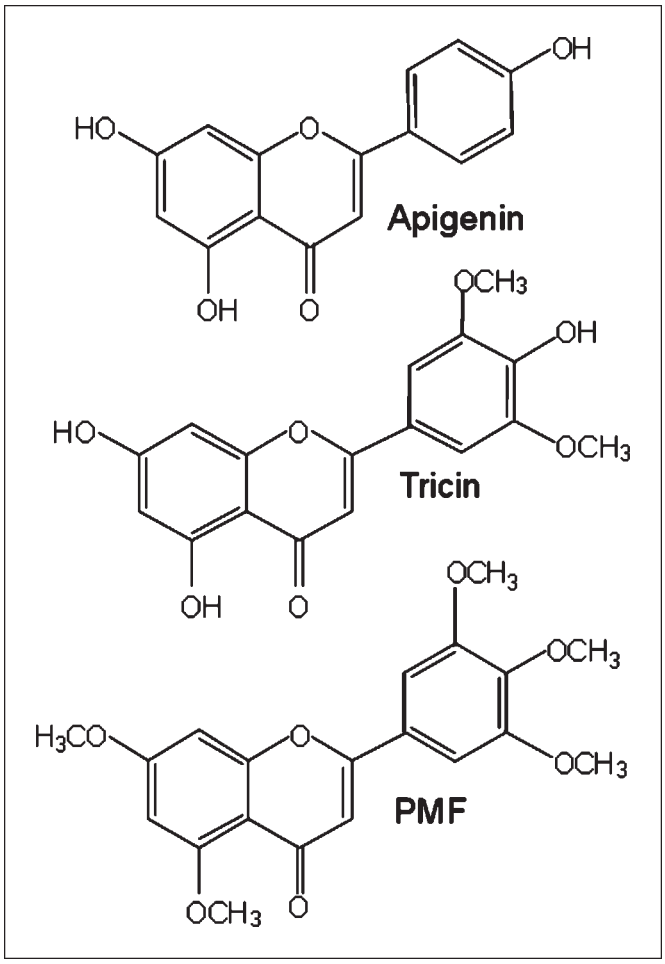

Fig. 1. Chemical structures of flavones used in this study.

3′,4′,5′,5,7-pentamethoxyflavone (PMF; for structures, see Fig. 1), and their effects were compared with those of tricin. Apigenin, des-dimethoxy tricin, occurs in many leafy vegetables and fruits and is reported to possess cancer chemopreventive properties (8). PMF, fully *O*-methylated tricin, has been found in the leaves of *Murraya paniculata*, a constituent of traditional Indonesian herbal medicines (9), and fruits of the Brazilian *Neoraputia magnifica* (10), both plants being members of the Rutaceae plant family. The only pharmacologic property of PMF reported thus far is its ability to reverse P-glycoprotein–mediated multidrug resistance in cancer cells (11). The hypothesis was tested that apigenin is less, and PMF more, capable than tricin of interfering with adenoma development, as a consequence of the relative number of methyl ether moieties harbored by these flavones. Three experimental paradigms were used to discover differences in chemopreventive efficacy between apigenin, tricin, and PMF. First, we assessed their ability to retard adenoma formation in Apc^{Min} mice *in vivo*. Adenoma number and burden were measured in mice that had received apigenin or PMF for their lifetime, and results were compared with those previously reported in this model for tricin (4). Second, the intrinsic ability of the three flavones to compromise adenoma cell growth was investigated *in vitro* in APC10.1 cells derived from adenomas in the Apc^{Min} mouse (12) and *in vivo* in Apc^{Min} mice that received flavones with their diet for the final month of their life. Third, the ability of the flavones to compromise PGE-2 generation, a potential mechanism of their chemopreventive action, was compared in human colorectal cancer cells *in vitro*. In addition, differences between the three flavones in affinity for COX enzymes, which catalyze arachidonic acid metabolism to generate PGE-2, were explored *in silico*. Overall, the work was designed to help define criteria that aid prioritization of flavones for colorectal cancer chemoprevention agent development.

Materials and Methods

Materials

PMF was synthesized as described before (13) by condensing 2′-hydroxy-4′,6′-dimethoxyacetophenone with 3,4,5-trimethoxybenzoyl chloride in pyridine. The resulting trimethoxybenzoate was subjected to Baker-Venkataraman migration to obtain a β-diketone, which was cyclized to the flavone. The identity of PMF was confirmed by the appropriate melting point and spectrometric/spectroscopic data, and it was >99% pure by high-performance liquid chromatography analysis (14). Apigenin was purchased from Apin Chemical Co. Tricin was custom synthesized by Syncom. For the cell culture experiments, flavones were dissolved in DMSO, and the final DMSO concentration in cellular incubations was 0.1%. All other chemicals were purchased from Sigma-Aldrich Chemical Co.

Animals and interventions

Breeding colonies were established in the Leicester University Biomedical Services facility using C57BL/6J Min/+ (Apc^{Min}) mice originally obtained from The Jackson Laboratory. Ear tissue from newborn mice was genotyped for the presence of the mutation using PCR as described previously (15). Experiments were carried out under animal project license PPL 80/2167, granted to Leicester University by the UK Home Office. The experimental design was vetted by the Leicester University Local Ethical Committee for Animal Experimentation and met the standards required by the United Kingdom Coordinating Committee on Cancer Research guidelines (16). Apc^{Min} mice (14-25 per group) received standard American Institute of Nutrition-93G (AIN-93G) diet or AIN-93G diet supplemented with flavone. In the case of the long-term intervention study, animals received apigenin at a dietary concentration of 0.2%, or PMF at 0.05% or 0.2%, from week 4 to the end of the their life (week 16). Both agents were investigated in two separate experiments with their own controls. In the study to assess the effect of flavones on proliferation of established adenomas, Apc^{Min} mice received standard AIN-93G diet until week 12 after weaning and then diet containing apigenin, tricin, or PMF (0.2%) up to week 16. Dietary doses of 0.2% and 0.05% equate to approximately 300 and 75 mg/kg murine body weight, respectively. Animals were culled by cardiac exsanguination under halothane anesthesia in week 16. The intestinal tract was removed and flushed with PBS. Intestinal tissue was cut open longitudinally, and multiplicity, location, and size of adenomas were recorded as described previously (15) using a magnifying glass (×5). Polyp volume was derived from polyp diameter; consistent with their histologic appearance, a hemispherical shape was assumed for the small bowel polyps and a spherical shape for colon polyps. Tumor burden is the sum of polyp volume per animal. The small intestines and colons of some animals were made into Swiss roles for histochemical analysis (see below).

Experiments in cells

APC10.1 cells (obtained from Dr. C. De Giovanni, Department of Experimental Pathology, University of Bologna, Bologna, Italy) from subculture 2 to 10 were seeded (density, 2.5×10^3 per well) and cultured in DMEM containing glucose (1 g/L) and 20% (v/v) FCS (Life Technologies) for 24 h to adhere to the dish. PMF, tricin, or apigenin

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was added to the medium to yield final concentrations of 0.1, 1, 5, 10, or 20 μmol/L, and cells were incubated with flavones for up to 6 d, and control cells with vehicle only. Cells were counted daily (Z2 Coulter Particle Count and Size Analyzer, Beckman Coulter), and growth curves were plotted. IC_{50} values, calculated on day 6 when cells were still in exponential growth phase, are the mean ± SD from three independent experiments, with each incubation conducted in triplicate. These values reflect intrinsic growth-inhibitory potency of the flavones not influenced by differential metabolic removal, as in an orientation experiment APC10.1 cells during 6 d of incubation failed to metabolize the flavones.

For assessment of effect of flavones on PGE-2 production, HCA-7 cells, derived from a mucinous adenocarcinoma of the colon, were purchased from the European Collection of Animal Cell Cultures. Cells from subculture 20 to 30 were seeded (90-mm Petri dishes, Nunc, Fisher Scientific). Initial seeding density, chosen such that cells were ~75% confluent at the time of analysis, was 3 or 2×10^6 for incubation periods of 6 or 24 h, respectively. Cells were incubated in DMEM containing Glutamax I, glucose (4.5 g/L), and 10% (v/v) FCS with or without tricin, apigenin, or PMF (0.1-20 μmol/L) for 6 or 24 h.

Immunohistochemistry

To evaluate the effect of the flavones on cell proliferation, formalin-fixed small intestine and colon tissues from Apc^{Min} mice were examined. Cell proliferation was assessed using a rabbit polyclonal Ki-67 antibody (NCL-Ki-67p, Novocastra, Leica Biosystems Newcastle Ltd.). Briefly, paraffin-embedded sections (4 μm) mounted on Vectabond-coated slides were dewaxed (65°C, 20 min) and hydrated by a graded series of alcohol rinses. The antigen was unmasked by microwaving sections (20 min) in Tris-EDTA buffer (pH 10). Endogenous peroxidase activity was inactivated by incubation of slides in hydrogen peroxide (3%) for 10 min; nonspecific binding was blocked with protein block solution provided with the NovoLink detection kit (Novocastra). Sections were incubated with primary antibody (dilution, 1:2,000) overnight at 4°C. After washing (PBS) the sections, staining was detected using the NovoLink kit. All slides were scored by two independent observers blinded to the treatment group. The proliferation index (PI) was quantitated as percentage of Ki-67–positive epithelial cells from 30 crypts and 10 villi of small bowel and 30 crypts of colon or adenomas per section, randomly selected, as described previously (17).

Prostaglandin assay

Aliquots (1 mL) of HCA-7 cell supernatant were removed for determination of PGE-2 by immunoassay, done according to the manufacturer's instructions using a PGE-2 immunoassay kit (Cayman Chemical Co.). In this assay, PGE-2 competes with a fixed amount of alkaline phosphatase–labeled PGE-2 for binding sites on a mouse monoclonal antibody coated onto the microplate. Following a wash step, bound enzyme activity (as reflected by absorbance at 405 nm) was quantified on a FLUOstar OPTIMA plate reader (BMG Labtechnologies GmbH). The intensity of the color was inversely proportional to the concentration of PGE-2 in the sample tested. PGE-2 values were corrected to cellular protein levels (18), thus allowing interpretation in terms of "cellular" PGE-2. In a control experiment omitting cells, flavones did not affect PGE-2 stability.

In vivo, PGE-2 is rapidly converted to its 13,14-dihydro-15-keto metabolite, which in turn degrades avidly to PGA products (19). 13,14-Dihydro-15-keto PGE-2 and its breakdown products (referred to in Results as "PGE-2 metabolite") were measured in plasma of mice that had consumed flavones by enzyme immunoassay using the prostaglandin E metabolite EIA kit from Cayman Chemical Co., which involves conversion of all PGE-2–derived species to a stable derivative.

Molecular modeling

Molecular structures of apigenin, tricin, and PMF were generated and energy minimized using HyperChem 8.0 (Hypercube, Inc.). The ligands were then docked into the indomethacin-bound structure of COX-2 (PDB entry: 4COX; ref. 20) and the ibuprofen-bound structure

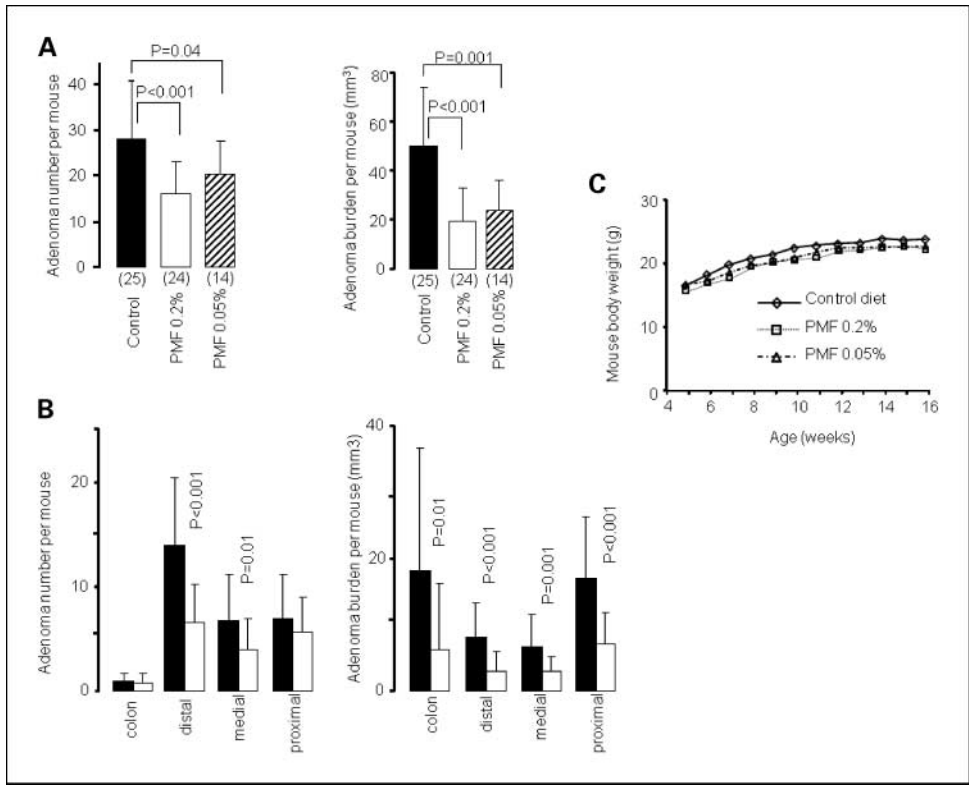

Fig. 2. Adenoma number (*left*) and burden (*right*) in the whole intestinal tract (*A*) or the colonic, distal, medial, or proximal subsections of the intestine (*B*), and whole body weight (*C*), in Apc^{Min} mice, which received PMF from week 4 to the end of the experiment (week 16). Mice were either on control diet (*black columns* in *A* and *B*, *rhombi* and *solid line* in *C*) or on diet adulterated with PMF at a concentration of 0.2% (*white columns* in *A* and *B*, *squares* and *dotted line* in *C*) or 0.05% (*hatched columns* in *A*, *triangles* and *broken line* in *C*). Numbers of animals were 25, 24, and 14 in the control, 0.2% PMF, and 0.05% PMF groups, respectively. *A* and *B*, *columns*, mean; *bars*, SD. Statistical comparison was by Student's *t* test; *P* values in *A* and *B* are indicated above bars. SD values in *C* have been omitted for the sake of clarity of presentation; they were between 13% (0.2% PMF group in week 5) and 17% of the mean (0.2% PMF group in week 16). For details of animal experiments, see Materials and Methods.

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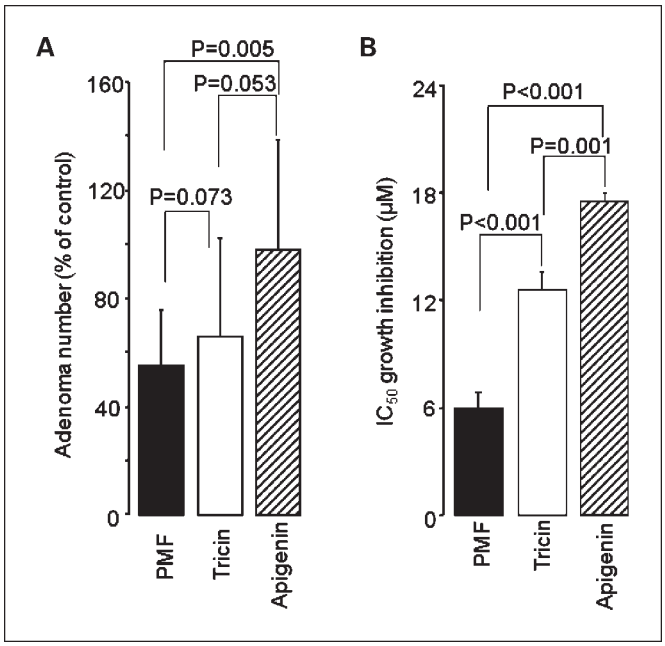

Fig. 3. Effect of apigenin (*hatched column*), tricin (*white column*), or PMF (*black column*) on adenoma development in Apc^{Min} mice *in vivo* (*A*) when administered at 0.2% with the diet, and on the growth of APC10.1 cells *in vitro* (*B*). Results in *A* stem from three experiments conducted at separate times, and adenoma reduction by flavones is presented as percentage of respective adenoma number in mice on control diet. Animal group numbers in the three studies were as follows: 20 controls and 15 on apigenin, 15 controls and 21 on tricin, and 25 controls and 24 on PMF; the results for tricin have been reported before (4). Cells (in *B*) were exposed to flavones for 6 d, and IC_{50} values were calculated from three separate experiments. *Columns*, mean; *bars*, SD. Statistical comparison was by Student's *t* test (*A*) or one-way ANOVA with post hoc Tukey's test and Bonferroni correction (*B*). Respective *P* values are indicated above bars.

of COX-1 (PDB entry: 1EQG; ref. 21) using the molecular docking program GOLD 4.0 (22). In a control experiment, indomethacin and ibuprofen were redocked into 4COX and 1EQG, respectively. All docking solutions for COX-1 and COX-2 were ranked according to the GOLD fitness function.

Statistical analyses

Mean and SD values were calculated using Microsoft Excel software. Statistical significance was evaluated using Statistical Package for the Social Sciences version 16 (Windows XP). Effects of PMF on adenoma development or of flavones on adenoma cell proliferation *in vivo* or on PGE-2 levels in cultured cells were compared by Student's *t* test. Effects of flavones on cell growth *in vitro* were subjected to one-way ANOVA with post hoc Tukey's test and Bonferroni correction. *P* values of <0.05 were considered significant.

Results

Effect of apigenin and PMF on adenoma development in Apc^{Min} mice

Apc^{Min} mice received apigenin or PMF in their diet at 0.2%, the concentration at which tricin has been shown to retard adenoma numbers by 33% (4). Apigenin had no efficacy in this model, as mice on apigenin (n = 15) developed overall 32.4 ± 21.8 intestinal adenomas per mouse compared with control mice (n = 20), which presented with 33.5 ± 14.1 adenomas. Likewise, adenoma burden was unaffected by apigenin (data not shown). These results are consistent with a recently published investigation of apigenin in the Apc^{Min} mouse (23). In contrast, PMF potently reduced adenoma development (Fig. 2). Total adenoma multiplicity and burden in mice on PMF (0.2%) were decreased by 43% and 61%, respectively, compared with animals on normal diet (Fig. 2A). Although the ameliorating effect of PMF on adenoma number was most marked in the distal section of the intestine, PMF failed to affect the number of colonic adenomas (Fig. 2B). The reducing effect of PMF on adenoma burden was similar along the whole intestinal tract, including the colon (Fig. 2B). PMF at the lower concentration of 0.05% reduced tumor number by 28% and tumor burden by 52% (Fig. 2A) compared with control mice, thus less potently than at the higher 0.2% dose. Intervention with PMF at either 0.2% or 0.05% did not affect mouse body weight (Fig. 2C).

The effects of lifetime administration of PMF, apigenin, or tricin at 0.2% in the diet on Apc^{Min} tumor number *in vivo* were compared in terms of reduction in adenoma number expressed as percentage of control (Fig. 3A), including data for tricin published previously (4). PMF was significantly more potent than apigenin, whereas the differences between PMF and tricin or between tricin and apigenin were just below levels of significance (Fig. 3A). Adenoma burden could not be compared between the three flavones, as it has not been investigated for tricin (4). The rank order for potency *in vivo* of the three flavones was PMF > tricin > apigenin.

Effect of apigenin, tricin, and PMF on APC10.1 cell growth

Inhibition of the growth of preneoplastic cells is an important chemopreventive mechanism. Therefore, we compared the effects of apigenin, tricin, and PMF on the growth of APC10.1 adenoma cells. Figure 3B shows the IC_{50} values for growth inhibition. PMF was approximately twice as potent as tricin, and almost thrice more potent than apigenin, in terms of cell growth inhibition. The rank order of growth-inhibitory potency in APC10.1 cells *in vitro* was PMF > tricin > apigenin, paralleling that observed for ability to compromise adenoma development in Apc^{Min} mice *in vivo* (Fig. 3A).

Effect of apigenin, tricin, and PMF on adenoma proliferation in Apc^{Min} mice

Adenoma cell proliferation as reflected by immunohistochemical staining for Ki-67 was studied in Apc^{Min} mice that received either PMF (0.2% or 0.05% in the diet) for their lifetime or the three flavones at 0.2% in the diet for 4 weeks from week 12 when adenomas were already established. The 4-week exposure design allows comparison of effects of intervention with flavones on cell proliferation in late-stage adenomas. Rolled-up intestine was subjected to immunohistochemical inspection for staining with Ki-67, which is a granular component of the nucleolus expressed exclusively in proliferating cells and used as a prognostic marker in human neoplasias. The PI in adenomatous crypts of mice that received PMF for their lifetime was significantly decreased at both dietary doses, in the case of the 0.2% dose by 11%, compared with controls (Fig. 4A and B). After intervention for 4 weeks only, adenoma number was not affected by any of the flavones. The PI in adenoma cells in mice on tricin or PMF was reduced modestly but significantly, by 2.9% or 3.4%, respectively, when compared with that in mice on

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control diet (Fig. 4B). In contrast, apigenin did not affect the PI. The magnitude of the reduction seen with tricin and PMF after intervention for only 4 weeks is unlikely to have physiologic consequences, suggesting that mice need to be exposed to flavones for longer periods to achieve a substantial decrease in adenoma proliferation rate. The decrease, although small, suggests that the rank order of effect on proliferation in adenoma tissue is PMF = tricin > apigenin. Proliferation of cells in normal intestinal crypts or villi was not affected by any of the interventions.

Levels of apoptosis in adenomas or normal intestinal tissue of Apc^{Min} mice as reflected by staining for cleaved caspase-3 were extremely low, confounding the detection of meaningful differences between mice on control diet and those that had ingested flavones.

Effect of PMF on PGE-2 generation in HCA-7 cells

The ability of PMF to interfere with the COX pathway and affect PGE-2 generation was studied in HCA-7 cells *in vitro*. HCA-7 cells were used in this experiment because they generate abundant PGE-2 and express all components of the COX pathway. PMF inhibited PGE-2 production with IC_{50} values of 0.8 or 5.8 μmol/L after incubation for 6 or 24 hours, respectively (Fig. 5). COX-2 expression was not affected by PMF in these cells (result not shown). The PGE-2 inhibitory potency of PMF is similar to that exerted by the putative cancer chemopreventive red grape constituent resveratrol in HCA-7 cells (24), albeit an order of magnitude inferior to that reported for selective COX inhibitors, exemplified by celecoxib, in HT29 cells (25). We have previously shown that apigenin and tricin can ameliorate PGE-2 generation in HCA-7 cells under conditions identical to those used here for PMF (26). The IC_{50} values for tricin and apigenin after incubation for 6 hours were approximately 10 and 30 μmol/L, respectively, and the respective values after incubation for 24 hours were approximately 20 and 40 μmol/L (26). So the rank order of ability to compromise PGE-2 generation *in vitro* is therefore PMF > tricin ≥ apigenin.

PMF affected PGE-2 generation also in Apc^{Min} mice *in vivo*. In 12 mice that had received PMF at 0.2% with their diet for their lifetime, plasma PGE-2 metabolite concentrations were reduced to 60.2 ± 21.1% ($P = 0.02$) compared with mice on control diet, whereas PMF at 0.05% had no effect (result not shown).

Molecular modeling of affinity of apigenin, tricin, and PMF to COX

In the light of the differential effects of the flavones on PGE-2 generation, the hypothesis was tested *in silico* that they possess differential affinities to COX enzymes. Ligand docking suggests that all three flavones can occupy the active site of COX-2 (Fig. 6) or COX-1 (data not shown). The structures of the docked complexes imply that the binding mode for apigenin and tricin is very similar (Fig. 6A), whereas PMF shows a different orientation in the binding site (Fig. 6B). For PMF, tricin, and apigenin, the GOLD fitness scores, which reflect goodness of fit of the docked COX-2 enzyme complex, were 57.2, 52.8, and 51.3, respectively. Although these values are lower than those for the potent COX-2 inhibitor indomethacin, which is 63.7, they suggest that PMF docks into COX-2 with higher affinity than tricin or apigenin. The GOLD fitness scores for docking of the three flavones in COX-1 displayed similar differences (data not shown). Therefore, the relative order of affinity to COX *in silico* is PMF > tricin > apigenin, consistent with ability to compromise PGE-2 generation. Results of a study of COX enzyme inhibition by the three flavones in a cell-free system using COX-2 and COX-1 enzyme preparations from sheep placenta and sheep seminal vesicles, respectively,

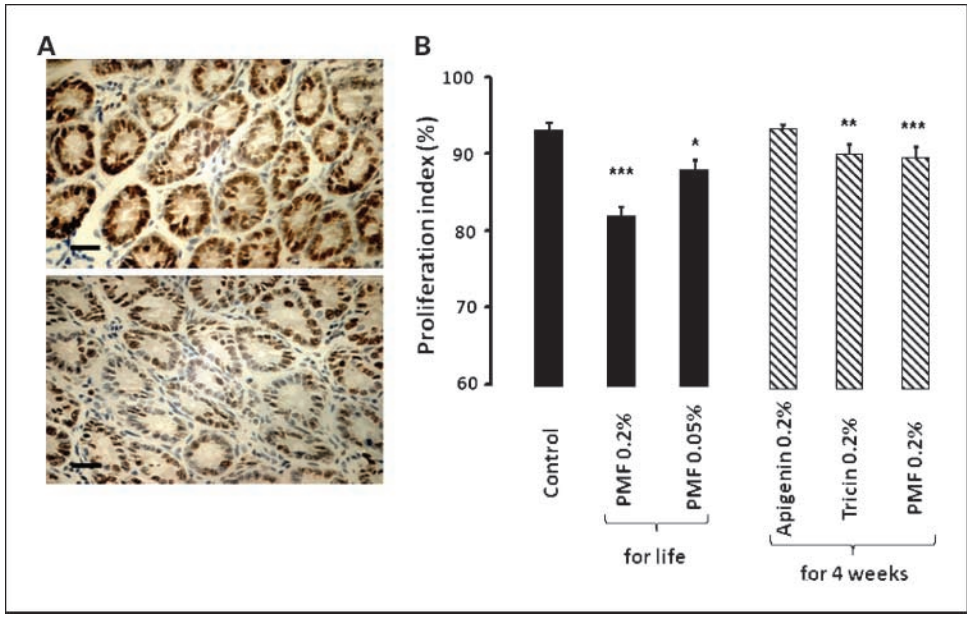

Fig. 4. Representative immunohistographs of adenomas stained for Ki-67 (*A*) in Apc^{Min} mice that had received either control diet (*top*) or diet containing PMF at 0.2% in the diet (*bottom*) from week 4 to the end of the experiment (week 16), and effect of dietary flavones on proliferation of adenoma cells in Apc^{Min} mice (*B*) that had received either PMF (0.05% or 0.2% in the diet) from week 4 to the end of the experiment (*black columns*) or apigenin, tricin, or PMF (each at 0.2% in the diet) from week 12 for 4 wk (*hatched columns*). *A,* bars, 25 μm. Note that the ordinate in *B* commences at 60%. *Columns*, mean (n = 6-8 mice); *bars*, SD. Statistical comparison was by Student's *t* test. *, $P < 0.006$; **, $P = 0.0003$; ***, $P = 0.0001$, with respect to controls.

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were inconclusive. The flavones inhibited enzyme activity differentially when assessed using a kit in which the COX peroxidase reaction is monitored with a cosubstrate generating a chemiluminescent species. However, chemical interaction of tricin with the cosubstrate metabolite quenched its chemiluminescence, confounding meaningful assay interpretation.

Discussion

The results described above define structural molecular features that determine the intestinal cancer chemopreventive activity of these three flavones in Apc^{Min} mice. Among the flavones under study here, the rank order of potency as adjudged by magnitude of inhibition of (*a*) adenoma development in the Apc^{Min} mouse model, (*b*) proliferation of Apc^{Min} adenoma cells *in vitro* or in the intact animal *in vivo*, and (*c*) PGE-2 generation in cells *in vitro* was PMF > tricin > apigenin. This rank order is inversely related to number of *O*-methyl ether functions harbored by the cogeneric flavone molecules, which is five, three, or zero, respectively. Differences between the flavones in their ability to inhibit the generation of PGE-2 were mirrored by their affinity to COX enzymes as reflected by molecular docking studies. The results raise the possibility that the presence of *O*-methyl ether moieties in flavones predisposes for ability to arrest intestinal adenoma cell growth, interfere with adenoma formation, and inhibit PGE-2 generation. The superiority of flavones containing methoxy functionalities over their hydroxyl-harboring counterparts as gastrointestinal anticarcinogens *in vivo* is shown here for the first time, although pharmacologic advantages that they may offer have already been reported (7). The methoxylated flavones 5,7-dimethoxyflavone and 5,7,4′-trimethoxyflavone were markedly more potent inhibitors of the growth of SCC-9 human oral carcinoma cells than their corresponding unmethylated analogues chrysin and apigenin, although this potency difference did not extend to other cancer cell types derived from the human pharynx, esophagus, or breast (7). 5,7-Dimethoxyflavone and 5,7,4′-trimethoxyflavone also exhibited systemic availability in rats superior to that of the corresponding hydroxy congeners, most likely a consequence of enhanced metabolic stability (7). Analytic work that accompanied the pharmacologic evaluation of the flavones described here suggests that mean steady-state plasma concentrations of parent flavone in mice that had received PMF, tricin, or apigenin at 0.2% in the diet are 1.2, 0.5, and 0.09 μmol/L (14, 27), respectively, consistent with the differential presence in the molecule of metabolic stability-imparting methoxy moieties. Thus, the rank order of bioavailability is PMF > tricin > apigenin, paralleling differences in efficacy.

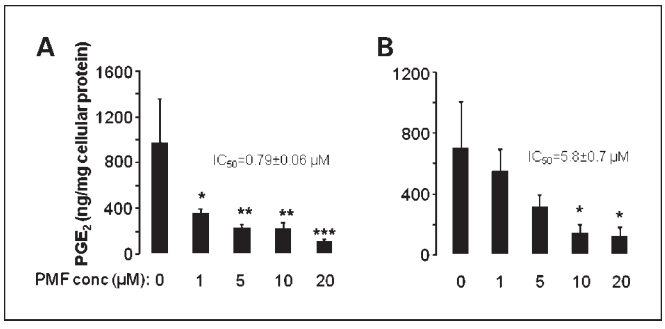

Fig. 5. PGE-2 concentrations in HCA-7 cells incubated with PMF for 6 h (*A*) or 24 h (*B*). *Columns*, mean of three experiments each done in triplicate; *bars*, SD. *, $P < 0.02$; **, $P = 0.004$; ***, $P = 0.001$, by Student's *t* test.

It is not clear whether methoxy moieties augment the chemopreventive efficacy of polyphenols other than flavones. Methylation of hydroxy in isoflavones has been associated with decreased rather than increased biological activity (28). On the other hand, methylation of the two hydroxyl functions of curcumin generated a molecule with antiproliferative and apoptogenic properties in human colonic HCT116 cells *in vitro* superior to those of curcumin (29). Future studies need to define the role that *O*-methylation may play as a chemopreventive pharmacophore in polyphenols.

Importantly, the work described here identifies PMF as a novel agent with dose-related and potent adenoma-retarding activity in the Apc^{Min} mouse. Apc^{Min} mouse survival was not measured in this study, as the generally accepted experimental design in this paradigm entails assessment of tumor number and/or burden in weeks 16 to 18, when adenomas in control mice are fully formed. However, interference with Apc^{Min} adenomagenesis is undoubtedly accompanied by increase in survival time, as borne out for example by a study of flurbiprofen (30): A dose, which reduced adenoma number by two thirds and burden by a third, extended murine survival to at least week 26, whereas only 30% of control mice survived that long. Although the value of the Apc^{Min} mouse as predictor of colorectal cancer chemopreventive activity in humans is a subject of debate, results obtained in this model (31, 32) correctly forecast the efficacy of COX inhibitors, such as sulindac and celecoxib, to retard adenoma recurrence in humans (33, 34). To our knowledge, there has been no other natural product polyphenol described thus far, which has displayed adenoma-retarding efficacy in the Apc^{Min} mouse model superior to that observed here for PMF. The activity of PMF in this model strongly advocates further investigation, especially assessment of its safety, with the ultimate aim to explore its suitability for advancement to clinical development as a colorectal cancer chemopreventive agent. The lack of detrimental effect of intervention with PMF on mouse body weight augurs favorably for its safety.

It is likely that PMF, in analogy to many other naturally occurring cancer chemopreventive agents, exerts its adenoma-reducing activity via a variety of anticarcinogenic mechanisms, which will have to be discovered in future studies. Guided by results obtained previously for tricin (4, 26), we focus here on one mechanism—inhibition of prostaglandin production. Compromising generation of cocarcinogenic and tumor-promotory prostaglandins by interference with components of the COX pathway is now considered a prime mechanism by which naturally occurring multitargeted cancer chemopreventive agents can prevent intestinal malignancies (6). PMF inhibited PGE-2 production potently in colon cancer cells *in vitro* and Apc^{Min} mice *in vivo*, and it is conceivable that interference with the COX pathway is one of the mechanisms by which PMF retards adenoma development. Assessment of the ability of the three flavones to dock with COX enzymes *in silico* suggests that, of the three flavones, PMF has the highest affinity to COX enzymes.

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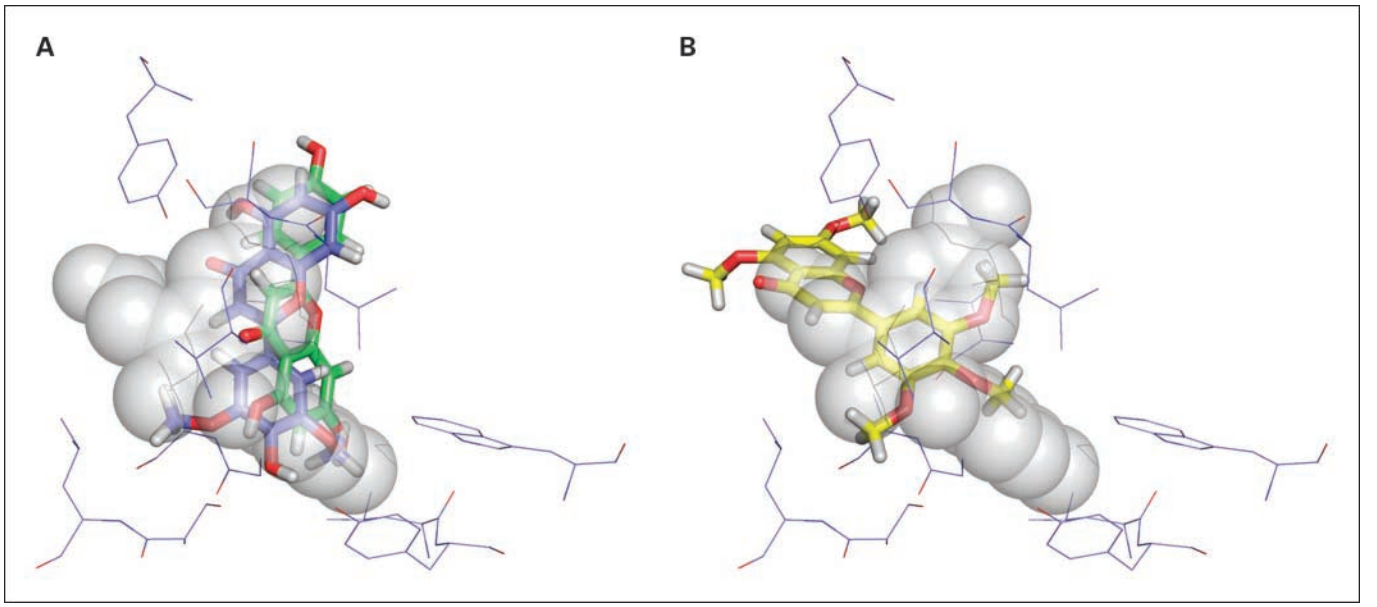

Fig. 6. Molecular docking of apigenin, tricin (*A*), and PMF (*B*) into the indomethacin-bound COX-2 enzyme. Residues in the proximity of the active site of COX-2 are indicated as blue lines. Flavones are shown in CPK-style stick representation with carbon atoms in green, blue, or yellow for apigenin, tricin, or PMF, respectively. For comparison, indomethacin in the active site of COX-2 is represented by semitransparent spheres. Figures were generated using PyMOL (35).

In summary, the work presented here suggests that *O*-methyl moieties augment the colorectal cancer chemopreventive properties of flavones. The flavonoid ring harbors nine positions, which can bear hydroxy or methoxy functions, and not surprisingly numerous polymethoxyflavones occur in the plant kingdom. Thus, it may be apposite to compare their growth-inhibitory potency in APC10.1 cells to tentatively identify potentially efficacious analogues and subsequently examine the most potent ones in the Apc^{Min} mouse model *in vivo*. Based on its preclinical adenoma-retarding efficacy documented here, PMF is a promising candidate for further development as a putative colorectal cancer chemopreventive agent. Ever increasing experimental costs make it essential to undertake research that helps rationalize the choice of molecules for chemopreventive agent development. Further work of the type outlined here, which helps identify molecular structural features important for cancer chemopreventive activity, will be extremely useful to maximize and optimize our armamentarium of cancer preventive interventions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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