Preclinical Colorectal Cancer Chemopreventive Efficacy and p53-Modulating Activity of 3′,4′,5′-Trimethoxyflavonol, a Quercetin Analogue

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Abstract

Some naturally occurring flavonols, exemplified by quercetin, seem to possess experimental cancer chemopreventive efficacy. Modulation of p53 is a mechanism thought to contribute to their activity. The hypothesis was tested that a synthetic flavonol, 3′,4′,5′-trimethoxyflavonol (TMFol), can interfere with tumor development and p53 expression in two models of colorectal carcinogenesis, ApcMin mice and human-derived HCT116 adenocarcinoma-bearing nude mice. Mice received TMFol with their diet (0.2%) from weaning to week 16 in the case of ApcMin or from either day 7 before ("TMFol early") or day 7 after ("TMFol late") tumor inoculation in HCT116 mice. The ability of TMFol to affect tumor proliferation or apoptosis, as reflected by staining for Ki-67 or cleaved caspase-3, respectively, was studied in HCT116 tumors. TMFol tumor levels were measured by high-performance liquid chromatography. Consumption of TMFol reduced small intestinal adenoma burden in ApcMin mice by 47%, compared with control mice (P < 0.002). The TMFol early regimen approximately halved HCT116 tumor size (P < 0.05), decreased tumor proliferation, and increased apoptosis, whereas the TMFol late regimen had no significant effect when compared with controls. In tumor tissues from mice, in which TMFol reduced tumor development, p53 expression was increased 3-fold in ApcMin and 1.5-fold in HCT116 tumor–bearing mice (P = 0.02). TMFol increased p53 also in cells derived from these tumors. TMFol was detected in HCT116 tumors, but levels did not correlate with tumor burden. TMFol was not mutagenic in the Ames test. The results suggest that chemical modification of the flavonol structure may generate safe and efficacious cancer chemopreventive agents. Cancer Prev Res; 3(8); 929–39. ©2010 AACR.

Introduction

Results of clinical trials of certain drugs, such as aspirin (1) and tamoxifen (2), provide proof of principle that cancer chemoprevention is a viable option to reduce cancer incidence. Nevertheless, the requirement of long-term intervention in cancer chemoprevention and concerns about the safety on long-term use of such drugs in relatively healthy humans (3, 4) render the search for safe and efficacious agents highly suitable. Edible plants constitute an attractive source of novel potential cancer chemopreventive agents highly suitable. Flavonoids, exemplified by the flavonols quercetin in onions and apples, and epigallocatechin-3 gallate in tea and the isoflavone genistein in soya, are examples of phytochemicals with suspected cancer chemopreventive properties (5–7). Very little information exists about molecular features that confer pharmacologic activity on to the flavonoid scaffold. Such knowledge is required to help the rational discovery, or chemical design, of flavonoids with optimal cancer chemopreventive efficacy. Recent evidence suggests that the presence of methoxy moieties instead of, or in addition to, hydroxy in certain flavonoids augments cancer chemopreventive activity (8, 9). Prominent among the many mechanisms through which flavonoids are thought to exert their chemopreventive activity is the activation of wild-type p53 (10) and/or downregulation of its mutant counterpart (11). The tumor suppressor protein p53 is involved in DNA damage repair, cell cycle arrest, and apoptosis through transcriptional regulation of genes implicated in these pathways and by direct interaction with other proteins (12, 13). P53-inactivating mutations are present in >50% of all cancers, including colon cancer, leading to aggressive, treatment-resistant malignancies (14, 15). Small molecules, such as CP-31398, a styrylquinazoline developed by Pfizer, have been designed to target p53 and to
restore its growth suppressor function. CP-31398 reduced the growth of human colon tumor xenografts in nude mice (16) and potently compromised adenoma development in the Apc<sup>Min</sup> mouse, accompanied by a marked increase in adenomatous p53 levels (17). The Apc<sup>Min</sup> mouse is a model of colorectal carcinogenesis associated with an Apc mutation (18), and this model is frequently used in the preclinical identification of candidate colorectal cancer chemopreventive agents for clinical development.

Flavonols, such as quercetin (3′,4′,5,7-tetrahydroxyflavonol), have been arguably the focus of more pharmacologic investigations than most other flavonoids. Quercetin has shown cancer chemopreventive properties in rodent models of carcinogenesis of the colon, mouth, cervix, and lung (5, 19–21), and it has been subjected to a phase 1 clinical trial in cancer patients (22). Its des-hydroxy cogeners fisetin (3′,4′,7-trihydroxyflavonol; for structures, see Fig. 1) has recently been found to possess preclinical prostate cancer chemopreventive activity (23). However, a serious toxicologic impediment of many flavonols, which militates against their development as cancer chemopreventive agents, is their mutagenicity as reflected by the Ames test. Quercetin at levels of typically 20 μg or more per plate (24–26) and, to a lesser extent, fisetin at ~500 μg per plate (27), were shown to be mutagenic. There is also limited evidence for the carcinogenicity of quercetin in animals (28, 29). These toxicologic properties of quercetin mitigate against its further clinical development. Toxicophoric structural features considered responsible for the mutagenic potential of flavonols include phenolic hydroxy moieties in the A and B rings of the molecular scaffold (24, 26, 27).

Taking all these findings into consideration, we hypothesized that it might be possible to synthesize a flavonol with optimized pharmacologic and toxicologic properties. We surmised that a flavonol molecule devoid of hydroxy moieties in the A ring and bearing methoxy rather than hydroxy functionalities in the B ring may be nonmutagenic and possess cancer chemopreventive efficacy. To test this hypothesis, we synthesized 3′,4′,5′-trimethoxyflavonol (TMFol) and explored its preclinical cancer chemopreventive properties. Initially, we compared its ability to compromise the growth of APC10.1 cells with that of its two naturally occurring flavonol cogeners quercetin and fisetin. APC10.1 cells have been derived from adenomas of Apc<sup>Min</sup> mice (30).

Potent inhibition of the growth of APC10.1 cells in vitro has recently been suggested to predict the ability of a compound to interfere with adenoma development in the Apc<sup>Min</sup> mouse in vivo (31). As TMFol exerted compelling APC10.1 cell growth–inhibitory activity, we investigated its effect on adenoma development in Apc<sup>Min</sup> mice in vivo.

**Fig. 1.** Effect of quercetin (A), fisetin (B), or TMFol (C) on the growth of APC10.1 cells. Cells were counted after exposure to flavonols for 6 d. Columns, mean of three independent experiments, each conducted in triplicate; bars, SD. Chemical structures and IC<sub>50</sub> values for growth inhibition are inserted. IC<sub>50</sub> values are significantly different from each other (P < 0.001 by ANOVA).
Efficacy of TMFol in vivo was corroborated in a second colorectal carcinogenesis model, nude mice bearing the human colon adenocarcinoma–derived HCT116 xenograft. As TMFol compromised tumor development in vivo, we explored whether its efficacy is accompanied by the alteration of tumor p53 expression. We also measured TMFol levels in murine tumor tissue to relate activity to the presence of pharmacologically active agent. The potential mutagenicity of TMFol was investigated in the Ames reverse mutation assay using a panel of Salmonella Typhimurium strains, in which quercetin has shown mutagenic activity (24–27). Overall, the work was designed to discover safe and efficacious cancer chemopreventive agents structurally based on pharmacologically interesting plant constituents.

Materials and Methods

Chemicals and reagents

TMFol, synthesized as previously described (32), was >99% pure as determined by high-performance liquid chromatography (HPLC) analysis. 2′,5′,5,6,7,8-Hexamethoxyflavone used as an internal standard in the HPLC analysis was supplied by the National Cancer Institute Developmental Therapeutic Programme Open Compound Repository. HPLC fluorescence grade methanol was purchased from Fisher Chemicals, and water for analysis was purchased in a Nano-Pure water purification system. All other chemicals were obtained from Sigma Corp. Antibodies were purchased from DAKO (versus p21, and clone DO-7 versus p53) or Cell Signaling Technology (clone b21 Versus p21, and clone 1C12 versus p53). Mouse and rabbit secondary antibodies were purchased from Sigma.

Cell growth experiments

APC10.1 cells, kindly provided by Dr. C. De Giovanni (Cancer Research Section, University of Bologna, Bologna, Italy), were cultured in DMEM supplemented with 20% fetal bovine serum (Life Technologies). HCT116 human colon adenocarcinoma cells were obtained from the American Type Cell Collection and maintained in McCoy’s 5A (Life Technologies) medium supplemented with 10% fetal bovine serum.

APC10.1 or HCT116 cells from subculture 2 to 20 were seeded at a density of 2 × 10^5 onto 24-well plates and allowed to adhere overnight. TMFol, quercetin, or fisetin dissolved in DMSO were added to cellular suspensions to yield final concentrations of 0.1 to 5, 0.1 to 40, or 0.2 to 8 μmol/L, respectively. Cells were incubated for up to 6 days; control cells were incubated with vehicle only. The amount of DMSO added (0.1% final concentration) on its own did not interfere with cell growth. Cells were washed with PBS, harvested by trypsinization, and resuspended in cell culture medium (1 mL), which was diluted 10-fold with Isoton II buffer (Beckman Coulter). An aliquot (0.5 mL) of the cell suspension was counted using a Z2 Coulter Particle Counter with Size Analyzer (Beckman Coulter). Growth curve experiments were conducted in triplicate, and IC_{50} values were calculated from a plot of cell number on day 6 (percentage of vehicle control) versus agent concentration, using the linear phase of the curve.

Animals and TMFol dose

Breeding colonies were established using C57BL/6l 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 previously described (33). Female MF-1 outbred nude mice (30-40 g) were obtained from Harlan UK and ear punched for identification. Mice were kept in the Leicester University Biomedical Services facility at 20 to 23°C under conditions of 40% to 60% relative humidity and a 12-hour light/dark cycle. 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 (34). Mice received standard AIN93G diet (controls) or AIN93G diet supplemented with TMFol at 0.2%. Similar dietary doses have been commonly used in preclinical chemoprevention studies of other polyphenols including curcumin (33), resveratrol (35), and anthocyanins (36). For further justification of the dose, see Discussion.

Intervention in Apc^{Min} mice

Apc^{Min} mice (n = 19–21 per group with equal numbers of males and females) received diet or diet supplemented with TMFol from week 4 to the end of their life (week 16). Animals were killed in week 16 by cardiac exsanguination under halothane anesthesia. The intestinal tract was removed and flushed with PBS. Intestinal tissue was cut open longitudinally, and multiplicity, location, and size of adenomas in the small and large intestine were recorded using a magnifying glass (×5). Polymp diameter was measured using digital calipers. Consistent with their histologic appearance, a hemispherical shape was assumed for small bowel polyps (volume = 2/3πr^3), with r = radius, and a spherical shape was assumed for colon polyps (volume = 4/3πr^3). Tumor burden is defined as total polyp volume per animal as described before (37).

Intervention in HCT-116 tumor–bearing nude mice

HCT116 cells (1 × 10^6) suspended in a Matrigel-serum-free medium (1:1; 100 μL; Becton Dickinson) were injected s.c. into the right flank of female nude mice of 8 weeks of age under light halothane anesthesia. Mice (n = 14–15 per group) received TMFol commencing either 7 days before or 7 days after cell inoculation. Mice were weighed weekly, and tumor size was measured thrice per week using callipers. Tumor volume was calculated by the formula length × width^2/2, in which length is the larger and width the smaller diameter of the tumor (in millimeters; ref. 38). Animals were culled 3 weeks after tumor cell inoculation, when tumors in nontreated mice reached the maximal size (17 mm in length) permissible.
under U.K. animal welfare stipulations. Tumor tissue samples were snap frozen (liquid nitrogen) and stored at -80°C until analysis by Western blotting or HPLC, or fixed in 10% formalin and histologically processed until analysis by immunohistochemistry.

**Immunohistochemistry**

Formalin-fixed HCT116 tumor tissue was stained for Ki-67 using a rabbit polyclonal Ki-67p antibody from Novocastra (Leica Biosystems Newcastle Ltd) or for cleaved caspase-3 using cleaved caspase-3 Asp 175 polyclonal antibody from Cell Signaling Technology. Brieﬂy, paraffin-embedded sections (4 μm) mounted on polysine-coated slides were dewaxed (65°C, 20 min) and hydrated through a graded series of alcohol rinses. Antigen was unmasked by microwaving sections (20 min) in Tris-EDTA buffer (pH 9). Endogenous peroxidase activity was inactivated by incubation of slides with hydrogen peroxide (3%, 10 min); nonspeciﬁc binding was blocked with protein blocking solution (Novocastra). Sections were incubated with primary antibody (dilution 1:2,000 for Ki67, 1:200 for caspase-3) overnight at 4°C. After washing sections (PBS), the tissue-antibody reaction was visualized using a commercial kit (NovoLink, Novocastra). All slides were scored by two independent observers blinded to the treatment group. Proliferative and apoptotic indices were quantitated as the percentage of epithelial cells from 10 random ﬁelds of view per section that stained positive for Ki-67 or cleaved caspase-3, respectively. Representative ﬁelds were selected, and the total number of epithelial cells and the number of positively staining epithelial cells were counted (magnification, ×40; Leitz Orthoplan microscope, Leica DC 300 camera) for each sample. Differences in counts between the observers were <10%, and both noted the same differences between cohorts. Acquisition software was Adobe Photoshop version 7. Total numbers of epithelial cells counted on each slide were 2,312 ± 307 and 2,340 ± 154 (mean ± SD, n = 15) for Ki-67 and cleaved caspase-3 staining, respectively.

**Western blot analysis**

Tumor tissue from control mice or mice that received TMFol was homogenized with lysis buffer [1:4, w:v. PBS; 1% NP40; 0.5% sodium deoxycholate; 0.1% SDS; with protease inhibitors phenylmethylsulfonyl fluoride (1 mmol/L), aprotinin (2 μg/mL), and leupeptin (5 μg/mL); and phosphatase inhibitors sodium vanadate (1 mmol/L) and sodium fluoride (1 mmol/L)]. The homogenate was placed on ice (15 min) and centrifuged (20 min, 10,000 × g, 4°C). Cells were grown to 60% to 80% confluency, followed by exposure for 72 hours to TMFol. Cells were centrifuged (5 min, 10,000 × g, 4°C), and the cell pellet was suspended in lysis buffer. Protein concentration in tissue or cell lysate was determined using the Bio-Rad protein assay (Bio-Rad) with bovine serum albumin as standard. An aliquot of protein lysate (50 μg) was separated by SDS-PAGE and transferred for 1 hour onto a nitrocellulose membrane (Schleicher and Schuell GmbH). Blots were blocked for 2 hours with 5% skimmed milk in PBS/Tween 0.05% (PBS-T) and probed with a speciﬁc primary antiserum in PBS containing 0.05% PBS-T and 5% nonfat dry milk (4°C, overnight). After washing (PBS-T), blots were treated with horseradish peroxidase–conjugated secondary antibody for 1 hour and washed (5× for 5 min) in PBS-T. Proteins were detected by the enhanced chemiluminescence system (Geneﬂow).

**Assays for p21 and Bax luciferase activity and p21 real-time PCR**

HCT116 were seeded in triplicate (30,000/well, 12-well plate). A day later, 400 ng of reporter plasmid pGL2 (Promega Italia SRL) containing the ﬁreﬂy luciferase sequence under the control of the promoters for Bax (Bax-Luc) or p21 (p21-Luc) were transfected into cells using Lipofectamine 2000 (Invitrogen). An aliquot (50 ng) of Renilla luciferase expression vector (pRL-CMV, Promega) was cotransfected with the above plasmids to normalize for transfection efﬁciency. After 6 hours, the medium was replaced, and cells were exposed to TMFol (0-10 μmol/L) for 72 hours. Luciferase activity was measured with a Veritas microtiter plate luminometer (Turner Biosystems) and a Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions, and normalized to Renilla expression activity. Activity was expressed as fold increase compared with vehicle control.

Total RNA was isolated from HCT116 cells using the SV Total RNA Isolation System (Promega). The reverse transcription reaction was done with 1 μg of total RNA. RNA was reverse transcribed into cDNA, using a high capacity cDNA reverse transcription kit (Applied Biosystems), and ampliﬁed with Power SYBR Green PCR Master Mix (Applied Biosystems) and two real-time oligonucleotide primers, which were derived from published primer sequences for p21 (forward: 5’-GGCAGACCATGAC-\_AGTTTC-3’; reverse: 5’-CGGAATTGCAGTCCTCTTCG-3’) or glyceraldehyde-3-phosphate dehydrogenase (forward: 5’-CCC CAC ACA CAT GCA CIT ACC-3’; reverse: 5’-CCT AGT CCC AGG GCT TGT ATT-3’). Real-time PCR was done on a 7900HT Sequence Detection System (Applied Biosystems). Fold ampliﬁcation was calculated using the comparative threshold cycle method, as speciﬁed by the manufacturer.

**HPLC analysis**

Tissue sample preparation and HPLC analysis using ﬂuorescence detection were as described before (32) using a Varian Prostar HPLC system (Varian Ltd) consisting of a Varian ProStar 230 solvent delivery system, a ProStar 363 detector, and a 410 Varian autosampler. Separation was achieved on a Gemini C18 column (4.6 mm × 150 mm, 3 μm, Phenomenex Ltd) at a ﬂow rate of 0.75 mL/min, using an isocratic mobile phase of 69% methanol in 0.1 mol/L ammonium acetate buffer (pH 5.1). TMFol in tumor tissue from MF1 nude mice was quantiﬁed using 2’,5’,5,6,7,8-hexamethoxyﬂavone as an internal standard.
Reverse mutation assay
The Ames test was conducted at a facility operating to good laboratory practice (GLP) conditions by Safepharm Laboratories. *Salmonella* strains TA100, 102, and 98, obtained from the University of California at Berkeley, were exposed to TMFol dissolved in DMSO using the Ames plate incorporation method at five dose levels, both with and without a rat liver homogenate metabolism system (10% liver S9 with standard cofactors). The dose range for the experiment (50–5,000 μg/plate) was determined in a preliminary toxicity assay. Aliquots (0.1 mL) of bacterial culture were dispensed into test tubes followed by molten trace histidine-supplemented top agar (2 mL); solutions (0.1 mL) of TMFol, vehicle only, or positive control mutagen; and either S9 mix or phosphate buffer (0.5 mL). The tube content (final volume, 2.7 mL) was mixed and poured onto the surface of agar plates (9 cm diameter). The assay was conducted in triplicate for each strain and each concentration of TMFol or control mutagen.

Statistical analyses
Values shown in the results are the mean ± SD. Statistical significance was evaluated using the Statistical Package for the Social Sciences version 16 program (Windows XP). Effects of TMFol on adenoma number and burden were compared by Student's t test. Effects of TMFol on cell growth in vitro and xenograft tumor volume in vivo were subjected to one-way ANOVA with post hoc Bonferroni correction. P values of <0.05 were considered significant.

Results

Effect of flavonols on tumor cell growth in vitro
APC10.1 cells were exposed to quercetin, fisetin, or TMFol, and IC50 values were computed from the growth curves (Fig. 1). Of the three flavonols, TMFol was the most potent cell growth inhibitor, with an IC50 of about a tenth of that for quercetin and a fourth of that for fisetin. TMFol also inhibited the growth of human-derived HCT116 adenocarcinoma cells, characterized by an IC50 of 3.3 ± 1.0 μmol/L (mean ± SD, n = 3). In contrast, at the highest concentration tested (10 μmol/L), quercetin and fisetin reduced HCT116 cell growth nonsignificantly by approximately 6% and 8%, respectively, so that the IC50 values for growth inhibition were above 10 μmol/L for both flavonols. In the light of the growth-inhibitory potency of TMFol in intestinal cells in vitro, its ability to compromise tumor development in the ApcMin or HCT116 xenograft mouse models in vivo was considered worthy of investigation.

Effect of TMFol on adenoma development in ApcMin mice
ApcMin mice received TMFol at 0.2% with their diet. Tumor development was reflected by burden and number of adenomas observed in the mice at the end of the experiment in week 16. Consumption of TMFol almost halved adenoma burden in the small intestine, albeit failing to affect adenoma burden in the colon (Fig. 2A). Total adenoma number was not significantly reduced by TMFol; however, when the proximal subsection of the small intestine was considered separately, TMFol decreased both adenoma number and burden (Fig. 2B). In contrast, there was no significant effect on the middle or distal small intestine. The body weight of the ApcMin mice that received TMFol for their lifetime was indistinguishable from that of mice on the control diet (data not shown). Inspection of lung, liver, and kidney tissues failed to reveal significant pathologic abnormalities. Both observations tentatively intimate lack of toxicity of TMFol.

Effect of TMFol on HCT116 tumor growth in MF1 mice
Nude MF1 mice bearing HCT116 adenocarcinoma cells received TMFol at 0.2% with their diet. Tumor size was measured by caliper at 2- to 3-day intervals. Consumption of TMFol from a week before tumor inoculation ("TMFol early") halved tumor size beyond day 16 postinoculation (Fig. 3A). Dietary intervention with TMFol commencing a week after tumor inoculation ("TMFol late") had only a small, but not significant, tumor growth-inhibitory effect. Tumor tissue was investigated immunohistochemically for the proportion of proliferating or apoptotic cells, reflected by staining for Ki-67 or cleaved caspase-3, respectively. Ki-67 is a granular component of the nucleolus expressed exclusively in proliferating cells and used as a prognostic marker in human neoplasias. Consistent with in vivo tumor growth-inhibitory efficacy, TMFol significantly reduced cell proliferation in HCT116 tumors from mice on the TMFol early regimen, with a proliferation index of 72%, when compared with tumors in control mice, with a proliferation index of 86% (Fig. 3B). Proliferation was not reduced in tumors from mice on the TMFol late regimen. Tumor levels of apoptotic cells were elevated by 55% compared with control animals in tumors from mice on the TMFol early regimen, whereas there was no increase in apoptosis in tumors from mice on the TMFol late regimen (Fig. 3C).

Effect of TMFol on p53 expression in tumors
In adenomas from ApcMin mice, which had consumed TMFol, tissue expression of p53 was about three times the level observed in control mice (Fig. 4A). Similarly, p53 expression in tumor tissue from HCT116 tumor-bearing mice on the TMFol early regimen was increased by 50% over expression in controls (Fig. 4B). In contrast, p53 expression was not significantly increased over controls in tumors from mice on the TMFol late regimen. Expression of p53 was also upregulated in APC10.1 and HCT116 tumor cells in vitro, when exposed for 72 hours to TMFol at 5 or 10 μmol/L, respectively, compared with cells in control incubations (Fig. 4C and D). In preliminary experiments, HCT116 cells exposed to TMFol showed biochemical changes fully consistent with p53 upregulation. TMFol at 10 μmol/L significantly increased the expression of the p21 gene, by a factor of 3.2 ± 1.2, as measured by real-time PCR analysis, and of the p21 protein, determined...
by Western analysis, by a factor of 3.0 ± 0.6, compared with controls (P < 0.01 for both). Furthermore, in HCT116 cells transfected with suitable luciferase constructs, TMFol elevated the expression of the p53 target proteins p21 and Bax in a concentration-dependent fashion (Fig. 5).

**Analysis of TMFol levels in tumor tissue**

We wished to relate the ability of TMFol to retard HCT116 tumor development in nude mice with agent levels achieved in the target tissue. Tumor tissue was obtained at the end of the efficacy experiment described in Fig. 3 and subjected to HPLC analysis (Fig. 6). Levels of TMFol in the tumor were 146 ± 80 pmol/g tissue (mean ± SD, n = 6 mice), 146 nmol/L in molar concentration terms. When TMFol levels in individual mice were related to tumor burden, a correlation between the two parameters was not observed.

**Lack of mutagenicity of TMFol in the Ames test**

TMFol was subjected to the Ames reverse mutation assay to establish its mutagenic potential in vitro. Salmonella strains TA100, 102, and 98 were tested, the two former indicating bp substitution mutations and the latter frameshift mutations. TMFol at concentrations of up to 5 mg per plate failed to cause any visible increase in the frequency of revertant colonies either in the presence or absence of rat hepatic metabolic activating enzymes in any of the bacterial incubations (Table 1). In contrast, suitable model mutagens, both direct acting genotoxicants and chemicals requiring metabolic activation, caused expected revertant colony increases. These results show that TMFol at the concentrations used is not mutagenic in this system.

**Discussion**

The results described above provide proof of principle that judicious structural modification of the quercetin molecular scaffold can generate compounds that lack mutagenic activity in vitro and interfere with colorectal carcinogenesis in mice in vivo. Although quercetin has shown chemopreventive properties in some preclinical models of oral, cervical, lung, and colon carcinogenesis (5, 19–21), it was devoid of the ability to prevent adenoma development in the ApcMin mouse (39). Quercetin at dietary concentrations of 0.3% to 3% has even been shown to augment, rather than counteract, azoxymethane-induced formation of colonic aberrant crypts and adenocarcinomas in rodents (40, 41). Furthermore, the mutagenicity of quercetin (24–27) and limited evidence for its carcinogenicity (28, 29) render its further development as a cancer chemopreventive agent unlikely. Fisetin, which is less mutagenic than quercetin in the Ames test (27), has not yet been investigated for adenoma development–retarding...
ability in the Apc<sup>Min</sup> mouse, although it has shown promise as a putative prostate cancer chemopreventive agent (23). The synthesis of TMFol, the flavonol described here, was guided by a 2-fold rationale: to design mutagenicity out of the flavonol scaffold and to impart colorectal cancer chemopreventive properties onto the molecule. The latter expectation was based on the observation that in flavones, which are structurally closely related to flavonols, inclusion of methoxy moieties in the molecule led to the acquisition of superior cancer chemopreventive properties, when compared with the hydroxy-containing counterpart molecules. The benefit for cancer chemopreventive activity of presence of methoxy functionalities is illustrated by, for example, 5,7-dimethoxyflavone and 5,7,4′-trimethoxyflavone, which were superior to their hydroxy congeners in terms of not only systemic availability in rats but also ability to inhibit oral cancer cell proliferation in vitro (8). Moreover, 3′,4′,5′,5,7-pentamethoxyflavone and tricin (4′,5,7-trihydroxy-3′,5′-dimethoxyflavone) reduced adenoma development in Apc<sup>Min</sup> mice in vivo, whereas apigenin (4′,5,7-trihydroxyflavone) did not (9). 3′,4′,5′,5,7-Penta-methoxyflavone and tricin also inhibited adenoma cell growth in vitro much more potently than apigenin (9). These findings imply an intrinsic advantage of methoxylated flavones over hydroxylated ones in terms of growth-inhibitory properties.

Consistent with a chemoprevention-enhancing role of methoxy moieties in flavonoids, TMFol was shown here...
to interfere with tumorigenesis in two mouse models and to engage antiproliferative and proapoptotic mechanisms in vivo. The dietary dose chosen for the study described here equates to ~240 mg/kg per day. When extrapolated from mice to humans on the basis of body surface area comparison (42), this dose is 720 mg/m², thus 1.4 g per 80 kg human per day, a substantial but perhaps feasible dose. Intriguingly, in the Apc<sup>Min</sup> model, the adenoma development-reducing effect of TMFol was confined to the proximal intestine. This tissue specificity could arguably be the consequence of differences in levels of TMFol between intestinal sections, with TMFol concentrations inadequate for activity in the intestine beyond the proximal section. We have reported previously that the concentration of TMFol in the gastrointestinal mucosa of C57BL6/J mice, the Apc<sup>Min</sup> background strain, which received TMFol for a week at the same dietary dose as that used in the Apc<sup>Min</sup> mice described here, was 220 ± 68 nmoles/g tissue, 220 μmol/L in concentration terms (32). This value is 169-fold the IC<sub>50</sub> for growth inhibition by TMFol in APC10.1 cells as described here. TMFol levels in the intestinal mucosa of Apc<sup>Min</sup> mice were not studied in the present study, but one can assume that they were very similar to those observed in C57BL6/J mice. It needs to be pointed out in this context that results obtained in the Apc<sup>Min</sup> mouse, when serving as a model of human colorectal carcinogenesis, need to be interpreted with caution, as the model has some shortcomings, especially the facts that tumor development occurs mainly in the small intestine, not in the colon, as in humans, and that adenomas tend not to progress to the stage of carcinomas before animals are moribund. The large difference in TMFol levels between HCT116 xenograft tumor and murine gut mucosa reflects agent accessing tissues through the circulation in the former and mainly unabsorbed TMFol in the latter tissue. TMFol levels in the xenograft tumor were only a 15th of the IC<sub>50</sub> for TMFol-mediated growth inhibition in HCT116 cells in vitro, suggesting that HCT116 tumor cells in the intact animal environment in vivo are considerably more sensitive to the antiproliferative action of TMFol than HCT116 cells under cell culture conditions.

The results presented above show that TMFol consumption upregulated wild-type p53 expression in tumor target tissues in both murine models, a mechanism that may...
well contribute to the observed chemopreventive efficacy of TMFol. Accompanying preliminary results in HCT116 cells in vitro intimate that p53 upregulation by TMFol has functional consequences as adjudged by induction of downstream promoters. The study of CP-31398 (17) referred to in the introduction provides convincing support for the notion that increasing expression and activation of wild-type p53 reduces intestinal adenoma development in the ApcMin mouse, probably through the suppression of adenoma cell proliferation and induction of apoptosis. The p53-modulating ability of flavonoids in cells in vitro has been described before (10, 11, 43–46), although flavonoid-mediated upregulation of p53 in intact animals has to our knowledge been shown previously only for the flavone apigenin (4′,5,7-trihydroxyflavone) in mice bearing the 22Rv1 prostate tumor xenograft (45). TMFol at 10 μmol/L doubled p53 protein expression in HCT116 cells in vitro, and fisetin has been reported to have a similar effect (44). In contrast, quercetin at up to 40 μmol/L failed to affect p53 expression in this cell type (46), although it increased p53 phosphorylation status. In the light of the order of growth-inhibitory potency of the three flavonols in APC10.1 cells in vitro shown here (TMFol > fisetin > quercetin), and the fact that quercetin was devoid of activity in the ApcMin mouse, these differences in p53 modulatory potency tentatively hint at a mechanistic link between upregulation of p53 and cancer chemopreventive activity in mice. The mechanisms by which TMFol increased p53 expression in the ApcMin and HCT116 xenograft models as described here remain to be elucidated. Based on the literature pertaining to the p53-modulating effects of CP-31398 (cited in reference 17), these mechanisms are likely to be complex and model-dependent. It is worth emphasising that TMFol is likely to engage anticarcinogenic mechanisms other than p53 modulation, and these mechanisms need to be identified in future studies.

The mutagenic activity of flavonols has been associated with hydroxy moieties in rings A and B of the molecular structures. It is therefore important to understand how TMFol, with its unique hydroxylation pattern, affects p53 expression and activity. The data presented in the current study suggest that TMFol is a promising agent for cancer chemoprevention, with potential mechanisms of action including p53 upregulation and suppression of cancer cell proliferation.

**Fig. 5.** Effect of TMFol on luciferase reporter activity of p21-Luc (A) and Bax-Luc (B) constructs in HCT116 cells, into which suitable firefly luciferase sequences had been transfected. Columns, mean of three independent experiments; bars, SD; *, values were significantly different from controls (P < 0.01). For details of cells and transfections, see Materials and Methods.

**Fig. 6.** Representative HPLC chromatograms of extracts of HCT116 tumor xenograft tissue from mice on AIN93G diet without (A) or spiked with TMFol (500 ng/mL; B), or from mice that received AIN93G diet fortified with TMFol (0.2%) for their lifetime (C). For chromatographic conditions, see Materials and Methods. Is (internal standard) was 2′,5′,6,7′-hexamethoxyflavone.
scaffold (24, 25), and their methylation decreased mutagenic activity (24, 27). Consistent with these toxicophoric structural features, TMFol, which bears no hydroxy moieties in ring A and three methoxys in ring B, was shown here to be devoid of mutagenic properties in the Ames test. Furthermore, the observed absence of detrimental effects of TMFol on murine body weight or organ pathology augurs well for its safety profile. Nevertheless, the putative safety of TMFol needs to be elucidated carefully in further preclinical experiments.

In summary, the properties of TMFol described here support the notion that chemical modification of the flavonol structure based on existing, albeit scarce, information on the relationship between flavonoid structure and chemopreventive activity or toxicity may generate useful cancer chemopreventive agents. It is conceivable that, with further acquisition of knowledge of the important molecular targets of flavonoids and their structure-activity relationships, the flavonol structure can be optimized even more, beyond TMFol. Nevertheless, in the light of its favorable pharmacologic profile described here, TMFol deserves additional preclinical investigation to help adjudicate whether it is worthy of advancement to the stage of clinical development. TMFol should be studied for chemopreventive efficacy in other rodent carcinogenesis models. Furthermore, combinations of TMFol together with agents, such as cyclooxygenase inhibitors, which engage complimentary chemoprevention mechanisms, should be investigated.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References

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