BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.01185.x
www.brjpharmacol.org

RESEARCH PAPER

Tangeretin and its metabolite 4′-hydroxytetramethoxyflavone attenuate EGF-stimulated cell cycle progression in hepatocytes; role of inhibition at the level of mTOR/p70S6K

Z Cheng[1], S Surichan[1]*, K Ruparelia[2], R Arroo[2] and MR Boarder[1]

[1]*The Cell Signalling Laboratory, Leicester School of Pharmacy, De Montfort University, Leicester, UK, and* [2]*The Natural Products Group, Leicester School of Pharmacy, De Montfort University, Leicester, UK*

Correspondence
Professor MR Boarder, The Cell Signalling Laboratory, Leicester School of Pharmacy, De Montfort University, Leicester LE1 9BH, UK. E-mail: mboarder@dmu.ac.uk

*Present address: Research and Development Institute, Government Pharmaceutical Organisation, Bangkok 10400, Thailand.

Keywords
tangeretin; hepatocyte; S6K; p70S6K; cancer; mTOR; Akt

Received
9 June 2010
Revised
3 November 2010
Accepted
1 December 2010

BACKGROUND AND PURPOSE

The mechanisms by which the dietary compound tangeretin has anticancer effects may include acting as a prodrug, forming an antiproliferative product in cancer cells. Here we show that tangeretin also inhibits cell cycle progression in hepatocytes and investigate the role of its primary metabolite 4′-hydroxy-5,6,7,8-tetramethoxyflavone (4′-OH-TMF) in this effect.

EXPERIMENTAL APPROACH

We used epidermal growth factor (EGF)-stimulated rat hepatocytes, with [^{3}H]-thymidine incorporation into DNA as an index of progression to S-phase of the cell cycle, and Western blots for phospho-proteins involved in the cell signalling cascade.

KEY RESULTS

Incubation of tangeretin with microsomes expressing CYP1A, or with hepatocytes, generated a primary product we identified as 4′-OH-TMF. Low micromolar concentrations of tangeretin or 4′-OH-TMF gave a concentration-dependent inhibition of EGF-stimulated progression to S-phase while having little effect on cell viability. To determine whether time for conversion of tangeretin to an active metabolite would enhance the inhibitory effect we used long pre-incubations; this reduced the inhibitory effect, in parallel with a reduction in the concentration of tangeretin. The EGF-stimulation of hepatocyte cell cycle progression requires signalling through Akt/mTOR/p70S6K kinase cascades. The tangeretin metabolite 4′-OH-TMF selectively inhibited S6K phosphorylation in the absence of significant inhibition of upstream Akt activity, suggesting an effect at the level of mTOR.

CONCLUSIONS AND IMPLICATIONS

Tangeretin and 4′-OH-TMF both inhibit cell cycle progression in primary hepatocytes. The inhibition of p70S6K phosphorylation by 4′-OH-TMF raises the possibility that inhibition of the mTOR pathway may contribute to the anticancer influence of a flavonoid-rich diet.

Abbreviations

4′-OH-TMF, 4′-hydroxy-5,6,7,8-tetramethoxyflavone; EGF, epidermal growth factor; ERK, extracellular signal-related kinase; GSK-3, glycogen synthase kinase; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PI3K, phosphoinositide 3-kinase

Introduction

Anticancer drug therapy aims to exploit differences between tumours and normal tissues to achieve a selective cytotoxic effect (when cells are killed) or cytostatic effect (block of progress through the cell cycle and proliferation). Flavonoids such as tangeretin (5,6,7,8,4′-pentamethoxyflavone) are dietary compounds thought to contribute to the chemopreventive/anticancer effects of a diet rich in fruit and vegetables. Their anticancer effects include inhibition of metabolic activation of pro-carcinogens, antioxidant activity reducing DNA damage, and suppression of tumour growth by activating apoptosis or inhibiting intracellular signalling that promotes cell cycle progression. They may also have direct effects, or act as pro-anticarcinogens, becoming active after CYP1-dependent conversion selectively within cancer cells to a cytotoxic or cytostatic metabolite (Androutsopoulos *et al.*, 2008; 2009). Tangeretin is a potent antioxidant compound which has been reported to inhibit cell cycle progression through G1 in the human leukaemia cancer cell line HL60 (Hirano *et al.*, 1995; Pan *et al.*, 2002; Morley *et al.*, 2007), induce apoptosis and G2/M arrest in the erythroleukaemia cell line K562 (Sophie, 2005) and in cisplatin-resistant ovarian cancer cells (Arafa el *et al.*, 2009), and inhibit extracellular signal-related kinase (ERK) in human mammary ductal T47D carcinoma cells (Van Slambrouck *et al.*, 2005). These studies show the possible contribution of either or both cytotoxic and cytostatic effects to the anticancer potential of these compounds. Recent studies on human colon (COLO 205, HT-29) and breast cancer (MDA-MB-435) cell lines have emphasized the significance of their cytostatic effects (Pan *et al.*, 2002; Morley *et al.*, 2007). In addition anti-inflammatory effects have been reported, such as inhibition of induced cyclooxygenase 2 expression in human lung carcinoma cells (Chen *et al.*, 2007).

In a comparison between cancer cells and non-cancer cells, hepatocytes are of particular clinical interest. Following resection surgery for hepatic cancers the survival of the patient is in part determined by the timely restoration of liver mass. This occurs by proliferation of the remaining hepatocytes (Fausto *et al.*, 2006). An intervention which can target remaining cancer cells without inhibiting hepatocyte proliferation is clearly desirable. In a healthy liver there is very little hepatocyte proliferation – only following partial hepatectomy do they enter the cell cycle, an event dependent in part on stimulation with epidermal growth factor (EGF). This can be mimicked in cell culture, where progression of primary hepatocytes through G1 to S-phase of the cell cycle can be elicited by addition of EGF (Loyer *et al.*, 1996; Coutant *et al.*, 2002; Luo *et al.*, 2007), providing a model for liver regeneration (Fausto *et al.*, 2006). It is of considerable significance, therefore, to understand what influence anticancer treatments, such as the presence of tangeretin, may have on the proliferation of normal hepatocytes. If the hypothesis being considered is that tangeretin exerts its anticancer effect by acting as a prodrug, being converted to an active compound by CYP1 enzymes preferentially expressed in cancer cells, then it is particularly relevant to determine whether normal non-transformed hepatocytes are affected since they also express CYP1 enzymes.

Prior to the study reported here we identified 4′-hydroxy-5,6,7,8-tetramethoxyflavone (4′-OH-TMF) as a major product of tangeretin metabolism by CYP1A1 and CYP1A2, consistent with a previously reported pattern of metabolism (Nielsen *et al.*, 2000; Breinholt *et al.*, 2003). Here we have investigated the effects of this compound and the parent compound tangeretin on rat primary hepatocytes. The signalling pathways necessary for EGF-stimulated hepatocyte cell cycle progression include the ERK and phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) cascades (Coutant *et al.*, 2002; Thoresen *et al.*, 2003; Dixon *et al.*, 2005; Luo *et al.*, 2007; Scheving *et al.*, 2008). Akt directly phosphorylates glycogen synthase kinase (GSK-3), and indirectly through mTOR (in the rapamycin-sensitive mTORC1 complex) induces the phosphorylation of ribosomal S6 kinase (p70S6K). Activation of p70S6K is required for enhanced protein synthesis in G1 and progression to S-phase. Consistent with this pattern of events, rapamycin attenuates EGF-stimulated hepatocyte cell cycle progression (Coutant *et al.*, 2002) and inhibits p70S6K phosphorylation (Z. Cheng and M.R. Boarder, unpublished). In the present study, we showed that tangeretin and 4′-OH-TMF both inhibit cell cycle progression to S-phase in hepatocytes. 4′-OH-TMF (but not tangeretin) also potently inhibits p70S6K phosphorylation. This occurs without significant inhibition of Akt phosphorylation or activity, indicating that 4′-OH-TMF is a novel inhibitor of p70S6K activation in hepatocytes, acting at the level of mTORC1 or its activation. The anticancer influence of flavonoid-rich diets may in part be because they contain anticancer compounds which are PI3K/Akt/mTOR inhibitors (Brachmann *et al.*, 2009).

Methods

Cell preparation and culture

Hepatocytes were isolated from male Wistar-strain rats (200–300 g) as described previously (Luo *et al.*, 2007) and seeded at a density of 1×10^5 cells per well in collagen-coated 24-well plates, and 6×10^5 cells per well in collagen-coated six-well plates. Cells were cultured in William's medium E (WME) supplemented with 10% foetal calf serum and insulin (as the Sigma cell culture supplement: 10 $\mu g \cdot mL^{-1}$ insulin, 5.5 $\mu g \cdot mL^{-1}$ transferrin and 6.7 $ng \cdot mL^{-1}$ sodium selenite). After 4 h, the medium was replaced with serum-free WME. Cells were used within 48 h.

[^{3}H]-thymidine incorporation into DNA and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay

Cells were cultured under the conditions indicated, in 24-well plates for 24 h. They were then incubated for a further 24 h with or without 3 nM EGF, with 1 $\mu L \cdot mL^{-1}$ methyl-[^{3}H]-thymidine (37 $MBq \cdot mL^{-1}$: Amersham, Bucks, UK) for the final 4 h. Tangeretin (Apin Chemicals, Abingdon, UK) or 4′-OH-TMF was added in dimethyl sulphoxide (DMSO) as indicated, 15 min before EGF. Control experiments indicated that up to 0.5% DMSO had no measurable effect; it did not exceed 0.2% in the experiments reported here.

For the cell viability assay 250 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) at 2 $mg \cdot mL^{-1}$ was added

to 0.5 mL of the medium in 24-well plate 22 h after EGF addition. This was followed by a 2 h incubation. Following aspiration of medium 350 µL DMSO was added to the wells, and readings were taken from a plate reader at 540 nm.

Western blots

Hepatocytes were cultured in six-well plates under the conditions described above. Cells were pre-incubated with tangeretin or 4′-OH-TMF for 15 min before addition of EGF (3 nM final concentration) as indicated. Following stimulation, 100 µL per well lysis buffer was added (20 mM Tris-HCl, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 0.5% Triton X100, 1 mM phenylmethylsulphonylfluoride, 2 mM sodium orthovanadate; 1 mM β-mercaptoethanol, 20 $\mu g \cdot mL^{-1}$ aprotinin, 5 $\mu g \cdot mL^{-1}$ leupeptin, pH 7.6). Samples were equalized for protein content, then separated on 10% polyacrylamide gels. Blots were probed with anti-phospho-ERK (Promega, Southampton, UK), anti-phospho-Akt (Ser 473) and anti-phospho-GSK-3 (from Cell Signalling Laboratories, Herts, UK), and visualized using ECL$^+$ Plus (Amersham). The Western blots showed two bands for phospho ERK (corresponding to $p42^{mapk}$ and $p44^{mapk}$), two bands for phosphor-GSK-3 (the α- and β-forms) and two bands for phospho S6K (the p70 and p85 forms). For densitometry and pooling data across blots both bands were used for ERK and GSK-3 blots, while only the p70 band was used for the S6K blots, generating results specific for p70S6K.

Microsomal incubation and HPLC separation of tangeretin and metabolites

Cytochrome P450 microsomes prepared from insect cells transformed using a baculovirus vector expressing human CYP with co-expression of human NADPH-cytochrome P450 reductase were purchased from BD Biosciences (UK). Tangeretin (10 µM) was incubated with CYP microsomes (20 $pmol \cdot mL^{-1}$), NADPH (0.5 mM) and $MgCl_2$ (0.5 mM) in 10 mM phosphate buffer pH 7 at 37°C for 30 min when tangeretin and products were extracted by addition of cold methanol followed by injection onto a Phenomenex Luna 5 µ C18 (250 × 4.6 mm) column which was eluted (1 $mL \cdot min^{-1}$) with 60% solvent A (0.5% acetic acid and 1% acetonitrile in water) and solvent B (0.5% acetic acid and 4% acetonitrile in methanol) with a gradient of 60% solvent A and 40% solvent B rising to 89% solvent B over 15 min and held at this for 2 min, with detection at 327 nm. For LC/MS-MS the same Phenomenex column was used with an Agilent 1100 Trap XTC system.

Synthesis of 4′-OH-TMF

Ethanethiol sodium salt (0.05 g, 0.59 mmol) was added to a stirred solution of tangeretin (0.05 g, 0.13 mmol) in anhydrous *N*-dimethylformamide (5 mL). The reaction mixture was stirred under reflux for 3 h. The mixture was poured into an ice-water bath, acidified with 10% hydrochloric acid and extracted with ethyl acetate (3 × 15 mL). The combined organic extract was washed with saline solution, dried over anhydrous magnesium sulphate. Removal of the solvent *in vacuo* yielded the crude 4′-demethyltangeretin. Purification by column chromatography [SiO_2, dichloromethane with an increasing gradient of ethyl acetate (20–50%)] yielded 4′-demethyltangeretin as an off-white powder (0.032 g, 67%). The structure (see Figure 1) was established by ^1H and ^{13}C-NMR spectra recorded on a 400 MHz super-conducting Bruker Spectrometer (Karlsruhe, Germany) at 30°C. Thin layer chromatography was performed on aluminium sheets precoated with silica gel $60f_{254}$ (Merck, Darmstadt, Germany) observed under UV light (450 nm). Mass spectra were recorded on a Micromass Quattro II low resolution triple quadrupole mass spectrometer (EPSRC National Mass Spectrometry Service Centre, Swansea, UK).

Statistical tests

Data were analysed by GraphPad prism using ANOVA followed by Bonferroni's post test.

Results

Initial experiments identified a major metabolic product of the incubation of tangeretin with specific CYP enzymes. Figure 1B (upper panel) shows HPLC profiles following the incubation (30 min) of tangeretin with microsomes expressing CYP1A1, CYP1A2 or CYP1B1 enzymes, compared with control microsomes and a tangeretin standard. Incubation with CYP1-expressing microsomes led to an incubation time-dependent reduced tangeretin peak and the appearance of peaks with a shorter retention time. The rank order of rate of loss of area under the tangeretin peak was CYP1A1>CYP1A2>>>CYP1B1. Two major peaks from metabolites were seen; the one that formed first and eluted closest to tangeretin was characterized as 4′-OH-TMF, following the work of Nielsen and co-workers (Nielsen *et al.*, 2000; Breinholt *et al.*, 2002; 2003), by co-elution with the synthetic standard (the lower trace in Figure 1B shows an HPLC profile of the product of tangeretin incubation spiked with synthetic 4′-OH-TMF) and by LC/MS-MS, which gave an [M + H] for tangeretin of 373.1 and for the metabolic product of 359.1, consistent with it being identified as 4′-OH-TMF. The structure of this metabolite and the parent tangeretin are shown in Figure 1A. The 4′-OH-TMF was synthesized as described in *Methods* and used along with tangeretin in the work described here. We also incubated tangeretin with hepatocytes to investigate whether 4′-OH-TMF is formed. We found (Figure 1C) that after the 24 h incubation there was a peak on the HPLC trace, which co-eluted with 4′-OH-TMF, consistent with the formation of this metabolite within hepatocytes.

We tested tangeretin and 4′-OH-TMF for effects on the cell cycle progression of primary hepatocytes using [^3H]-thymidine incorporation into DNA as an index of progression to S-phase. We have previously shown that under the conditions of culture used here the maximum stimulant effect of EGF (3 nM) is observed when [^3H]-thymidine is present for the last 4 h of a 24 h stimulation period, suggesting that the onset of the S-phase occurs 20 h after exposure to EGF. As seen in Figure 2 (A,B) the unstimulated hepatocytes showed a low level of [^3H]-thymidine incorporation into DNA, with a substantial increase when EGF was present. The [^3H]-thymidine response to EGF was inhibited when tangeretin was added to the cells 15 min before EGF and was then present for the duration of a 24 h stimulation period

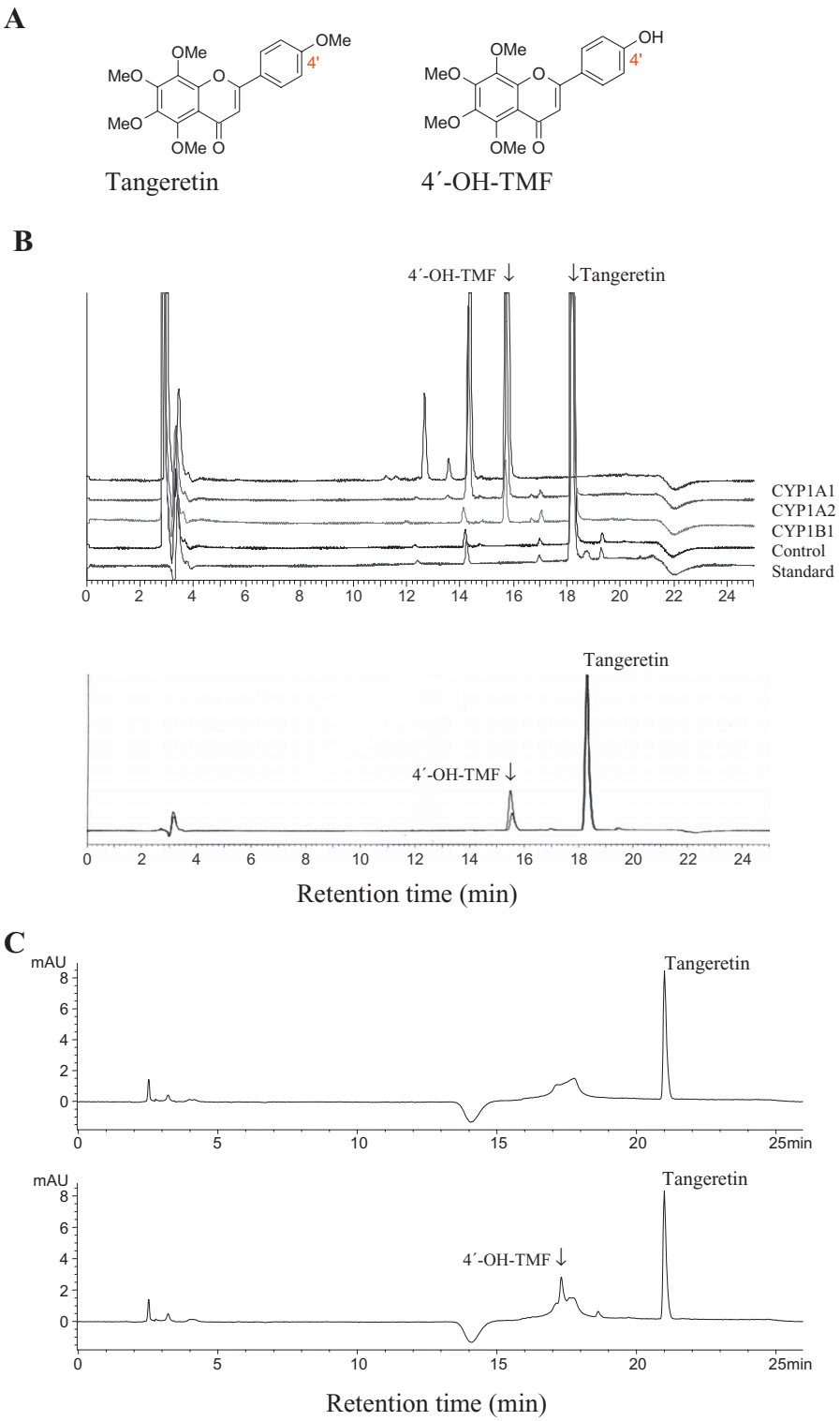

Figure 1

The HPLC profiles of tangeretin after incubation with microsomes expressing CYP1A1, CYP1A2 and CYP1B1 and with hepatocytes. (A) The structures of tangeretin and 4′-hydroxy-5,6,7,8-tetramethoxyflavone (4′-OH-TMF). (B) Upper trace: extracts from incubation (30 min) of 10 μM tangeretin with CYP-expressing microsomes, control microsomes and tangeretin standards were separated on a C18 analytical column as described in *Methods* and detected by absorbance at 327 nm. The tangeretin and 4′-OH-TMF peaks are indicated, and the structures of each are shown. Lower trace: HPLC profiles of the product of 10 μM tangeretin incubation (3 min) with CYP1A1-expressing microsomes, both with (larger peak at 4′-OH-TMF) and without (smaller peak) 1.5 μM synthetic 4′-OH-TMF added to the sample just before the HPLC run. (C) HPLC profiles of medium from incubation of 30 μM tangeretin after incubation with hepatocytes for 0 h (upper trace) or 24 h (lower trace).

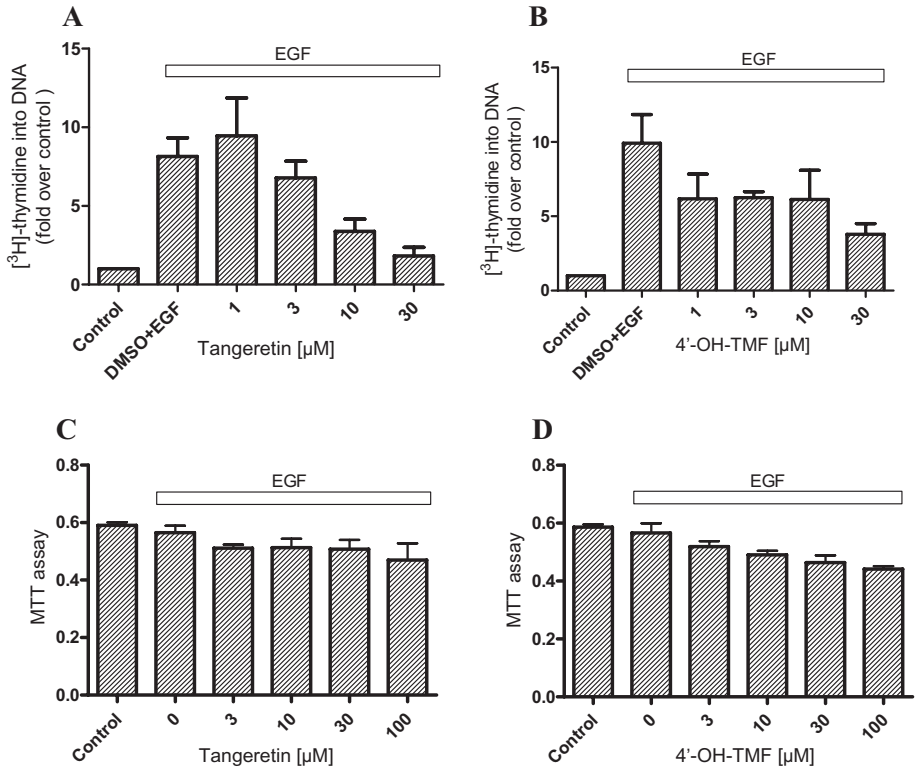

Figure 2

Effect in hepatocytes of tangeretin (A,C) and 4′-hydroxy-5,6,7,8-tetramethoxyflavone (4′-OH-TMF) (B,D) on epidermal growth factor (EGF)-stimulated [^{3}H]-thymidine incorporation into DNA, and cell viability. Tangeretin or 4′-OH-TMF to the final concentrations shown was added 15 min before 3 nM EGF followed by [^{3}H]-thymidine after 20 h and extraction of DNA at 24 h (A,B). One-way ANOVA showed that the effect of tangeretin and 4′-OH-TMF was significant ($P < 0.05$). Cell viability at 24 h was estimated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (C,D). Data for the [^{3}H]-thymidine assay were normalized to the unstimulated controls and pooled across separate experiment. Data are mean ± SEM ($n = 4$) in each case.

(Figure 2A). This inhibition was concentration dependent, with the onset between 1 and 3 μM tangeretin and an IC_{50} of about 5 μM; there was some variation between cell preparations but in all cases the response to EGF was almost completely inhibited by 30 μM tangeretin.

These results show that tangeretin effectively inhibited EGF-stimulated cell cycle progression to S-phase of primary hepatocytes. The tangeretin derivative 4′-OH-TMF also inhibited cell cycle progression (Figure 2B), but in this case the concentration–response relationship was more complex, with inhibition apparent at 1–3 μM but the degree of inhibition then remaining constant through to 10 μM, with further inhibition at 30 μM.

To investigate whether the reduction in the [^{3}H]-thymidine response in hepatocytes to either compound was due to a cytotoxic effect, resulting in a reduced number of cells, we used the MTT assay, which measures mitochondrial activity. The MTT assay is an index of the number of healthy cells and assumes the mitochondrial activity per cell is constant. Figure 2 (C,D) shows that EGF does not stimulate the MTT reading; under these conditions there is not a significant increase in the number of viable cells in response to EGF, despite a substantial increase in the [^{3}H]-thymidine response. This confirms that the cells progressed to S-phase but had not yet completed the proliferative cycle. Figure 2 shows the effect of tangeretin (C) and 4′-OH-TMF (D) on cell viability over the same period (24 h) used in the [^{3}H]-thymidine assay. In both cases there is a decline in cell viability as the concentration of drug increases, but this is small compared with the reduction in cell cycle progression seen with the [^{3}H]-thymidine assay.

If tangeretin is acting as a prodrug, being converted over time into a metabolic product such as 4′-OH-TMF, then a longer pre-incubation may allow accumulation of the metabolite and thereby enhance the cytostatic effect. To address this we pre-incubated hepatocytes with various concentrations of tangeretin for 15 min, 2 h, 6 h and 24 h prior to initiating a 24 h stimulation with EGF, followed by the [^{3}H]-thymidine and MTT assays. Figure 3A shows that at the lowest concentration of tangeretin tested (3 μM) its inhibitory effect on the [^{3}H]-thymidine response was prevented by the 24 h pre-incubation. However, inhibition was partially or completely retained with this longer pre-incubation time when higher concentrations of tangeretin (10 and 30 μM) were used. The longer pre-incubation times with tangeretin did not result in cell death at any concentration used (Figure 3B). Figure 3C shows that tangeretin was broken down by the cells after the 24 h pre-incubation. Tangeretin was added to cells and either removed with no incubation or incubated for 24 h (with incubation in the absence of cells as

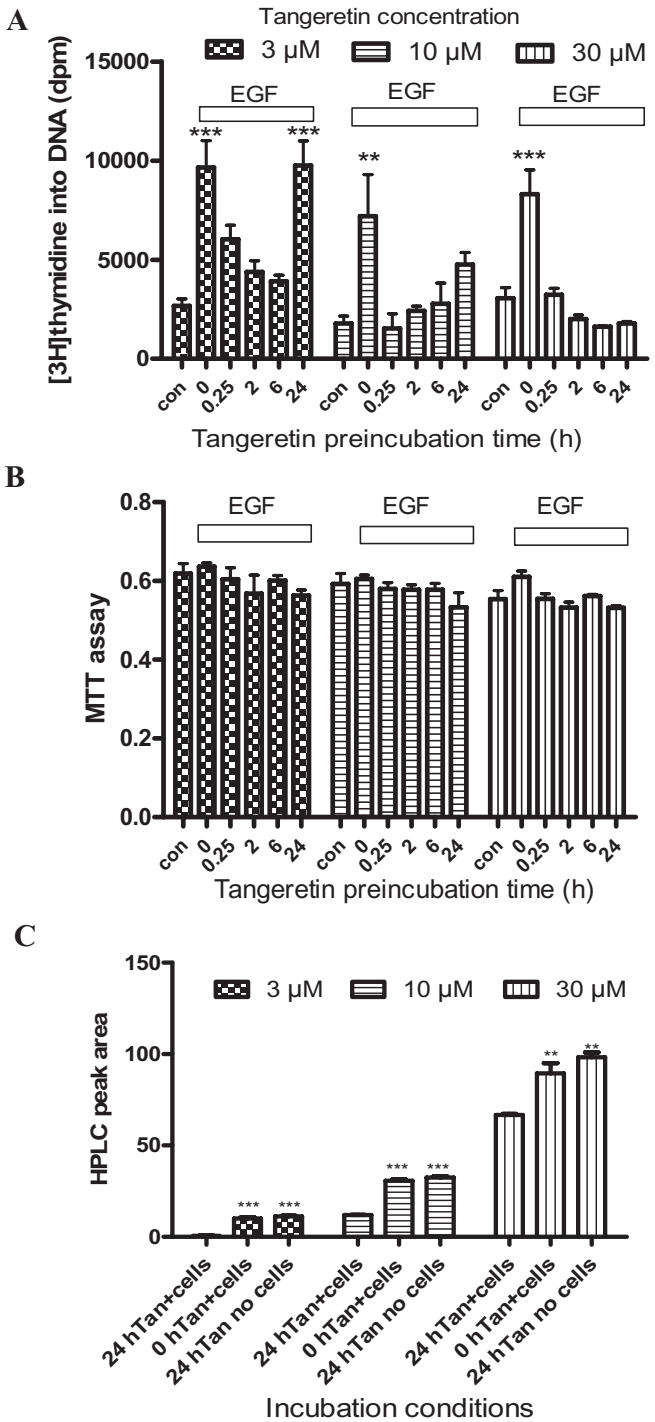

Figure 3

Effect of a long pre-incubation time with tangeretin (Tan) on [^{3}H]-thymidine and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) responses. Hepatocytes were pre-incubated with tangeretin at the concentrations shown for the times shown prior to stimulation with epidermal growth factor (EGF) (3 nm), before extraction of [^{3}H]-thymidine incorporation into DNA (A) or the MTT assay (B) 24 h later. Data are mean ± SEM (n = 4) from a single experiment representative of 3. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control (con; one-way ANOVA with Bonferroni's post test). (C) The loss of tangeretin in medium on incubation with hepatocytes for 24 h. The same three concentrations (3, 10 and 30 μM) of tangeretin were incubated, as above, with the cells for 24 h, added to the cells and immediately removed (0 h Tan + cells) or added to wells with medium but lacking cells (24 h Tan, no cells). The medium was then analysed for tangeretin by HPLC as described in *Methods*, and the area under the tangeretin peak was pooled across three separate experiments (data are mean ± SEM, n = 3; $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the 24 h incubation of each tangeretin concentration with cells using one-way ANOVA with Bonferroni's post test).

control). When the starting concentration was 3 μM there was no detectable tangeretin remaining after the 24 h incubation with cells, with 10 μM there was about 4 μM, and with a starting concentration of 30 μM there was about 20 μM left (Figure 2C). The residual inhibition of the [^{3}H]-thymidine response after 24 h pre-incubation shown in Figure 3A therefore approximately correlates with the remaining tangeretin.

To address the issue of how tangeretin and 4′-OH-TMF inhibit the stimulant effect of EGF on the cell cycle machinery in hepatocytes we looked at two sets of pathways previously shown to be involved in the cell cycle response. The first is the Ras/Raf/ERK pathway, which we assessed by Western blots for the phosphorylation of ERK1 and ERK2. The second pathway is the Akt/mTOR/GSK/S6K pathway, which we monitored by Western blots for the phosphorylation of Akt, GSK-3 and p70S6K. Figure 4A–D shows the results obtained for tangeretin in the absence (left hand blots) and presence (right hand blots) of EGF with the histograms beneath each blot showing pooled results from densitometric scans. After Western blot analysis for phospho-Akt, phospho-GSK, phospo-p70S6K and phospho-ERK, we saw no significant effect of increasing concentrations of tangeretin with and without EGF stimulation. It is notable (Figure 4C) that there is a trend towards reduced basal p70S6K phosphorylation in the presence of 30–100 μM tangeretin – overall this effect of tangeretin was not significant (one-way analysis of variables).

Figure 5 shows the results obtained when 4′-OH-TMF was used instead of tangeretin. It was found that 4′-OH-TMF tended to induce a modest but not statistically significant reduction in phospho-Akt immunoreactivity (A) in both the presence and absence of stimulation with EGF. Neither basal GSK phosphorylation (B) nor ERK (D) phosphorylation was affected by the tangeretin derivative. However, there was a major reduction in the phosphorylation of p70S6K (C) in the absence (left) of EGF, which was substantially inhibited by 3 μM 4′-OH-TMF, the lowest concentration used, and abolished by 30 and 100 μM 4′-OH-TMF. In the presence of EGF, 4′-OH-TMF also significantly inhibited p70S6K phosphorylation, although this was less pronounced at the lowest concentration of 4′-OH-TMF.

For both the tangeretin and 4′-OH-TMF studies in Figures 4 and 5 non-phospho (i.e. pan) Western blots were run as loading controls and to monitor any changes in total protein levels. No changes in pan-Western blots for the treatments shown in these two figures were seen for Akt, GSK, ERK or S6K (illustrated for S6K in Figure S1).

We considered the possibility that tangeretin had no effect on the signalling pathways because it has insufficient

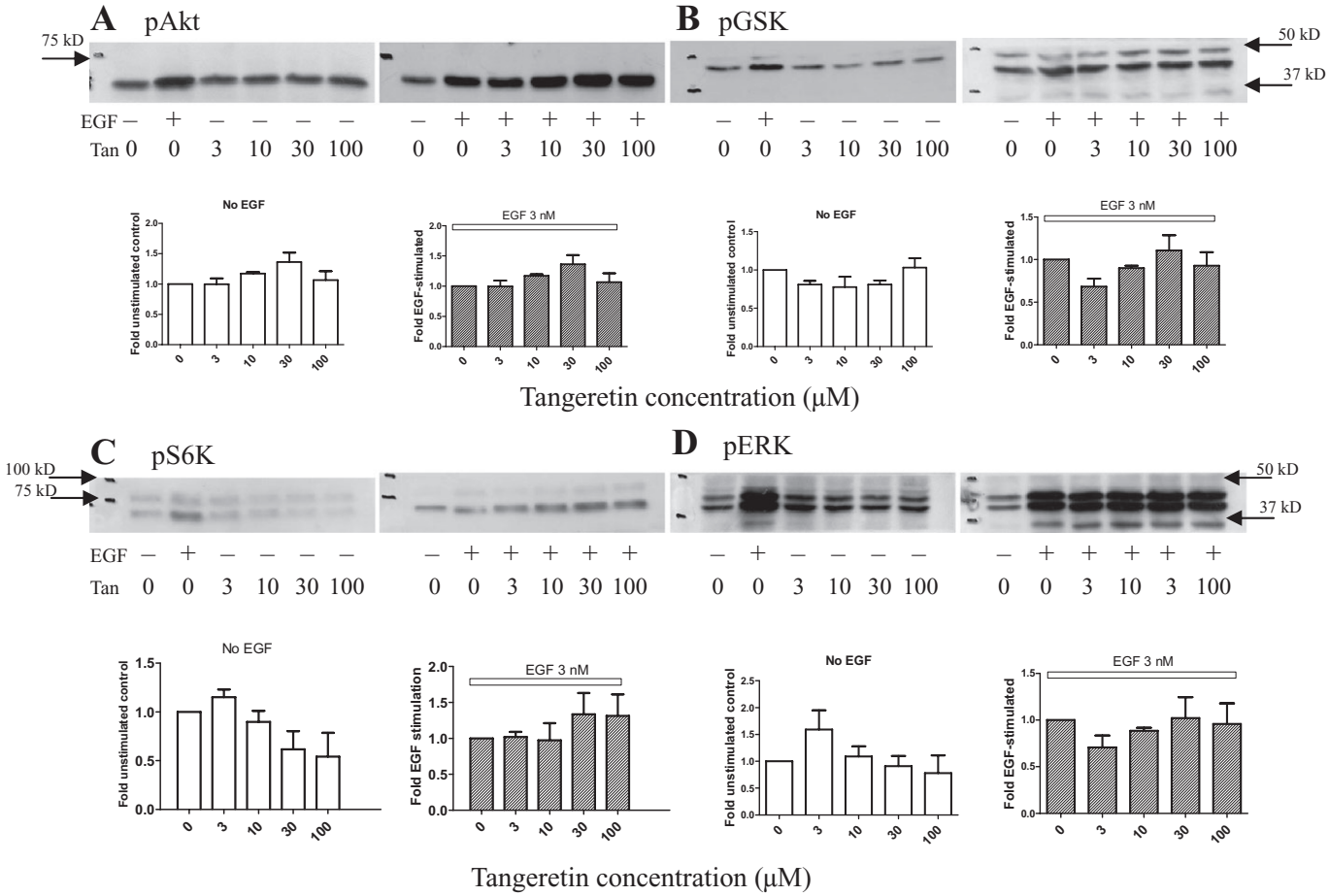

Figure 4

Effect of tangeretin on basal and epidermal growth factor (EGF)-stimulated phosphorylation of Akt, glycogen synthase kinase (GSK-3), S6K and extracellular signal-related kinase (ERK). Hepatocytes in six-well plates were stimulated with 3 nM EGF for 10 min, after a 15 min pre-incubation with tangeretin (Tan) at the concentrations shown (3–100 μM). Cell extracts were then subject to Western blot analysis for phospho-Akt (A), phospho-GSK (B), phospho-S6K (C) and phospho-ERK (D). In each case the left hand blots show the effect of tangeretin on unstimulated cells and the right hand blot shows the effect on cells stimulated with EGF. Sample blots are shown (with molecular weight markers as appropriate), and beneath these the results of scanning of blots from three to four repeated experiments are pooled (mean ± SEM). For the phospho-S6K blots the histograms are for p70S6K (i.e. the lower band only).

time, in these short incubation experiments, to be converted to an active metabolite. We therefore undertook studies in which hepatocytes were pre-incubated with tangeretin for 15 min, 2 h, 6 h and 24 h. We found that even with the longer pre-incubation times tangeretin had no effect on these kinase cascades (not shown).

Discussion

When the hepatocytes were incubated with tangeretin, the identification of 4′-OH-TMF as a primary metabolite of CYP1A1, CYP1A2 and CYP1B1, consistent with studies of others (Nielsen *et al.*, 2000; Breinholt *et al.*, 2003), was followed by a peak on HPLC analysis that co-eluted with 4′-OH-TMF. This encouraged us to investigate the hypothesis that tangeretin exerts effects on hepatocytes by a mechanism that is dependent on its metabolism to 4′-OH-TMF. The major enzymes in the liver are CYP3A4 (which does not metabolize tangeretin) and small amounts of CYP1A2 (which can metabolize tangeretin, but not as much as CYP1A1); there is very little or no CYP1A1 or CYP1B1 in the liver. Hence, hepatocytes were thought to have a limited ability to metabolize tangeretin to 4′-OH-TMF. However, the results presented here, obtained from HPLC analysis of the product derived after a 24 h incubation of tangeretin with hepatocytes, do nevertheless show a significant degree of this biotransformation in the cultured liver cells. This finding was followed by the synthesis of 4′-OH-TMF, enabling us to investigate the effect of both tangeretin and 4′-OH-TMF on hepatocyte cell cycle progression. Our results showed that tangeretin is able to inhibit EGF- stimulated cell cycle progression to S-phase in a concentration-dependent manner. This occurred without a commensurate loss of viable cells, as indicated by only minor falls in the readings from the MTT assay, leading us to

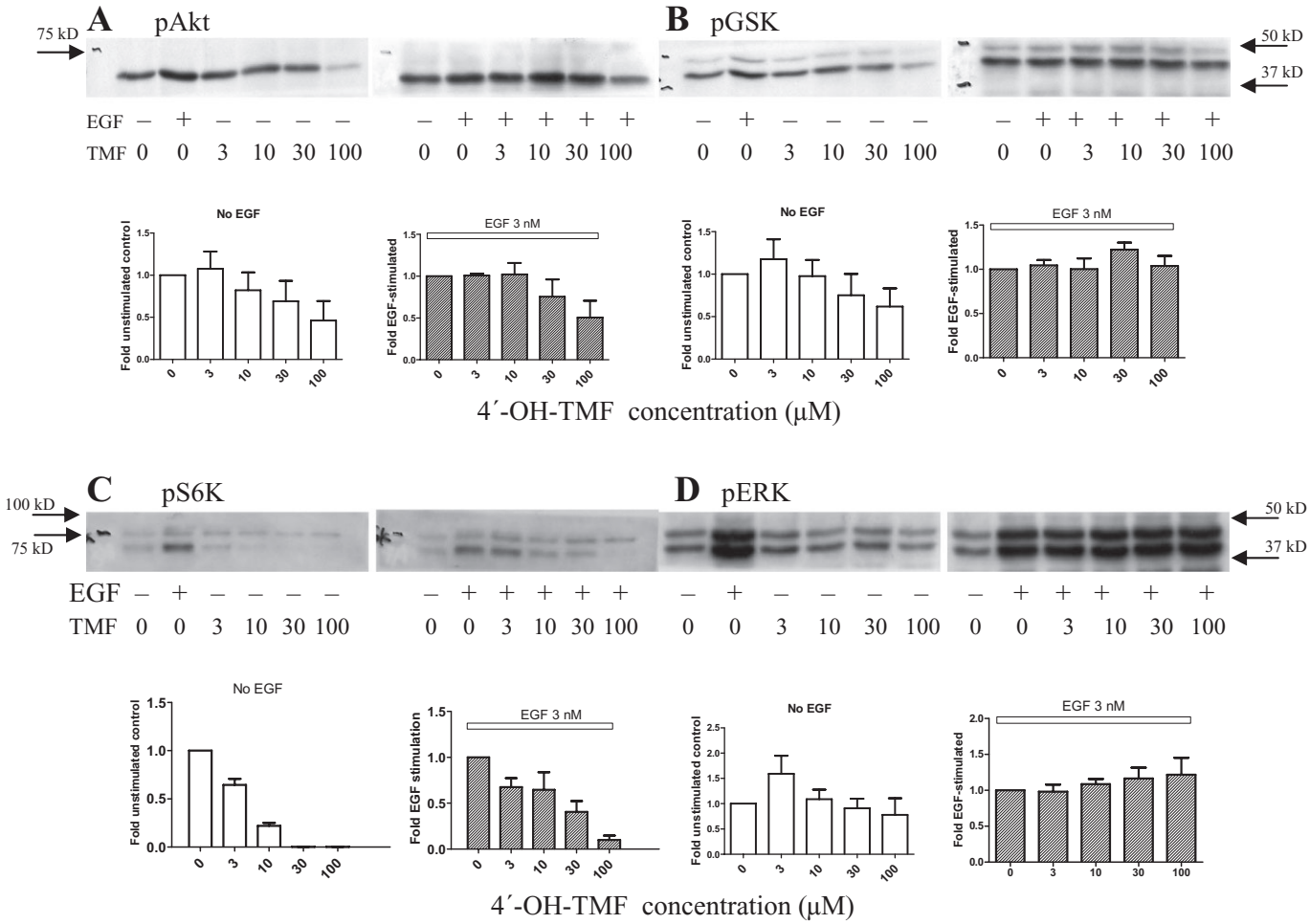

Figure 5

Effect of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (4′-OH-TMF) on basal and epidermal growth factor (EGF)-stimulated phosphorylation of Akt, glycogen synthase kinase (GSK-3), S6K and extracellular signal-related kinase (ERK). Hepatocytes in six-well plates were stimulated with 3 nM EGF for 10 min, after a 15 min pre-incubation with 4′-OH-TMF (TMF) at the concentrations shown (3–100 μM). Cell extracts were then subject to Western blot analysis for phospho-Akt (A), phospho-GSK (B), phospho-S6K (C) and phospho-ERK (D). In each case the left hand blots show the effect of 4′-OH-TMF on unstimulated cells and the right hand blot shows the effect on cells stimulated with EGF. Sample blots are shown with molecular weight markers as appropriate, and beneath these the results of scanning of blots from three to four repeat experiments are pooled (mean ± SEM). For the phospho-S6K blots the histograms are for p70S6K (i.e. the lower band only was used for the densitometry). (C) Effect of 4′-OH-TMF was significant (one-way ANOVA, $P < 0.05$).

conclude that tangeretin is cytostatic rather that cytotoxic, consistent with the work of others on cancer cells (Pan *et al.*, 2002; Morley *et al.*, 2007). To our knowledge, this is the first time that such an effect of tangeretin has been reported on non-transformed cells. As non-cancer cells hepatocytes may be unusual in that, as indicated above, they have substantial CYP activities; if tangeretin acts as a prodrug then the presence of CYP1A2 and its conversion to 4′-OH-TMF may account for its cytostatic effects. It is possible, therefore, that of all the non-transformed cells hepatocytes are unusually sensitive to tangeretin and other compounds with a similar mode of action.

The hypothesis that the marked cytostatic effect of tangeretin was due to its metabolite 4′-OH-TMF was indicated by the observation that this metabolite also inhibited EGF-stimulated DNA synthesis when applied to hepatocytes. However, the concentration–inhibition relationship was more complex with the metabolite. Notably, at most concentrations, tangeretin was more effective than its product. This suggests that the effects of tangeretin are not due to its conversion to 4′-OH-TMF within hepatocytes. However, it is possible that the greater polarity of 4′-OH-TMF makes it less cell membrane permeable and this would reduce its effect when added to the medium. Whereas following the addition of tangeretin it would form within the cells and could accumulate to a high intracellular level.

If the latter is the case then a longer pre-incubation time with tangeretin should increase its inhibitory effect. Accordingly, we pre-incubated the cells with different concentrations of tangeretin for various times up to 24 h, and then stimulated with EGF. Firstly, we noted that this procedure did not induce a cytotoxic effect. Secondly, that the cytostatic

effect of tangeretin was diminished, rather than increased, with the longer pre-incubation, and this reduction in effect was related to the decline in the concentration of tangeretin within the medium. This argues against the formation of an inhibitory metabolite trapped within cells.

To further pursue the mode of action of both tangeretin and 4′-OH-TMF we undertook short-term incubations with and without EGF stimulation followed by Western blot analysis for two signalling pathways known to be required for progression through G1 to S-phase in hepatocytes. These are the Ras/ERK pathway, which we monitored by Western blots for ERK phosphorylation, and the PI3K/Akt/mTOR/S6K pathway. Following PI3K activation the mTOR complex, mTORC2, is activated and phosphorylates Akt at serine 473. Akt directly phosphorylates GSK-3. The phosphorylation and activation of p70S6K by contrast is by Akt-dependent activation of mTORC1, the rapamycin-sensitive form of mTOR. The mTORC1 then phosphorylates and activates S6K. The role of mTOR in cancer cell signalling has recently been extensively explored (Laplante and Sabatini, 2009; 2010; Efeyan and Sabatini, 2010), and there are a number of studies on rapamycin and mTOR in hepatocytes (Coutant *et al.*, 2002; Luo *et al.*, 2007; Parent *et al.*, 2007; Chen *et al.*, 2009). Importantly for this study, we have previously shown that in hepatocytes the band on the GSK-3 phospho-Western blots used here is Akt-dependent (Luo *et al.*, 2007), but not inhibited by rapamycin, whereas p70S6K phosphorylation is rapamycin-sensitive (Z. Cheng and M.R. Boarder, unpubl. obs.). This pattern of events indicates that GSK-3 phosphorylation can be used as a measure of Akt activity, and S6K phosphorylation as a measure of mTORC1 activity.

In this study, we have followed the effects of tangeretin and 4′-OH-TMF on this sequence of events with phospho-Western blots for Akt, GSK-3 and p70S6K, and also monitored ERK phosphorylation. The salient observations were: (i) there were no effects on ERK phosphorylation; (ii) tangeretin weakly inhibited basal p70S6K, with no effect on EGF-stimulated p70S6K or Akt and GSK phosphorylation; and (iii) 4′-OH-TMF inhibited basal and EGF-stimulated p70S6K phosphorylation with no significant reductions in Akt-, GSK- or ERK-phosphorylation. The pattern of effects seen here, namely loss of p70S6K activation without similar effects on upstream Akt (both measured as Akt phosphorylation and Akt activity indicated by GSK phosphorylation), indicate inhibition by 4′-OH-TMF at the level of mTORC1. This could be as an inhibitor of the mTORC1 complex itself, which would indicate a rapamycin-like action. Alternatively it may indicate inhibition of a pathway upstream of mTORC1 but downstream of Akt. One possibility is that the intervention occurs at the level of the tuberous sclerosis complex, which mediates the action of Akt as an activator of mTORC1 (Efeyan and Sabatini, 2010).

It was notable that the inhibitory effect of 4′-OH-TMF on p70S6K phosphorylation was greatest under basal conditions, and weaker with EGF-stimulated cells. We know that basal S6K phosphorylation is dependent on continuing mTOR activity within the time of these experiments, because inhibition with rapamycin leads to loss of the phospho-p70S6K band (not shown). The greater effect of 4′-OH-TMF on p70S6K under basal conditions suggests that there may be some competitive aspect to the mechanism of action. It is also of interest to note that tangeretin has a (small) inhibitory influence on p70S6K phosphorylation, but no effect on the EGF-stimulated response. This similarity to 4′-OH-TMF with respect to a greater effect on unstimulated cells could be used to support the hypothesis that its anticancer effect is induced by biotransformation to 4′-OH-TMF and inhibition of p70S6K activation.

However, there are a number of observations that do not support this hypothesis. Notably, the cytostatic effect of tangeretin is seen under conditions of EGF stimulation in which we were unable to detect much effect on p70S6K. Further, if the cytostatic effect of both compounds were due to inhibition of p70S6K phosphorylation then 4′-OH-TMF would be more potent at inhibiting cell cycle progression than tangeretin. Instead, tangeretin is more potent as a cytostatic agent than 4′-OH-TMF, while not effective at blocking S6K phosphorylation. Issues such as differential cell permeability may complicate the interpretation of some of these issues. Nevertheless, the results presented here do not provide unequivocal support for the hypothesis that in hepatocytes, with limited expression of tangeretin-metabolizing CYP enzymes, the cytostatic effect of tangeretin is induced by conversion to 4′-OH-TMF and inhibition of p70S6K phosphorylation. Interestingly, in a study on cisplatin-resistant human ovarian cancer cells (Arafa el *et al.*, 2009), it was found that tangeretin down-regulates Akt phosphorylation. This is fundamentally different from our findings obtained in the present study in hepatocytes and indicates that we can expect cell type-specific responses to these and related agents.

The observation that tangeretin inhibits non-transformed hepatocytes may prove significant for the management of post-resection patients, when hepatocyte proliferation is crucial for restoration of liver mass. We also showed that the primary metabolite 4′-OH-TMF is cytostatic in hepatocytes. This is likely to be explained, at least in part, by our report here that 4′-OH-TMF is an inhibitor of proliferative signalling at the mTOR level. Currently inhibitors of the PI3K/Akt/mTOR pathway are of considerable interest in cancer therapeutics (Brachmann *et al.*, 2009). The flavone demethylation discussed here is mainly catalysed by the extrahepatic cytochromes P450 CYP1A1 and CYP1B1. The CYP1A1 is thought to play a role in the aetiology of breast (Subramanian *et al.*, 2008) and lung (Planchard *et al.*, 2009) cancer. CYP1B1 is notably found in a range of malignant tumours, including cancers of breast, colon, lung, oesophagus, skin, lymph node, brain and testis (Murray *et al.*, 1997). With respect to the bioavailability of dietary flavones, there are indications that these can reach levels of 2–6 μM in liver, kidney and lung (Walle, 2007), placing them within reach of the concentrations used in the present study. However, it remains to be seen whether in cancer cells, with their high CYP1 potential for conversion of tangeretin to 4′-OH-TMF, a flavonoid-rich diet exerts its anticancer influence by biotransformation to inhibitors of the mTOR pathway.

Acknowledgements

Authors are thankful to Professor AG Brenton and team at EPSRC National Mass Spectrometry Service Centre at Swansea for carrying out analyses of synthesized compounds, and the

Laxton Bequest, the Edith Murphy Foundation and the UK Human Tissue Bank for financial support.

Conflict of interest

All authors declare that there is no conflict of interest relating to this paper.

References

Androutsopoulos V, Arroo RR, Hall JF, Surichan S, Potter GA (2008). Antiproliferative and cytostatic effects of the natural product eupatorin on MDA-MB-468 human breast cancer cells due to CYP1-mediated metabolism. Breast Cancer Res 10: R39.

Androutsopoulos VP, Ruparelia K, Arroo RR, Tsatsakis AM, Spandidos DA (2009). CYP1-mediated antiproliferative activity of dietary flavonoids in MDA-MB-468 breast cancer cells. Toxicology 264: 162–170.

Arafa el SA, Zhu Q, Barakat BM, Wani G, Zhao Q, El-Mahdy MA *et al.* (2009). Tangeretin sensitizes cisplatin-resistant human ovarian cancer cells through downregulation of phosphoinositide 3-kinase/Akt signaling pathway. Cancer Res 69: 8910–8917.

Brachmann S, Fritsch C, Maira SM, Garcia-Echeverria C (2009). PI3K and mTOR inhibitors: a new generation of targeted anticancer agents. Curr Opin Cell Biol 21: 194–198.

Breinholt VM, Offord EA, Brouwer C, Nielsen SE, Brosen K, Friedberg T (2002). In vitro investigation of cytochrome P450-mediated metabolism of dietary flavonoids. Food Chem Toxicol 40: 609–616.

Breinholt VM, Rasmussen SE, Brosen K, Friedberg TH (2003). In vitro metabolism of genistein and tangeretin by human and murine cytochrome P450s. Pharmacol Toxicol 93: 14–22.

Chen KH, Weng MS, Lin JK (2007). Tangeretin suppresses IL-1beta-induced cyclooxygenase (COX)-2 expression through inhibition of p38 MAPK, JNK, and AKT activation in human lung carcinoma cells. Biochem Pharmacol 73: 215–227.

Chen P, Yan H, Chen Y, He Z (2009). The variation of AkT/TSC1-TSC1/mTOR signal pathway in hepatocytes after partial hepatectomy in rats. Exp Mol Pathol 86: 101–107.

Coutant A, Rescan C, Gilot D, Loyer P, Guguen-Guillouzo C, Baffet G (2002). PI3K-FRAP/mTOR pathway is critical for hepatocyte proliferation whereas MEK/ERK supports both proliferation and survival. Hepatology 36: 1079–1088.

Dixon CJ, White PJ, Hall JF, Kingston S, Boarder MR (2005). Regulation of human hepatocytes by P2Y receptors: control of glycogen phosphorylase, Ca2+, and mitogen-activated protein kinases. J Pharmacol Exp Ther 313: 1305–1313.

Efeyan A, Sabatini DM (2010). mTOR and cancer: many loops in one pathway. Curr Opin Cell Biol 22: 169–176.

Fausto N, Campbell JS, Riehle KJ (2006). Liver regeneration. Hepatology 43 (Suppl. 1): S45–S53.

Hirano T, Abe K, Gotoh M, Oka K (1995). Citrus flavone tangeretin inhibits leukaemic HL-60 cell growth partially through induction of apoptosis with less cytotoxicity on normal lymphocytes. Br J Cancer 72: 1380–1388.

Laplante M, Sabatini DM (2009). mTOR signaling at a glance. J Cell Sci 122: 3589–3594.

Laplante M, Sabatini DM (2010). mTORC1 activates SREBP-1c and uncouples lipogenesis from gluconeogenesis. Proc Natl Acad Sci U S A 107: 3281–3282.

Loyer P, Cariou S, Glaise D, Bilodeau M, Baffet G, Guguen-Guillouzo C (1996). Growth factor dependence of progression through G1 and S phases of adult rat hepatocytes in vitro. Evidence of a mitogen restriction point in mid-late G1. J Biol Chem 271: 11484–11492.

Luo Y, Dixon CJ, Hall JF, White PJ, Boarder MR (2007). A role for Akt in epidermal growth factor-stimulated cell cycle progression in cultured hepatocytes: generation of a hyperproliferative window after adenoviral expression of constitutively active Akt. J Pharmacol Exp Ther 321: 884–891.

Murray GI, Taylor MC, McFadyen MC, McKay JA, Greenlee WF, Burke MD *et al.* (1997). Tumor-specific expression of cytochrome P450 CYP1B1. Cancer Res 57: 3026–3031.

Morley KL, Ferguson PJ, Koropatnick J (2007). Tangeretin and nobiletin induce G1 cell cycle arrest but not apoptosis in human breast and colon cancer cells. Cancer Lett 251: 168–178.

Nielsen SE, Breinholt V, Cornett C, Dragsted LO (2000). Biotransformation of the citrus flavone tangeretin in rats. Identification of metabolites with intact flavane nucleus. Food Chem Toxicol 38: 739–746.

Pan MH, Chen WJ, Lin-Shiau SY, Ho CT, Lin JK (2002). Tangeretin induces cell-cycle G1 arrest through inhibiting cyclin-dependent kinases 2 and 4 activities as well as elevating Cdk inhibitors p21 and p27 in human colorectal carcinoma cells. Carcinogenesis 23: 1677–1684.

Parent R, Kolippakkam D, Booth G, Beretta L (2007). Mammalian target of rapamycin activation impairs hepatocytic differentiation and targets genes moderating lipid homeostasis and hepatocellular growth. Cancer Res 67: 4337–4345.

Planchard D, Loriot Y, Goubar A, Commo F, Soria JC (2009). Differential expression of biomarkers in men and women. Semin Oncol 36: 553–565.

Scheving LA, Stevenson MC, Zhang X, Russell WE (2008). Cultured rat hepatocytes upregulate Akt and ERK in an ErbB-2-dependent manner. Am J Physiol Gastrointest Liver Physiol 295: G322–G331.

Sophie L (2005). Tangeretin induces caspase dependetn apoptosis and G2/M arrest in the erythroleukaemia cell line, MCF-10. Blood 106: 4435.

Subramanian A, Salhab M, Mokbel K (2008). Oestrogen producing enzymes and mammary carcinogenesis: a review. Breast Cancer Res Treat 111: 191–202.

Thoresen GH, Guren TK, Christoffersen T (2003). Role of ERK, p38 and Pl3-kinase in EGF receptorMediated mitogenic signalling in cultured rat hepatocytes: Requirement for sustained ERK activation. Cell Physiol Biochem 13: 229–238.

Van Slambrouck S, Parmar VS, Sharma SK, De Bondt B, Fore F, Coopman P *et al.* (2005). Tangeretin inhibits extracellular-signal-regulated kinase (ERK) phosphorylation. FEBS Lett 579: 1665–1669.

Walle T (2007). Methoxylated flavones, a superior cancer chemopreventive flavonoid subclass? Semin Cancer Biol 17: 354–362.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Hepatocytes in six-well plates were with a 15 min pre-incubated for 15 min with tangeretin (Tan) (A) or 4′-hydroxy-5,6,7,8-tetramethoxyflavone (4′-OH-TMF) (B) at the concentrations shown (3–100 μM). They were then incubated without epidermal growth factor (EGF) (– sign) or with 3 nM EGF (+ sign) for 10 min. Cell extracts were then subject to western blot for pan-S6K. Sample blots are shown.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.