# Preclinical Colorectal Cancer Chemopreventive Efficacy and p53-Modulating Activity of 3',4',5'-Trimethoxyflavonol, a Quercetin Analog

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# Abstract

Naturally occurring flavonols, exemplified by quercetin, possess experimental cancer chemopreventive efficacy. Modulation of p53 is a mechanism thought to contribute to their activity. Many flavonols are mutagenic. 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 carcinogenesis, Apc<sup>Min</sup> mice and human-derived HCT116 colorectal adenocarcinoma-bearing nude mice. Mice received TMFol admixed in with their diet (0.2%) from weaning to week 16 in the case of  $Apc^{Min}$ , or from either day 7 prior to ("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 HPLC. Consumption of TMFol reduced small intestinal adenoma burden in Apc<sup>Min</sup> mice by 47%, compared to control mice (P<0.002). The TMFol early regimen approximately halved HCT116 tumor size (P<0.05), decreased tumor proliferation and increased apoptosis, whilst the TMFol late regimen had no significant effect, when compared to controls. In tumor tissues from mice, in which TMFol reduced tumor development, p53 expression was increased, 3-fold in Apc<sup>Min</sup> and 1.5-fold in HCT116 tumor-bearing mice (P=0.02). TMFol increased p53 also in vitro in cells derived from these tumors. The level of TMFol in HCT116 tumors was 0.15nmol/g. 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.

#### 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 propitious. Edible plants constitute an attractive source of novel potential cancer chemopreventive agents, as the safety record of dietary constituents tends to be good. Flavonoids, exemplified by the flavonols guercetin 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 as to molecular features which confer pharmacological 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 moleties instead of, or in addition to, hydroxy in certain flavonoids augments cancer chemopreventive activity (8,9). Prominent among the many mechanisms via which flavonoids are thought to exert their chemopreventive activity is activation of wildtype p53 (10) and/or down-regulation 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 more than 50% of all cancers, including colon cancer, leading to aggressive, treatment-resistant malignancies (14,15). Small molecules, such as CP-31398, a styrylquinazoline from 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  $Apc^{Min}$  mouse, accompanied by a marked increase in adenomatous p53 levels (17). The  $Apc^{Min}$  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, exemplified by quercetin (3',4',5,7-tetrahydroxyflavonol), have been arguably the focus of more pharmacological 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 cogener 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 toxicological impediment of many flavonols, which militates against their development as cancer chemopreventive agents, is their mutagenicity. Quercetin (24-26) and, to a lesser extent, fisetin (27), were shown to be mutagenic in the Ames test. There is also limited evidence for the carcinogenicity of quercetin in animals (28,29). These toxicological properties of quercetin militate against its further clinical development. Toxicophoric structural features considered responsible for the mutagenic potential of flavonols include phenolic hydroxy moieties in both the A and B rings of the molecular scaffold (24,26,27).

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Taking all these findings into consideration, we hypothesized that it might be possible to synthesize a flavonol with optimized pharmacological and toxicological 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 non-mutagenic and possess cancer chemopreventive efficacy. In order 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 strong APC10.1 cell growth-inhibitory activity, we investigated its effect on adenoma development in Apc<sup>Min</sup> mice in vivo. 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 alteration of tumor p53 expression. We also measured TMFol levels in murine target tissues, to relate activity to presence of pharmacologically active agent. The potential mutagenicity of TMFoI was investigated in the Ames reverse mutation assay using a panel of Salmonella Typhimurium strains, in which guercetin 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 described previously (32), was >99% pure as determined by HPLC analysis. 2',5',5,6,7,8-Hexamethoxyflavone used as an internal standard in the HPLC analysis was supplied by the NCI Developmental Therapeutic Programme Open Compound Repository (NCI, Bethesda, USA). HPLC fluorescence grade methanol was purchased from Fisher Chemicals (Loughborough, UK), and water for analysis was generated in a Nano-Pure water purification system (Barnstead, UK). All other chemicals were obtained from Sigma Chemical Corp. (Poole, UK). P53 antibodies were purchased from Dako (clone DO-7) (Ely, UK) and from Cell Signaling Technology (clone 1C12) (Hitchin, UK). 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, Italy), were cultured in Dulbecco's modified Eagle medium supplemented with 20% fetal bovine serum (Gibco, Paisley, UK). HCT116 human colon adenocarcinoma cells were obtained from the American Type Cell Collection (ATCC, Manassas, VA) and maintained in McCoy's 5A (Gibco) medium supplemented with 10% fetal bovine serum.

APC10.1 or HCT116 cells from subculture 2-20 were seeded at a density of 2x10<sup>3</sup> 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 0.1-5,1-40 or 0.2-8µM, 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 phosphate buffered saline (PBS), harvested by trypsinization and resuspended in cell culture medium (1 ml), which was diluted 10-fold with Isoton II buffer (Beckman Coulter, High Wycombe, UK). Cells in an aliquot (0.5ml) of the suspension were counted using a Z2 Coulter Particle Counter with Size Analyser (Beckman Coulter). Growth curve experiments were conducted in triplicate, and IC<sub>50</sub> values were calculated from a plot of cell number at 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/6J Min/+ (*Apc<sup>Min</sup>*) mice originally obtained from the Jackson Laboratory (Bar Harbor, ME). Ear tissue from newborn mice was genotyped for the presence of the mutation, using PCR as described previously (33). Female MF-1 outbred nude mice (30–40g) were obtained from Harlan UK (Bicester, Oxon, UK) and ear-punched for identification. Mice were kept in the Leicester University Biomedical Services facility at 20–23°C under conditions of 40–60% relative humidity and a 12h 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 UKCCCR guidelines (34). Mice received standard AIN93G diet (controls) or AIN93G diet supplemented with TMFol at 0.2%. This dietary dose equates to approximately 240mg/kg per day. When extrapolated from mice to humans on the basis of body surface area comparison (35), this dose is 720mg/m<sup>2</sup>,

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thus 1.4g per 80kg human per day. Similar dietary doses have been commonly used in preclinical chemoprevention studies of other polyphenols including curcumin, resveratrol and anthocyanins.

# Intervention in Apc<sup>Min</sup> mice

*Apc<sup>Min</sup>* mice (n=19-21 per group) 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 (x5). Polyp volume was derived from polyp diameter assuming a hemispherical shape for small bowel polyps and a spherical shape for colon polyps, consistent with their histological appearances. Tumor burden was calculated as the sum of polyp volume per animal.

# Intervention in HCT-116 tumor-bearing nude mice

HCT116 cells ( $10^6$ ) suspended in matrigel:serum free medium (1:1;  $100\mu$ L; Becton Dickinson, Worthing, UK) were injected subcutaneously into the right flank of nude mice under light halothane anesthesia. Mice (n=14-15 per group) received TMFol commencing either seven days prior to, or seven days post, cell inoculation. Mice were weighed weekly, and tumor size was measured three times per week using callipers. Tumor volume was calculated by the formula: length x width<sup>2</sup>/2, where length is the larger and width the smaller diameter of the tumor (in mm). Animals were culled three weeks after tumor cell inoculation, when control tumors reached maximal size (17mm in length) permissible under 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, Newcastle UK) or for cleaved caspase-3 using cleaved caspase-3 Asp 175 polyclonal antibody from Cell Signaling Technology (Hitchin, UK). Briefly, paraffinembedded sections (4µm) mounted on polysine-coated slides were dewaxed (65°C, 20min) and hydrated through a graded series of alcohol rinses. The antigen was unmasked by microwaving sections (20min) in Tris-EDTA buffer (pH 9). Endogenous peroxidase activity was inactivated by incubation of slides with hydrogen peroxide (3%, 10min); nonspecific binding was blocked with protein block solution (Novocastra). Sections were incubated with primary antibody (dilution 1:2000 for Ki67, 1:200 for caspase 3) overnight at 4°C. After washing (PBS) sections, 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 which 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 x 40; Leitz Orthoplan microscope, Leica DC 300 camera) for each sample. Differences in counts between the observers were less than 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 2312±307 and 2340±154 (mean±SD, n=15) for Ki-67 and cleaved caspase 3 staining, respectively.

#### Western blot analysis

Tumor tissue from control mice or mice which received TMFol was homogenized with lysis buffer (1:4, w:v. PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, with protease inhibitors PMSF 1mM, aprotinin 2µg/ml, leupeptin 5µg/ml and phosphatase inhibitors sodium vanadate 1mM and sodium fluoride 1mM). The homogenate was placed on ice (15min) and then centrifuged (20min, 10,000xg, 4°C). Cells were grown to 60-80% confluency, followed by exposure for 72h to TMFol. Cells were centrifuged (5min, 10,000xg, 4°C), and the cell pellet was suspended in lysis buffer. Protein concentration in tissue or cell lysate was determined using the Biorad protein assay (Biorad, Hemel Hempstead, UK) with bovine serum albumin as standard. An aliquot of protein lysate (50µg) was separated by SDS–PAGE and transferred for 1h onto a nitrocellulose membrane (Schleicher & Schuell, NH, USA). Blots were blocked for 2h 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% non-fat dry milk (4°C, overnight). After washing (PBS-T), blots were treated with horseradish peroxidase-conjugated secondary antibody for 1h and washed (5X for 5min) in PBS-T. Proteins were detected by the enhanced chemiluminescence system (Geneflow, Staffordshire, UK).

#### **HPLC** analysis

Tissue sample preparation and HPLC analysis using fluorescence detection were as described before (32) using a Varian Prostar HPLC system (Varian, UK) consisting of a Varian ProStar 230 solvent delivery system, a Pro-Star 363 fluorescence detector, and a 410 Varian autosampler. Separation was achieved on a Gemini C<sub>18</sub> column (4.6 mm x 150 mm, 3 µm, Phenomenex, UK) at a flow rate of 0.75 mL/min, using an isocratic mobile phase of 69% methanol in 0.1 M 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 GLP conditions by Safepharm Laboratories (Shardlow, Derbyshire, UK). *Samonella* 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 5 dose levels, both with and without a rat liver homogenate metabolism system (10% liver S9 with standard co-factors). The dose range for the experiment (50-5000µg/plate) was determined in a preliminary toxicity assay. Aliquots (0.1ml) of bacterial culture were dispensed into test tubes followed by molten trace histidine-supplemented top agar (2ml), solutions (0.1ml) of TMFol, vehicle only or positive control mutagen and either S9 mix or phosphate buffer (0.5ml). The tube content was mixed and distributed onto the surface of agar plates. 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 (SPSS) version 16 programme (Windows XP). Effects of TMFol on adenoma number and burden were compared by Students t-test. Effects of TMFol on cell growth *in vitro* and xenograft tumor volume *in vivo* were subjected to one-way analysis of variance (ANOVA) with post-hoc Bonferroni correction. P values <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 IC<sub>50</sub> values were computed from the growth curves (Fig. 1). Of the three flavonols, TMFol was the most potent cell growth inhibitor, with an IC<sub>50</sub> of about a tenth of that for quercetin and a fourth of that for fisetin. In an orientation experiment TMFol also inhibited the growth of human-derived HCT116 adenocarcinoma cells, characterized by an IC<sub>50</sub> of  $3.3\mu$ M, compared to IC<sub>50</sub> values of >10 $\mu$ M for both quercetin and fisetin (mean of n=2, data not shown). In the light of the growth-inhibitory potency of TMFol in intestinal cells *in vitro*, its ability to compromise tumor development in the *Apc<sup>Min</sup>* or HCT116 xenograft mouse models *in vivo* was considered worthy of investigation.

# Effect of TMFol on adenoma development in Apc<sup>Min</sup> mice

*Apc<sup>Min</sup>* 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 *Apc<sup>Min</sup>* mice which received TMFol for their lifetime was indistinguishable from that of mice on the control diet (result not shown). Inspection of lung, liver and kidney tissues failed to reveal significant pathological abnormalities. Both observations are consistent with 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 calliper at 2-3 day intervals. Consumption of TMFol from a week prior to tumor inoculation ("TMFol early") halved tumor size beyond day 16 post-inoculation (Fig. 3A). Dietary intervention with TMFol commencing a week post 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, TMFoI significantly reduced cell proliferation in HCT116 tumors from mice on the TMFoI early regimen, with a proliferation index of 72%, when compared to tumors in control mice, with a proliferation index of 86% (Fig. 3B). Proliferation was not reduced in tumors from mice on the TMFoI late regimen. Tumor levels of apoptotic cells were elevated by 55% compared to control animals in tumors from mice on the TMFoI early regimen, whilst there was no increase in apoptosis in tumors from mice on the TMFoI late regimen (Fig. 3C).

#### Effect of TMFol on p53 expression in tumors

In adenomas from  $Apc^{Min}$  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 up-regulated in APC10.1 and HCT116 tumor cells *in vitro*, when exposed for 72h to TMFol at 5 or 10µM, respectively, compared to cells in control incubations (Fig. 4C,D). In preliminary experiments, HCT116 cells exposed to TMFol showed biochemical changes fully consistent with p53 upregulation: TMFol at 10µM significantly increased expression of *p53*, *p21* and *Bax* genes in HCT116 cells, as measured by RTPCR analysis, and it increased the expression of the p53 target proteins p21 and Bax 4- and 2-fold, respectively, in HCT116 cells transfected with suitable luciferase constructs (results not shown).

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# Analysis of TMFol levels in tumor tissue

We wished to relate the observed efficacy of TMFol to retard tumor development in the mouse models with agent levels achieved in the target tissues. HCT116 tumour tissue was obtained from nude mice at the end of the efficacy experiment described in Fig. 3 and subjected to HPLC analysis (Fig. 5). Tumor levels of TMFol were 146±80pmoles/g tissue (mean±SD, n=6 mice), 146nM in molar concentration terms. 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±68nmoles/g tissue, 220µM in concentration terms (32). TMFol levels in the *Apc<sup>Min</sup>* mice in the present study were probably of a similar order of magnitude. The large difference in TMFol levels between xenograft tumor and murine gut reflects agent available in tissue *via* the circulation in the former and mainly unabsorbed TMFol in the latter tissue.

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

TMFol was subjected to the Ames reverse mutation assay in order to establish its mutagenic potential *in vitro*. *Salmonella* strains TA100, 102 and 98 were tested, the two former indicating base-pair substitution mutations and the latter frameshift mutations. TMFol at concentrations of up to 5mg/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 demonstrate that TMFol at the concentrations employed 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 which lack mutagenic activity in vitro and interfere with colorectal carcinogenesis in mice in vivo. Whilst 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  $Apc^{Min}$  mouse (36). Quercetin at dietary concentrations of 0.3-3% has even been demonstrated to augment, rather than counteract, azoxymethane-induced formation of colonic aberrant crypts and adenocarcinomas in rodents (37,38). 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, even though it has shown promise as a putative prostate cancer chemopreventive agent (23). The synthesis of TMFol, the flavonol described here, was guided by a two-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 to 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 (PMF) and tricin (4',5,7-trihydroxy- 3',5'-dimethoxyflavone) reduced adenoma development in Apc<sup>Min</sup> mouse in vivo, whilst apigenin (4',5,7-trihydroxyflavone) did not (9). PMF 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 pro-apoptotic mechanisms in vivo. 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 TMFoI between intestinal sections, with TMFoI concentrations inadequate for activity in the intestine beyond the proximal section. Intestinal mucosa levels of TMFol in C57BL/6J mice, the Apc<sup>Min</sup> background strain, which received TMFol at 0.2% in their diet (32), were 169-fold the  $IC_{50}$  for growth inhibition by TMFol in APC10.1 cells. Although TMFol concentrations were not measured in specific intestinal subsections, the high concentration observed in the whole intestine renders differences in TMFol levels between intestinal sections unlikely as explanation for its selective activity in the proximal intestine. TMFol levels in the HCT116 xenograft tumor were only a fifteenth 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

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environment *in vivo* are considerably more sensitive to the antiproliferative action of TMFol than HCT116 cells under cell culture conditions.

The results presented above demonstrate that TMFol consumption upregulated wild-type p53 expression in tumor target tissues in both murine models, a mechanism which 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 promotors. 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 Apc<sup>Min</sup> mouse, probably via suppression of adenoma cell proliferation and induction of apoptosis. The p53-modulating ability of flavonoids in cells in vitro has been described before (10,11,39-42), although flavonoid-mediated upregulation of p53 in intact animals has to our knowledge been shown previously only for the flavone apigenin (4',5,7trihydroxyflavone) in mice bearing the 22Rv1 prostate tumor xenograft (41). TMFol at 10µM doubled p53 protein expression in HCT116 cells in vitro, and fisetin has been reported to have a similar effect (40). In contrast, quercetin at up to 40µM failed to affect p53 expression in this cell type (42), although it increased p53 phosphorylation status. In the light of the order of growth-inhibitory potency of the three flavonols in vitro shown here (TMFol>fisetin>quercetin), and the fact that quercetin was devoid of activity in the  $Apc^{Min}$  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 Apc<sup>Min</sup> and HCT116 xenograft models as described here, remain to be elucidated.

On the basis of the literature pertaining to the p53-modulating effects of CP-31398 (cited in 17), these mechanisms are likely to be complex and model-dependent. It is worth emphasising that TMFoI is likely to engage anti-carcinogenic 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 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 pharmacological profile described here, TMFol deserves additional preclinical investigation to help adjudge whether it is worthy of advancement to the stage of clinical development. TMFol should be studied for chemopreventive efficacy in other rodent carcinogenesis models. It might also be propitious to investigate

combinations of TMFol together with agents, such as cyclooxygenase inhibitors, which engage complimentary chemoprevention mechanisms.

# **Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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### **Figure legends**

**Fig. 1.** Effect of quercetin (A), fisetin (B) or TMFoI (C) on the growth of APC10.1 cells. Cells were counted after exposure to flavonols for 6 days. Values are the mean $\pm$ SD of three independent experiments, each conducted in triplicate. 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).

**Fig. 2.** Effect of consumption of TMFol on burden (top) and number (bottom) of adenomas in the total small intestine or colon (A) and in small intestinal subsections (B) of *Apc<sup>Min</sup>* mice. Mice received AIN93 diet (control) or AIN93 diet fortified with TMFol (0.2 %) from weaning (week 4) until week 16, when they were killed, and adenoma number and burden were measured. Values are the mean±SD of n=19 (control) or 21 mice (TMFol). Statistical comparison was by Students t-test; P values above bars indicate significant differences between mice on TMFol and controls.

**Fig. 3.** Effect of consumption of TMFoI (0.2% in the diet) on growth of the HCT116 adenocarcinoma xenograft in nude MF1 mice (A), and on proliferation and apoptosis in HCT116 tumor tissue as reflected by staining for Ki-67 (B) and cleaved caspase 3 (C). Mice received TMFoI from either a week prior to (squares in A, "TMFoI early" in B, C), or a week post tumor inoculation (triangles in A, "TMFoI late" in B,C). Tumor volume in A was measured by calliper (see Materials and Methods). B and C show photomicrographs of tumor tissue stained for KI-67 (B) or cleaved caspase 3 (C),

bars reflect densitometry readings. Note that in B ordinate commences at 60%. Bars in the photomicrographs (B,C) are 50µm. Values are the mean±SD of n=14 (TMFol late) or 15 mice (control and TMFol early) in A and of 8-10 per group in B and C. Asterisks in A indicate that tumor volume in the TMFol early group (squares) was significantly smaller than that in control mice (P values: 0.036, 0.049 and 0.007 for days 16, 19 and 21, respectively), P values above bars denote statistical difference in B and C. Statistical analysis was by one-way ANOVA with posthoc Bonferoni correction.

**Fig. 4.** p53 Protein expression in adenomas of  $Apc^{Min}$  mice (A) or in HCT116 tumor xenografts from nude MF1 mice (B) which received TMFoI (0.2%) with their diet, and in APC10.1 (C) or HCT116 cells (D) which were exposed to TMFoI at the indicated concentrations for 72h before harvest. Tumour tissue was obtained from the  $Apc^{Min}$  (A) or HCT116 xenograft mice (B) described in Figs 2 and 3. Analysis was by Western blotting. Bars reflect densitometric evaluation of bands, and values are the mean±SD of 6 (control) and 12 (TMFoI) (A) or 14-15 mice (B), and of 3 (C) or 4 (D) independent cell exposure experiments, each conducted in triplicate. Asterisk with appropriate P values indicate that the difference between intervention and control was significant, analyses were by Students t-test (A) or by one-way ANOVA with posthoc Bonferoni correction (B,C,D).

**Fig. 5.** Representative HPLC chromatograms of extracts of HCT116 tumor xenograft tissue from mice on AIN93G diet without (A) or spiked with TMFol (500ng/ml) (B), or from mice which received AIN93G diet fortified with TMFol (0.2%) for their lifetime (C). For chromatographic conditions see Materials and Methods. is=internal standard, which was 2',5',5,6,7,8-hexamethoxyflavone.

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Fig. 1



Figure 2







Figure 4





Figure 5



TMFol concentration (µg/plate)	Number of revertants	s per plate without or v	vith S9 (in brackets)
	S Typhimurium strain		
-	TA100	TA102	TA98
0	124±5 (129±3)	232±19 (271±8)	12±1 (24±6)
50	119±9 (112±5)	226±17 (267±9)	11±4 (19±6)
150	122±1 (113±10)	217±13 (270±4)	15±3 (20±6)
500	111±11 (112±5)	216±15 (258±15)	14±1 (23±8)
1500	115±14 (104±5)	208±11 (270±8)	13±2 (20±4)
5000	118±34 (102±16)	231±23 (271±10)	11±2 (21±6)
Positive controls (µg/plate):			
<i>N</i> -Ethyl- <i>N</i> '-nitro- <i>N</i> - nitroso-guanidine (3)	413±58		
2-Aminoanthracene (	1) (1039±964)		
Mitomycin C (0.5)		1147±10	
1,8-Dihydroxy- anthraquinone (10)		(1607±75)	
4-Nitroquinoline-1- oxide (0.2)			222±20
Benzo( <i>a</i> )pyrene (5)			(263±52)

# **Table 1.** Effect of TMFol in the reverse mutation assay using SalmonellaTyphimurium