Inhibition of Prostate Cancer Cell Growth by 3', 4', 5'-Trimethoxyflavonol (TMFol)

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Abstract

Purpose TMFol (3', 4', 5'-trimethoxyflavonol) is a synthetic analogue of the naturally occurring flavonols fisetin and quercetin, which have been considered of potential usefulness in the management of prostate cancer. We investigated whether TMFol may have preclinical features superior to those of its two flavonol congeners.

Methods The ability of the three flavonols to compromise prostate cancer cell survival was tested in four prostate cancer cell types 22Rv1, TRAMP C2, PC-3 and LNCaP. The effect of TMFol on prostate cancer development *in vivo* was investigated in nude mice bearing the 22Rv1 or TRAMP C2 tumours.

Results TMFol inhibited cell growth *in vitro* in all four prostate cancer cell types more potently than fisetin and quercetin. It also interfered with TRAMP C2 tumour development *in vivo*, whilst fisetin and quercetin at equivalent doses were without activity in this model. Likewise, TMFol slowed the growth of the 22Rv1 tumour *in vivo*. Efficacy in either model was accompanied by induction of apoptosis, although *in vitro* only TRAMP C2 cells, but not 22Rv1, underwent apoptosis when exposed to TMFol.

Conclusions The results support the notion that among the three congeneric flavonols, quercetin, fisetin and TMFol the latter may be the most suitable candidate agent for potential development in prostate cancer management.

Introduction

Naturally occurring flavonoids have long been considered potentially useful in the prevention and/or treatment of malignancies, including prostate cancer [1]. The flavonol fisetin (3',4',7-trihydroxyflavonol, for structure see Fig. 1), which occurs in apples, onions and other fruits and vegetables, has been described to possess anti-prostate cancer activity in cells *in vitro* and in 22Rv1 human prostate tumours grown in nude mice *in vivo* [2]. A mechanism by which fisetin is thought to interfere with prostate cancer cell survival is induction of apoptosis [2, 3]. The fisetin analogue quercetin (3',4',5,7-tetrahydroxyflavonol), the biological properties of which have been widely studied [4], at concentrations of 10-100 μ M has also exhibited growth-arresting properties in human-derived prostate cancer cells [5]. We recently synthesized the flavonol TMFol (3', 4', 5'-trimethoxyflavonol) [6] as a "structurally optimized" analogue of fisetin and quercetin, which lacks hydroxy moieties in the A ring of the flavonol scaffold and bears methoxy, rather than hydroxyl, functionalities in ring B. TMFol possessed colorectal cancer chemopreventive efficacy in the *Apc^{Min}* mouse model, which was superior to that of quercetin or fisetin [7]. Furthermore, TMFol lacked the mutagenic properties of its flavonol congeners. In the light of these findings we wished to explore whether TMFol exhibits anti-prostate cancer activity similar, or superior, to that of fisetin. TMFol was compared with fisetin and quercetin in terms of ability to compromise the growth of four prostate cell types *in vitro*. Furthermore, its effect on the development of two prostate adenocarcinoma xenografts in nude mice *in vivo*, the murine TRAMP C2 and human-derived 22Rv1 tumours, was studied. Levels of TMFol were measured in TRAMP tumour and prostate tissues. The overall aim of the work was to contribute to the body of knowledge which might help prioritise a flavonol for development to the stage of clinical evaluation for the management of prostate cancer.

Methods

Chemicals and reagents

Quercetin and fisetin were purchased from Sigma-Aldrich (Poole, UK). TMFol was synthesised as described previously [6]. Cleaved caspase-3 antibody was sourced from Cell Signaling Technology (Hitchin, UK) while the Novolink polymer detection system was obtained from Leica Biosystems (Newcastle, UK). Laboratory chemicals were purchased from Fischer Scientific (Loughborough, UK) and Sigma-Aldrich (Poole, UK), unless otherwise specified.

Cell culture

Cells were sourced from the American Type Culture Collection (ATCC) or the European Collection of Cell Cultures (ECACC) except 22Rv1 cells, which were kindly provided by Dr. Imran Ahmad (Beatson Institute for Cancer Research, Glasgow, UK). LNCaP cells were originally derived in 1977 from a left supraclavicular lymph node metastasis of a 50-year-old Caucasian, PC-3 cells were derived in 1979 from a grade IV bone prostate cancer metastasis of a 62-year-old Caucasian, 22Rv1 cells were derived in 1999 from a human prostate cancer xenograft serially propagated in mice after castration-induced regression and relapse of the parental CWR22 xenograft, TRAMP C2 cells were derived in 1996 from a heterogeneous 32 week-old primary tumour in the prostate of a PB-Tag C57BL/6 (TRAMP) mouse. LNCaP, PC-3 cells and 22Rv1 cells were maintained in RPMI-1640 medium (Invitrogen, Paisley, UK) supplemented with 10 % foetal calf serum without antibiotics; TRAMP C2 cells in DMEM high glucose with L-glutamine (Sigma-Aldrich, Poole, UK) supplemented with 5 % foetal calf serum, 5 % Nu-Serum IV (BD Biosciences, California, USA), 5 µg/mL insulin and 10 nM dihydrotestosterone. The doubling times of the cells used were 25 h for PC-3 and TRAMP C2 cells, 36 h for the LNCaP and 40 h for the 22Rv1 cells. Cells were incubated at 37°C at 5 % CO₂. Cells from subcultures 3 to 30 were used in this study. They were tested for mycoplasma at 6 month intervals throughout the study period.

Cell proliferation

Cells were seeded ($3 \times 10^3 - 1 \times 10^4$) in 2 mL of medium onto 24 well plates in three replicates and allowed to adhere overnight. The LNCaP cells were allowed 48 h to adhere before treatment. TMFol, quercetin or fisetin, dissolved in DMSO, were added to cell incubates at different concentrations. Control cells were incubated with DMSO only (0.1 % final concentration). The cells were incubated for up to 6 days. They were washed with phosphate buffered saline (PBS) and harvested by trypsinisation. Trypsin was inactivated by cell culture medium. Cells were counted using a Z2 Coulter Particle Counter with Size Analyser (Beckman Coulter, UK). Growth curve experiments were performed in triplicate; IC_{50} values were calculated from the linear equation generated from the plot of cell number (as percentage of the vehicle control) against drug concentration on day 6.

Annexin V-FITC assay for measurement of apoptosis

An Annexin V- FITC Apoptosis Detection Kit (BD Biosciences, New Jersey, USA) was used to detect apoptosis. Cells were seeded at a density of $2 - 5 \times 10^5$ in 12 mL of medium. Cells were allowed to adhere overnight before the medium was removed and replaced with medium containing TMFol (10 μ M) or DMSO for 72 h. Cells that had detached in the medium during the incubation time were removed and reserved. Adherent cells were washed with PBS, trypsinised and then pooled with the detached cell media. Cells were centrifuged at $350 \times g$ for 5 min at 4°C and the pellet was resuspended in 10 mL fresh medium supplemented with FCS. Cells were incubated for 30 min at 37°C before being re-centrifuged, and the pellet formed was then resuspended in 1 mL annexin buffer. FITC-conjugated Annexin V (0.05 μ g/test) and propidium iodide (0.05 μ g/test) were added, followed by a 15 min incubation in the dark at room temperature. All samples were then analysed by flow cytometry using a FACS Aria II (Becton Dickinson, New Jersey, USA) with the BD FACS Diva analysis software (version 6.1.2).

Cell Cycle Analysis

Cells seeded at a density of 8×10^5 in 12 mL of medium were treated with 10 μ M TMFol or DMSO for 48 and 72 h. Cells were harvested by trypsinisation and centrifuged for 5 min at 400 x g. The supernatant was discarded and cells were fixed in 2 mL of ice-cold 70 % ethanol overnight or up to one week at 4°C. After fixation the cell suspension was centrifuged at 400 x g for 10 min, cells were resuspended overnight at 4°C in 800 μ L of PBS and RNase A at a final concentration of 10 μ g/mL. Propidium iodide at a final concentration of 20 μ g/mL was subsequently added and incubated for a minimum of 2 - 3 h at 4°C before FACS analysis on a FACS Aria II (Becton Dickinson, New Jersey, USA). Data were analysed using the ModFit software version LT3.3.

Animals and TMFol dose

Male MF-1 outbred nude mice (30–40 g, 6 weeks of age) 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 12 h 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 [8]. Mice received standard powdered AIN93G diet (controls) or powdered AIN93G diet supplemented with equimolar doses of flavonols at 0.731 mmol/kg, i.e. TMFol at 0.2 %, quercetin at 0.184 % or fisetin at 0.174 %.

TRAMP C2 cells (5×10^6) or 22Rv1 cells (2×10^6) suspended in matrigel:serum free medium (1:1; 100 μ L; Becton Dickinson, Worthing, UK) were injected subcutaneously into the right flank of male nude mice of 8 weeks of age under light halothane anaesthesia. TRAMP C2-bearing mice received diet adulterated with quercetin, fisetin (each n=5) or TMFol (n=15), or control diet (n=15) while the 22Rv1-bearing mice received diet with TMFol or control diet (each n=10). Mice commenced diet seven days prior to cell inoculation. Mice were weighed weekly, and tumour size was measured twice per week using callipers. Tumour volume was calculated by the formula: length x width²/2, where length is the larger and width the smaller diameter of the tumour (in mm) [9]. Animals were culled

approximately 7 weeks after tumour cell inoculation, when tumours in untreated mice reached the maximal size (~17mm in length) permissible under UK animal welfare stipulations. Tumour tissue samples were snap-frozen (liquid nitrogen) and stored at -80°C until analysis by HPLC or fixed in 10% formalin and histologically processed until analysis by immunohistochemistry.

Immunohistochemistry

Formalin-fixed TRAMP C2 and 22Rv1 tumour tissue were stained 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 10 mM citrate buffer (pH 6.0). Endogenous peroxidase activity was inactivated by incubation of slides with hydrogen peroxide (3 %, 10 min); nonspecific binding was blocked with protein blocking solution (Novocastra, Newcastle upon Tyne, UK). Sections were incubated with primary antibody (dilution 1:200) overnight at 4°C. After washing sections (PBS), the tissue-antibody reaction was visualized using a commercial kit (NovoLink, Novocastra, Newcastle upon Tyne, UK). The slides were scored by two independent observers blinded to the treatment group. Apoptotic index was quantitated as the percentage of epithelial cells from 10 random fields of view per section which stained positive for 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 x40; 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.

HPLC analysis

Tissue sample preparation and HPLC analysis using fluorescence detection were as described before [6] 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 C₁₈ 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 tumour tissue from MF1 nude mice was quantified using 2',5',5,6,7,8-hexamethoxyflavone as an internal standard.

Statistical Analyses

All values are presented as the mean $\pm$ SD. Statistical significance was evaluated using the Statistical Package for the Social Sciences (SPSS) version 20 programme. Effects of TMFol on xenograft tumour volume *in vivo* and protein expression *in vitro* were compared by Students t-test. Effects of TMFol on cell growth *in vitro* were subjected to one-way analysis of variance (ANOVA) with post-hoc Bonferroni correction. P values <0.05 were considered significant.

Results

In order to compare the effect of TMFol, fisetin and quercetin on prostate cancer cell growth, agents were incubated with cells originating from a murine (TRAMP C2) or three human prostate cancers (22Rv1, LnCaP, PC-3). TMFol consistently inhibited cell growth more potently than its congeners, with IC₅₀ values for TMFol between a fourth and a sixteenth of those established for fisetin or quercetin (Table 1). Many phytochemicals including flavonoids can generate hydrogen peroxide in *in vitro* incubations by way of reaction with constituents of the cell culture medium [11]. To ascertain that artefactual H₂O₂ generation did not contribute to the growth inhibition observed, we conducted an orientation experiment in which incubations with TMFol and 22Rv1 cells were complemented with 200U/mL catalase. This addition did not alter the degree of growth inhibition observed in the absence of catalase, militating against a role for in TMFol-induced cytostasis.

Next we wished to find out whether the growth delay caused by TMFol in prostate cancer cells *in vitro* translates into antitumour activity in mice bearing prostate cancer xenografts. Nude mice bearing TRAMP C2 or 22Rv1 tumours, representative of the tumour cell panel used *in vitro*, received TMFol at 0.2% with their diet. TMFol reduced tumour development modestly but

significantly in both models (Fig 2). In contrast, TRAMP C2 tumours in mice which received fisetin or quercetin with their diet at an isomolar dose grew at the same rate as those in animals on control diet (Supplemental Fig 1). As observed previously in the *Apc^{Min}* mouse model [7], the body weight of the mice which received TMFol was indistinguishable from that of mice on control diet, consistent with the notion that the intervention does not adversely affect the mice.

In the light of these results, the question arose as to whether TRAMP C2 and 22Rv1 cells respond to TMFol in an identical fashion or whether there were differences in response. Cell cycle analysis after incubation of TRAMP C2 cells with TMFol (10 μ M) showed an increase of the cell fraction in G2/M phase after incubation for 48 h and a reduction of cell number in G1 phase after 72 h compared to controls (Fig. 3). In contrast, TMFol did not affect 22Rv1 cell cycle distribution. Furthermore, TMFol induced apoptosis 16–18 fold in TRAMP C2 cells *in vitro* as reflected by annexin V staining, but failed to do so in 22Rv1 cells (Fig. 4). In order to ascertain that the observed induction of apoptosis in cells *in vitro* was not method-dependent, we conducted an orientation experiment in which, consistent with the results obtained with annexin V staining, the presence or absence of apoptosis induction was confirmed by analysis of caspase 3 cleavage by western blot. The apoptotic index, as adjudged by cleaved caspase-3 expression, in tumour tissue from either type of prostate cancer-bearing mouse which had received TMFol, was significantly increased over animals on control diet (Fig. 5). The increase was 2.2 ± 0.9 fold ($P < 0.05$, $n = 5$) for the TRAMP C2 and 1.5 ± 0.1 fold ($P < 0.05$, $n = 10$) for the 22Rv1 tumours.

Chemical analysis of tumour and prostate tissues obtained from mice bearing the TRAMP C2 tumour which had received TMFol suggests steady state levels of 0.92 ± 0.81 and 5.3 ± 2.7 nmoles/g tissue (mean $\pm$ SD, $n = 15$), respectively. Tumour levels of TMFol in individual mice were not correlated with tumour burden.

Discussion

The results presented above support the notion that among the three congeneric flavonols quercetin, fisetin and TMFol the latter may be the most suitable candidate for potential development in prostate cancer management. TMFol inhibited the growth of four prostate tumour-derived cells *in vitro* more potently than fisetin or quercetin. It also interfered with prostate tumour growth *in vivo* in two xenograft mouse models. In one of these models, the TRAMP C2 tumour, the three flavonols were compared side-by-side in terms of their ability to compromise tumour development. In this model quercetin and fisetin had no effect on tumour growth, in contrast to TMFol. Previously, fisetin has been shown to inhibit the growth of the 22Rv1 prostate cancer xenograft *in vivo* [2]. The efficacy of TMFol observed here in the 22Rv1 model seems to be broadly similar to that demonstrated for fisetin by Khan et al [2], although it is important to note that administration of fisetin in that experiment was by the intraperitoneal route of 1mg/animal twice weekly rather than via diet supplementation.

The differential activity of TMFol as compared to those of quercetin and fisetin against the TRAMP C2 tumour *in vivo* may not only be related to differences between them in intrinsic antineoplastic activity, but also to differences in metabolic stability. Although a side-by-side pharmacokinetic analysis of these three molecules has not been performed, their chemical structures predict probable pharmacokinetic differences. The molecular scaffold of quercetin and fisetin bears hydroxyl moieties rendering them susceptible to avoid metabolic conjugation, which is likely to severely confound their bioavailability. In contrast, TMFol harbours methoxy instead of hydroxy moieties, which impart lower propensity towards metabolic transformation on the molecule, ensuring superior bioavailability. Here we show that TMFol can be recovered from TRAMP C2 tumour tissue at levels which are about half of its growth-inhibitory IC_{50} value established in TRAMP C2 cells *in vitro*. In a recent pharmacokinetic study, TMFol was detectable in tissues up to 24 hours post intragastric administration of a dose of 240 mg/kg, and the C_{max} of TMFol in prostate tissue (18 nmol/g) was reached only after 6 hours post dosing [12]. All these results are commensurate with considerable metabolic stability of TMFol.

The results presented here suggest that TMFol may exert its prostate tumour growth-inhibitory properties *in vivo* by induction of apoptosis. The apoptotic index measured in tumour tissue derived from either model was raised in mice on TMFol as compared to those on control diet. It is likely that an indirect host-mediated mechanism contributed to this activity, as *in vitro* TMFol was able to induce apoptosis only in TRAMP C2 cells, but not in 22Rv1 cells. The mTOR inhibitor temsirolimus is an example of a drug which induced apoptosis in multiple myeloma-bearing mice *in vivo* but not in myeloma cells *in vitro* [13]. Further investigations need to be performed to shed light on the mechanisms by which TMFol can modulate prostate carcinogenesis targets.

In summary, the results presented here cautiously support prioritisation of TMFol over other flavonols for further investigation of the notion that such flavonoids are beneficial in the management of prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest to disclose.

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Table 1 Effect of TMFol, fisetin or quercetin on the growth of four prostate cancer-derived cells. The IC₅₀ values, which are the mean of triplicate determinations, were obtained in cell cultures after agent exposure for 6 days. The values quoted for 22Rv1 cells have been published previously [10].

Cell line	IC ₅₀ (μM ± SD)		
	TMFol	Fisetin	Quercetin
LNCaP	5.6 ± 0.3	39.7 ± 2.4	31.6 ± 5.1
PC-3	4.1 ± 0.6	37.5 ± 2.2	31.6 ± 2.5
TRAMP C2	1.7 ± 0.1	27.3 ± 3.4	23.0 ± 2.0
22Rv1 ^[10]	3.1 ± 0.3	25.9 ± 10.8	15.5 ± 6.2

Figure legends

Fig. 1. Structures of fisetin, quercetin and TMFol.

Fig. 2. Effect of TMFol on male MF-1 nude mice bearing 22Rv1 or TRAMP C2 prostate cancer cell xenografts. Mice received either AIN93G diet (control) or AIN93G diet that contained TMFol (0.2%) from seven days prior to cell inoculation until week 7 after cell inoculation. Tumour size was measured by calliper as described under Methods. Values are the mean ± SD of 10 (22Rv1) or 15 (TRAMP C2) animals per group. Asterisks indicate that values are significantly different from control ($p < 0.05$).

Fig. 3. Effect of TMFol on cell cycle distribution in prostate cancer cells. 22Rv1 and TRAMP C2 prostate cancer cells were exposed to TMFol (10 μ M) for 48 or 72h, harvested and stained with propidium iodide. Cell cycle distribution was determined by flow cytometry as described under Methods using the ModFit LT3.3 software. Values are the mean $\pm$ SD of 3 experiments. Asterisks indicate data values are significantly different from controls ($p < 0.05$).

Fig. 4. Effect of TMFol on apoptosis in prostate cancer cells. 22Rv1 and TRAMP C2 cells were exposed to TMFol (10 μ M) for up to 96h and harvested and stained with FITC-conjugated annexin V and propidium iodide. Apoptosis was determined by flow cytometry as described under Methods. Values represent the number of apoptotic cells as a percentage of the overall total cell number. Bars are the mean of 3 experiments $\pm$ SD. Asterisks indicate data is statistically significant from vehicle control ($p < 0.05$).

Fig. 5. Effect of dietary TMFol (0.2 %) on cleaved caspase-3 expression in prostate tumour tissue from mice bearing the TRAMP C2 or 22Rv1 tumours. Paraffin-embedded tumour tissue sections of the xenografts were evaluated by immunostaining with cleaved caspase-3 antibody and visualising with a NovoLink kit. Photomicrographs are representative of a control and a treated mouse. Arrows mark cells stained for cleaved caspase 3. 20X magnification. Scale bar = 25 μ m. For details see Methods. Digits indicate the mean of the apoptotic indices ($\pm$ SD) normalised to the control of 15 (TRAMP C2) or 10 mice (22Rv1). Asterisks indicate data is statistically significant from the control ($p < 0.05$).

Supplemental Fig. 1. Effect of dietary quercetin and fisetin on male nude MF-1 mice bearing the TRAMP C2 prostate cancer. Mice implanted with TRAMP C2 cells received either AIN93G diet (control) or AIN93G diet with quercetin (0.184 % w/w) or fisetin (0.174 % w/w) from seven days prior to cell inoculation until week 7 after cell inoculation. Tumour size was measured by calliper as described under Methods. Values are the mean $\pm$ SD of 5 animals per group.