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Effects of dietary flavonoids on major signal transduction pathways in human epithelial cells

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Abstract

Flavonoids (FVs) are an important class of plant compounds postulated to be one of the constituents responsible for the beneficial effects of fruits and vegetables on health, including heart disease and cancer. At pharmacological levels, various naturally-occurring flavonoids have been shown to be cancer-protective in a variety of animal models and flavonoid derivatives, such as flavopyridol, are being assessed as chemotherapy drugs in clinical trials. This report has investigated the effects of the most common dietary FVs on several major signalling pathways in biopsies of human epithelial cells using primary cultures freshly isolated from biopsies and has obtained evidence for the previously unrecognised importance of stress kinase responses induced by kaempferol (KF), apigenin (AP) and luteolin (LU). KF, AP and LU all activated ATM/ATR (mutated in ataxia-telangiectasia and related) kinases and the p38 stress kinase and this was associated with induction of GADD45 and cell cycle arrest in G2, but not induction of apoptosis. These effects were not due to general toxicity since they were reversible on removal of FV. The inductions of ATM/ATR and p38 were functionally important since caffeine, an inhibitor of ATM/ATR, and the p38-specific inhibitor, SB203580, prevented induction of GADD45 and growth arrest by these three flavonoids. In contrast, although quercetin (QU) activated ATM (but not ATR), it did not activate p38 kinase, GADD45 or p53. QU may interfere with one of the lipoxygenase (LOX) pathways since the growth inhibitory effects of QU (but not the other three flavonoids) could be reversed by addition of LOX metabolites, particularly 12- and 15-hydroxyeicostetraenic acids.

Keywords: Flavonoids; Signalling pathways; Diet; Kinases; Lipoxygenases

1. Introduction

Flavonoids (FVs)[*] are polyphenolic compounds found ubiquitously in foods of plant origin. The basic structure of FVs allows a large number of substitutions in the three rings, including phenolic hydroxyls, *O*-sugars, methoxy groups, sulphates and glucuronides, thus producing an extremely diverse range of derivatives. The main dietary sources of FVs are fruits, vegetables, red wine and tea: QU derivatives represent about 60% of total FV intake, with KP, AP, LU and myricetin derivatives comprising the remainder [1–4]. The dietary intake of FVs has been significantly associated in epidemiological studies with a reduced risk of heart disease and, possibly, cancer [5–8] (reviewed in [9–13]). Cancer-protective effects of various FVs, administered at pharmacological but non-toxic levels either as agyclones or as glycosides, have also been reported in several chemical- or UV-induced animal cancer models [12–22] (recently reviewed in [23]). There is also interest in using flavonoid derivatives therapeutically as anticancer drugs [24] and one semi-synthetic flavone derivative, flavopyridol, has shown promise in clinical trials [25–27].

It is therefore important to understand the biological effects of FVs in human cells and to elucidate their

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Abbreviations: AP, apigenin; ATM, ataxia-telangiectasia mutated; AKT/PKB, protein kinase B; ATR, ATM and rad3-related; CHO, Chinese hamster ovary; EGF, epidermal growth factor; ERK, extracellular regulated kinase; FV, flavonoids; HETE, hydroxyeicosatetraenoic acid; JNK, Jun N-terminal kinase; KF, kaempferol; LOX, lipoxygenase; LU, luteolin; MAP, mitogen-activated protein; MEM, minimal essential medium; NE, normal epithelial cells; PCR, polymerase chain reaction; PI-3-K, phosphatidyl inositol-3-phosphate kinase; QU, quercetin; RT, reverse transcription; SCC, squamous carcinoma cell.

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molecular mechanisms of action and their structure/function relationships. FVs have been shown to have a diverse range of effects on signalling enzymes of various kinds, using partially purified enzymes or cell extracts (recently reviewed in [23]): they have antioxidant properties [28] and can interfere with various kinase signal transduction pathways regulating cell growth, cell cycle control or apoptosis [29–35], as well as LOX and cycloxygenase (COX) pathways responsible for production of biological regulators such as leukotrienes and prostaglandins [36–39]. Moreover, studies with intact cells have shown that various FVs can inhibit mitogenic signalling pathways, such as activation of the mitogen-activated protein kinase (MAP) kinase pathway by epidermal growth factor (EGF) receptor and ErbB1 by silymarin [40,41], or inhibition of Ras-mediated MAP kinase activation by apigenin [42]. FVs also cause a G1 or G2 cell cycle arrest in different contexts [43–49] and this is associated with inhibition of cell cycle kinases, CDK2 or CDK1 [43–47,50]. Other biological effects of FVs include inhibition of enzymes involved in estrogen metabolism [51,52] and angiogenesis [53].

In this report, we have investigated the effects of common dietary FVs on several major regulatory/signal transduction pathways in human normal and malignant epithelial cells, using cells freshly isolated from biopsies in primary culture in order to provide as relevant an experimental model as possible. The novel conclusion from these studies is the previously unrecognised importance of stress kinase responses induced by three of the most common dietary FVs, KP, AP and LU, in particular the ATM/ATR-p38 stress kinase pathway controlling G2 cell cycle checkpoints; whereas QU appears to affect LOX-mediated pathways.

2. Materials and methods

2.1. Cells and culture conditions

The primary cultures of NE and SCC were isolated from biopsies of normal oral mucosa and oral carcinomas using the 3T3 feeder layer method of Rheinwald and Beckett [54] and have been characterised previously [55,56]. The SCC cultures are immortal and invasive, and are resistant to suspension-induced death [57]. NE and SCC cells were maintained on irradiated 3T3 feeders: NE cells in FAD+ medium (1:3 Ham's F12/Dulbecco's minimal essential medium (MEM) with 10% foetal calf serum and insulin, EGF, transferrin, cholera toxin, hydrocortisone and adenine); and SCCs in 10H medium (Dulbecco's MEM plus 10% foetal calf serum without added growth factors except hydrocortisone). However, all cells were transferred into 10H medium for 1 day before experiments. NE cells were used within the first two passages from frozen stocks, before their growth rate deteriorated significantly. 3T3 cells were maintained in 10C medium (Dulbecco's MEM plus 10% donor calf serum) and irradiated with 60 Gy from a Co^{60} source before use. 3T3 feeders were removed by washing with 0.02% EDTA/PBS for short-term experiments with FVs or other chemicals. Chinese hamster ovary (CHO) cells and HeLa cells were grown in Dulbecco's MEM containing 10% foetal calf serum.

2.2. Chemicals

All chemicals were obtained from Sigma. All FVs were dissolved in DMSO and then diluted to give the required concentration.

2.3. DNA synthesis/proliferation assay

10^4 irradiated 3T3 cells were plated in 200 μL 10C medium per microtitre well. After 3 days, the medium was removed and oral epithelial cells were added (5000 in the case of NE cells, or 2500 SCC) in 150 μL 10H medium. After 18 hr, 50 μL of FV at 4 times the required final concentration in 10H medium was added and incubated for 3 days. Each well was then given 0.5 μCi of tritiated thymidine for 6 hr, the medium removed and the cells trypsinised and transferred onto a filter paper mat (printed filtermat A, Pharmacia) using a microtitre plate harvester (Skatron Combi Harvester, model 11900, Skatron, Norway, distributed by LKB). After adding scintillator, the mat was scanned and counted using a plate counter (model 1205 Betaplate™, Pharmacia). Four replicate wells were used for each condition. Control experiments showed that the irradiated feeders supported growth of the oral cells for the duration of the experiment, but contributed an insignificant background thymidine incorporation (less than 5% of that of the oral cells). The radioactivity incorporated into DNA was calculated as a percentage of untreated cultures.

2.4. Cell cycle analysis

Cells were plated at a density of 5×10^5 cells/10 mL medium in 90 mm dishes. After 3 days the irradiated 3T3 cells were removed, and FV added 1 day later. After a further 2 days, the cells were harvested, washed in cold PBS and fixed in 70% ethanol. Prior to FACS analysis, DNA was stained with propidium iodide (20 μg/mL) and the cell cycle distribution calculated from 2×10^4 cells with the CellQuest software using a FACScan flow cytometer (Becton Dickinson).

2.5. Determination of LOX mRNAs by RT/PCR

RNA was prepared from SCCs or human white blood cells using Trizol reagent (Life Technologies). cDNA synthesis and polymerase chain reaction (PCR) amplification were carried out using a Perkin-Elmer GeneAmp RNA PCR Core kit, with random hexamer primers used

for first strand cDNA synthesis. 750 and 75 ng RNA was used per reaction from SCC and human white blood cells, respectively. Specific DNA sequences corresponding to the different lipoxygenases [58–61] were amplified by PCR using 30 cycles of 1 min at 95° and 1 min at 60° with a final step of 72° for 7 min. Oligonucleotides used were; human 15S Lox, 5′-GAA-ACT-GCG-CAA-ACG-GCA-CCT-CCT-TAA-GGA-3′ and 5′-ATA-GTT-TGG-CCC-CAG-CCA-TAT-TCA-GAA-TTA-3′; human 15/2S Lox, 5′-GTG-CTG-CCC-CTG-CTG-GGG-CCC-CTG-GCC-CCG-3′ and 5′-TCT-TTT-CAT-CCA-GGC-AGT-GAG-GCC-AAC-CTG-3′; human 12S Lox, 5′-GTG-AGG-CTG-CGC-AAG-CAC-CAC-TGG-CTG-GTG-3′ and 5′-CCT-TAC-GGT-CTG-CAG-CGA-TGG-TCA-GGG-GTA-3′; human 12R Lox, 5′-AGC-GGT-ACG-CCT-TCT-TCC-CCA-A-3′ and 5′-GTT-GGG-GTT-GCG-ATG-CCT-CCG-CAC-CGG-AGG-3′; human 5S Lox, 5′-AGA-ATC-GAG-AAG-CGC-AAG-TAC-TGG-CTG-AAT-3′ and 5′-TTG-TGG-CAT-TTG-GCA-TCG-ATG-CTC-AAG-GGG-3′. Human glyceraldehyde-3-phosphate dehydrogenase oligonucleotides were used as controls to confirm the integrity of the cDNA and PCR reaction (not shown).

2.6. Assays for phosphorylation of ERKs 1&2, Akt, p38, JNK and cdc2

To prepare whole cell protein extracts, the cells were washed twice with ice-cold PBS and then scraped off in 0.2 mL of buffer (20 mM Hepes, pH 6.8, 5 mM EDTA, 10 mM EGTA, 5 mM NaF, 0.1 μg/mL okadaic acid, 1 mM DTT, 0.4 M KCl, 0.4% Triton X-100, 10% glycerol, 5 μg/mL leupeptin, 50 μg/mL PMSF, 1 mM benzamidine, 5 mg/mL aprotinin, 1 mM Na orthovanadate) and incubated on ice for 20 min, followed by centrifugation at $13,000 \times g$ in a microfuge for 10 min. The supernatant was stored at −70°. Up to 50 μg of protein sample in 40 μL of buffer was mixed with 20 μL loading buffer (187.5 mM Tris–HCl, pH 6.8, 30% glycerol, 6.9% SDS, 2.1 M β-mercaptoethanol, 0.1% bromophenol blue) before electrophoresis on a SDS/8%PAGE gel. The proteins were then blotted onto nitrocellulose (Amersham) using a Camlab (UK) semi-dry blotter, following the manufacturer's protocol. Western blots were incubated in the presence of TBS-T (50 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1% Tween 20) and 5% dried milk. Activated or total amounts of specific kinases were measured using the following antibodies: activated Jun N-terminal kinase (JNK), monoclonal antibody p-JNK (G-7) (cat. no. sc-6254, Santa Cruz Biotechnology); total JNK1, monoclonal antibody JNK1(F-3) (cat. no. sc-1648, Santa Cruz Biotechnology); activated and total p38 kinase, PhosphoPlus p38 MAP kinase (tyr182) polyclonal antibody kit (cat. no. 9210, New England Biolabs); activated MAP kinases (extracellular regulated kinase, ERK 1&2), polyclonal antibody (cat. no. V667, Promega); and total MAP kinase, ERK1 (C-16)-G polyclonal antibody (cat. no. sc-93-G, Santa Cruz Biotechnology). ERK5 was detected as described in [62] using polyclonal antibody ERK5 (C-20) (cat. no. sc-1284, Santa Cruz Biotechnology); activated protein kinase (Akt/PKB) and total Akt, PhosphoPlus Akt (ser 473) polyclonal antibody kit (cat. no. 9270, New England Biolabs); tyrosine-15 phosphorylated cdc2, polyclonal antibody Phospho-cdc2 (Tyr15) (cat. no. 9111, New England Biolabs); polyclonal antibody GADD45 (C-20) (cat. no. sc-792, Santa Cruz Biotechnology). In all cases bound primary antibody was detected using ECL chemiluminescent methodology (Amersham).

2.7. cdc2 kinase assays

cdc2 kinase assays were carried out using the Promega SignaTECT cdc2 Protein Kinase Assay System (cat. no. V6430). Briefly, 2×10^6 SCC were plated out on 1×10^6 irradiated 3T3 feeders and allowed to adhere overnight. Feeders were removed and FV added 1 day later. After 24 or 48 hr whole cell protein extracts were prepared by lysing the cells in 300 μL extraction buffer (50 mM Tris–HCl pH 7.4, 250 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM dithiothreitol, 0.1% Triton X-100, 10 μM leupeptin, 100 μg/mL aprotinin, 1 mM phenylmethylsulphonyl fluoride). After centrifugation at 13000 *g*, 10 μg of protein extract was assayed for cdc2 kinase activity exactly as described in the SignaTECT kit.

2.8. ATM/ATR kinase assays

Immunoprecipitation of ATM and ATR and kinase assays were carried out using a modification of a procedure previously described [63]. Briefly, cells were lysed in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Tween 20, 0.2% NP-40, 1 mM NaF, 1 mM sodium orthovanadate, 50 mM β-glycerophosphate, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride, 2 μg/mL pepstatin A, 5 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM dithiothreitol). After centrifugation at 13,000 *g*, a 500 μg aliquot of extract was incubated with 10 μg rabbit IgG and 40 μL of a 50% slurry of protein A Sepharose beads in lysis buffer for 1 hr at 4°. ATM or ATR proteins were then immunoprecipitated with anti-ATM (Ab-3, Calbiochem) or anti-ATR (Ab-2, cat. no. PC538, Oncogene Research Products) antibodies and protein A Sepharose beads for 18 hr at 4°. Immunoprecipitates were washed three times in lysis buffer, once in lysis buffer containing 0.5 M LiCl and three times in kinase buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1% Tween 20, 0.2% NP-40, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM DTT and 10 mM $MnCl_2$). Kinase reactions were initiated by resuspending the immunoprecipitates in kinase buffer containing 5 μM ATP, 5 μCi {γ-^{32}P}ATP and 0.5 μg p53 protein as substrate (cat. no. sc-4246, Santa Cruz Biotechnology) and incubated at 30° for 20 min. The kinase reactions were stopped with SDS/PAGE loading dye and proteins were separated by gel electrophoresis

(10%), transferred to nitrocellulose and analysed by autoradiography. The same blots or parallel blots from 5% SDS/PAGE gels were probed with the ATM or ATR antibodies as loading controls.

3. Results

3.1. Dose–response relationships

In order to obtain information as relevant as possible to human epithelial cells *in vivo*, in these studies we have used the primary cultures of biopsies of normal oral mucosa (NE) and squamous carcinomas (SCC) described previously [55,56]. These were all isolated using the 3T3 feeder layer culture system of Rheinwald and Beckett which has been shown to facilitate growth of keratinocytes at all stages of cancer progression and to maintain the characteristics of the original tumours [54]. In some experiments, for technical reasons, CHO or HeLa cells were also used. FVs were used at various doses up to the levels that cause growth arrest: the IC_{50} concentrations for growth arrest of the various cell types used are given in Table 1. No significant differences in sensitivity were observed between NE and SCC cultures (Table 1).

3.2. Effect of FVs on activation of ERKs 1&2

We first tested whether FVs interfered with activation of either the ERKs 1&2 MAP kinases in view of their importance in the regulation of epithelial cell growth by mitogens [64,65]. We also tested whether FVs activated these kinases directly in the absence of mitogens since this has been shown to occur with certain types of agents that induce oxidative, or other, stress responses, such as hydrogen peroxide [66,67]. Activation of ERKs 1&2 and AKT was then measured in cell lysates by Western blotting with antibodies that recognise either the total amount of kinase or the amount of activated (phosphorylated) kinase. We tested a range of FV concentrations extending up to the levels that cause cell cycle arrest (Table 1). Detailed time–course and dose–response experiments showed that ERKs 1&2 were strongly activated by 1× the IC_{50} concentration of AP, but this only occurred after a considerable time lag of 18–48 hr (Fig. 1A). In contrast, QU gave a slight activation of ERKs 1&2 only at 2–4× the IC_{50} concentration, but this occurred much earlier, peaking at about 4 hr (Fig. 1A). KF and LU also significantly activated ERKs 1&2 in SCCs during 24 hr of treatment in normal growth medium (Fig. 1B). This suggests that the activation of ERKs 1&2 by AP/KF/LU and QU may be mechanistically different.

Table 1
IC_{50} concentrations for growth inhibition of NE, SCC and CHO cells by different FVs

IC_{50} (μM)				
FV	NE	SCC	CHO	HeLa
Apigenin	23	26	19	7.3
Luteolin	18	22	5.1	5.8
Kaempferol	37	35	22	13
Quercetin	31	30	24	5

These values were measured using the tritiated thymidine proliferation assay (see Section 2).

We also tested whether any of the FVs affected the activation of ERKs 1&2 by EGF in low serum medium. In these experiments, SCCs were pretreated with 2× the IC_{50} concentration of FV for 18 hr in low serum and then with EGF for 15 min, before lysates were prepared and the ERK 1&2 assays performed. In fact, the levels of activation by AP or LU were similar whether or not the cells were treated with EGF and this was comparable to the level induced by EGF alone, although with KF there seemed to be an additive effect of FV and EGF (Fig. 1C). This suggests that perhaps that EGF, AP or LU can each activate ERKs 1&2 to a saturation point. QU did not inhibit the activation of ERKs 1&2 by EGF (Fig. 1C).

3.3. Effects of FVs on activation of ERK5 and AKT

Similar analyses were performed for ERK5, since it has been implicated in the regulation of epithelial cell proliferation by EGF [65]. In this case, ERK5 activation was measured by the change in its mobility in response to EGF [62]. ERK5 does not appear to be important for oral cell proliferation since it appears not to be activated by EGF in either NE or SCCs (Fig. 2A). However, none of the FVs affected ERK5 activation by EGF in HeLa cells in which the pathway is important [54,62] (Fig. 2A). We also extended this analysis to AKT activation/phosphorylation in view of the importance of the PI-3-kinase/AKT pathway in controlling cell survival [68]. Activation of AKT was then measured in cell lysates by Western blotting with antibodies that recognise either the total amount of kinase or the amount of activated (phosphorylated) kinase. However, none of the FVs reduced activation of AKT in SCCs in the presence of serum, except possibly QU (Fig. 2B).

3.4. Effects of FVs on stress kinases

We next tested the effects of the FVs on the two main stress kinase families, the JNK and p38 kinases, which are involved in regulation stress responses by a variety of agents [69]. Activation of these stress kinases in oral epithelial cells was measured in cell lysates by Western blotting with antibodies that recognise either the total enzyme or specifically only the activated (phosphorylated) form. Dose–response and time–course experiments with AP showed that activation of p38 began to be observed at levels of AP that begin to arrest the growth of SCCs (1× IC_{50} concentration) after long treatment times of 18–48 hr (Fig. 3A), whereas, activation of JNK was not observed

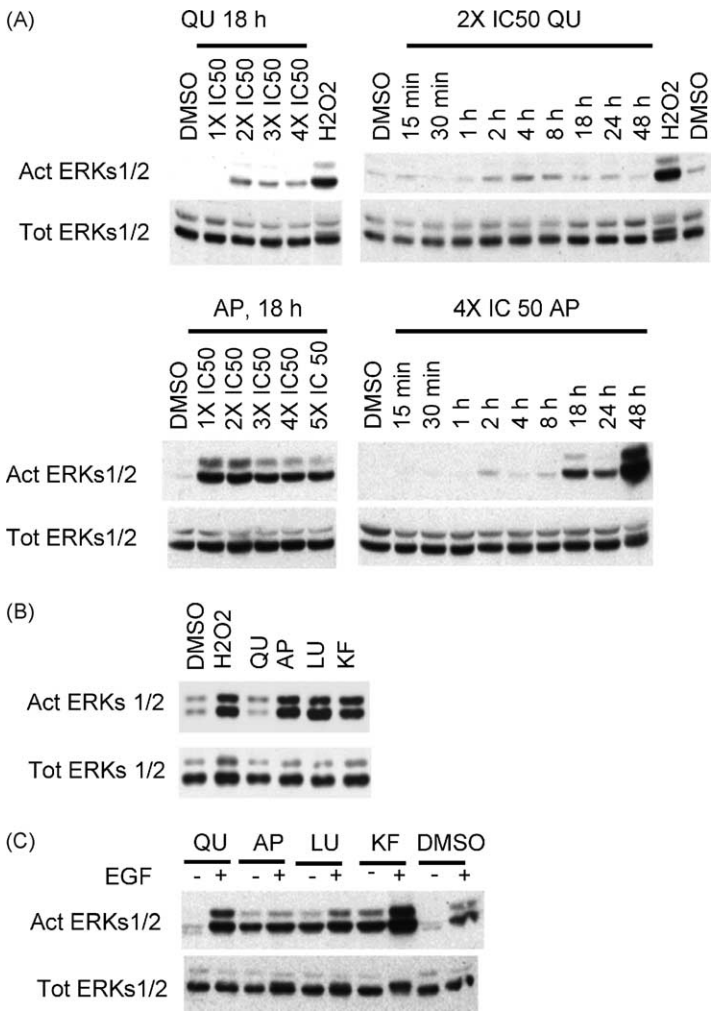

Fig. 1. Effects of FVs on activation of ERKs 1&2 (A) SCC were treated for the indicated times in normal serum-containing medium containing various concentrations of QU or AP (multiples of the IC_{50} concentrations—see Table 1) (or 1% H_2O_2 or solvent DMSO at the same concentration as that present in the FV samples) when whole cell extracts were prepared and Western blots performed using antibodies recognising specifically activated or total ERKs 1&2. (B) SCC cells were treated with twice the IC_{50} concentrations of QU, AP, LU, KF or H_2O_2, as described in (A). (C) SCCs were starved in medium containing 0.25% serum and twice the IC_{50} concentration of the indicated FV (or solvent DMSO) for 18 hr and then, where indicated, stimulated by addition of 10 ng/mL EGF for 15 min. Whole cell extracts were prepared and Western blots performed as described in (A).

until 3× the IC_{50} concentration (Fig. 3A). In contrast, QU did not significantly activate p38 even at 4× the IC_{50} concentration and JNK was only marginally activated at 2–4× IC_{50} concentrations (Fig. 3B). Further experiments confirmed that KF and LU induced p38 kinase activation selectively like AP (Fig. 3C).

3.5. Cell cycle arrest by flavonoids

The effects of FVs on cell cycle progression was investigated by cell cycle profiling, using CHO cells (which are frequently used for cell cycle studies because they can be arrested fairly homogeneously in G1 or G2 by various agents) as well as SCCs and NEs. KF, AP and QU all induced a dramatic G2/M arrest in CHO cells (Fig. 4A), and this was reversible on removal of FV (a typical result using AP is shown in Fig. 4B). KF, AP and QU also induced a less dramatic but reproducible arrest of SCC cells in G2/M (Fig. 4A) (and this was also the case with LU (data not shown)). AP, LU, KF and QU also inhibit the G2 cell cycle kinase, cdc2 (Fig. 5). All four FVs also arrested NE cells in G2/M (data not shown). No evidence of apoptosis was observed, either by formation of a sub-G0 population by propidium iodide staining and FACS analysis (Fig. 4A), DNA fragmentation in the form of a nucleosomal DNA 'ladder' or cleavage of poly-adenyl ribonuclease polymerase (PARP) (data not shown). Growth arrest of cells by DNA damage and other stress signals that arrest cells in G2 is often associated with induction of GADD45 [70] and this was also found in SCCs treated with AP, LU and KF, but not with QU (Fig. 4C). GADD45 can be induced by various mechanisms, including the p53 pathway which is activated by phosphorylation on serine 15 in response to DNA damage and various other signals that induce G2 cell cycle arrest [70]. In fact, AP, LU and KF induce p53 and activation of p53 by phosphorylation of

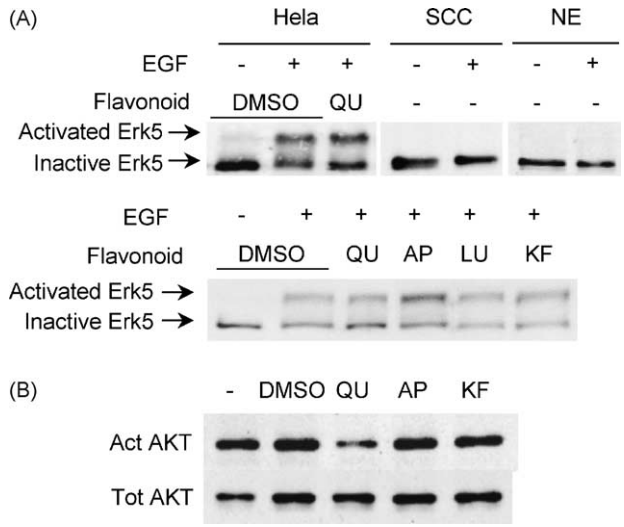

Fig. 2. Effects of FVs on ERK5 and AKT activation. (A) HeLa, SCC or NE cells were starved in medium containing 0.25% serum for 18 hr before the addition of 10 ng/mL EGF for 15 min. Where indicated, HeLa cells were also treated with six times the IC_{50} concentration of QU for 18 hr. Whole cell extracts were prepared and Western blots performed using an anti-ERK5 antibody. Activation of ERK5 in HeLa cells has been previously demonstrated by its change in mobility [62]. HeLa cells were starved in medium containing 0.25% serum and then twice the IC_{50} concentration of the indicated FV added for 18 hr before the addition of 10 ng/mL EGF for 15 min. The samples were then analysed for ERK5 as described in Section 2.6. (B) SCC were grown in normal medium and twice the IC_{50} concentration of the indicated FV for 18 hr. Whole cell extracts were prepared and Western blots performed using antibodies that recognise either specifically the activated (phosphorylated) form of AKT or total AKT.

serine 15 in wild-type p53 normal oral cells, whereas QU does not (see Section 3.6 and Fig. 9B). Thus, AP, LU and KF appear to induce a G2 cell cycle arrest by a different molecular mechanism to QU.

3.6. Functional importance of p38 kinase induction for the biological effects of AP, LU and KF

The functional involvement of p38 kinase in mediating the effects of KF and AP on GADD45 and cell cycle arrest was tested using the p38-selective inhibitor SB203580 [71] which we have previously confirmed selectively inhibits p38 kinase in SCCs [72]. These experiments showed clearly that pretreatment of SCC with SB203580 significantly reduced both the induction of GADD45 in SCC by KF and AP (Fig. 6A) and the G2 cell cycle arrest induced by these FVs in both CHO and SCC cells (Fig. 6B and C). However, SB203580 did not prevent the G2 arrest induced by QU (Fig. 6B and C).

3.7. Involvement of ATM/ATR kinases in mediating the biological effects of AP, LU and KF

One of the known mechanisms whereby irradiation and other stress agents can cause GADD45-mediated G2 cell cycle arrest is by inducing the ATM/ATR upstream kinase

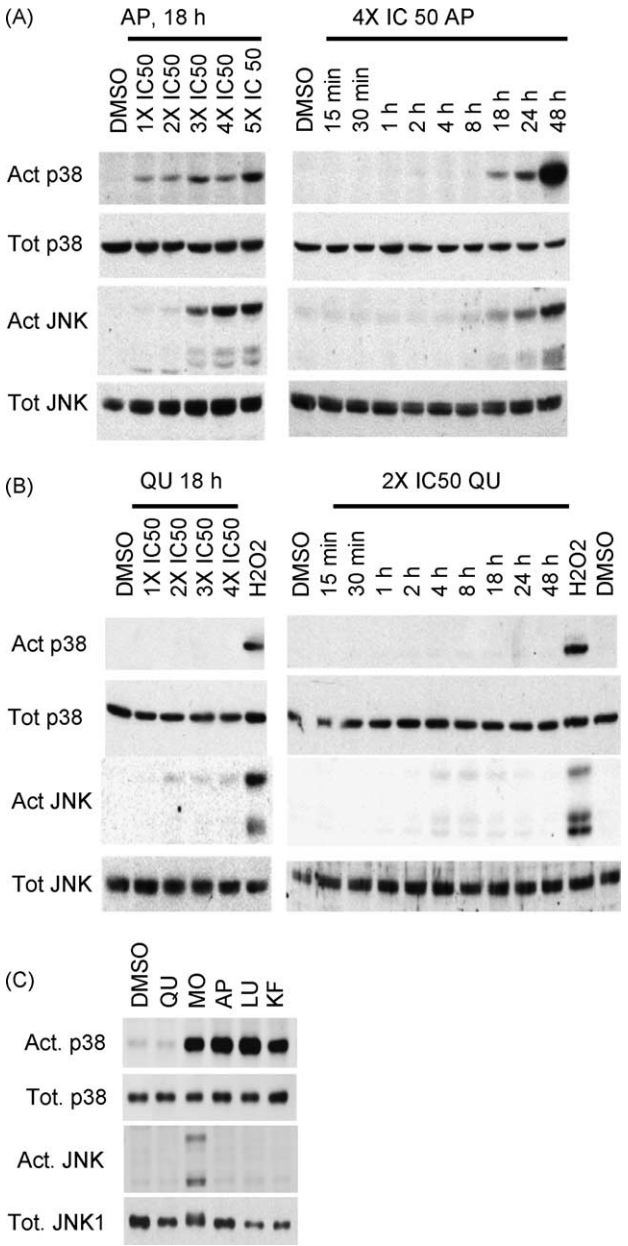

Fig. 3. Activation of stress kinases by FVs. SCCs were treated for the indicated time with various multiples of the IC_{50} concentrations of (A) AP or (B) QU. (C) SCCs were treated for 18 hr with twice the IC_{50} concentrations of QU, AP, LU or KF or another non-dietary FV, morin (MO) that has previously been shown to activate JNK [103]. Whole cell extracts were prepared and Western blots performed using antibodies that recognise the total amount of JNK or p38 kinase or the amount of activated (phosphorylated) kinases.

pathways (reviewed in [70,73,74]). We therefore tested whether this was the case also for dietary FVs. ATM and ATR kinase activities were measured directly in SCCs using an immunoprecipation/kinase assay using p53 as substrate [63]. As controls, SCCs were also irradiated with two levels of ionising radiation or UV in view of data in the literature that they induce ATM or ATR somewhat selectively [70,73,74]. These experiments showed that AP, KF and QU significantly induced activation of ATM, whereas

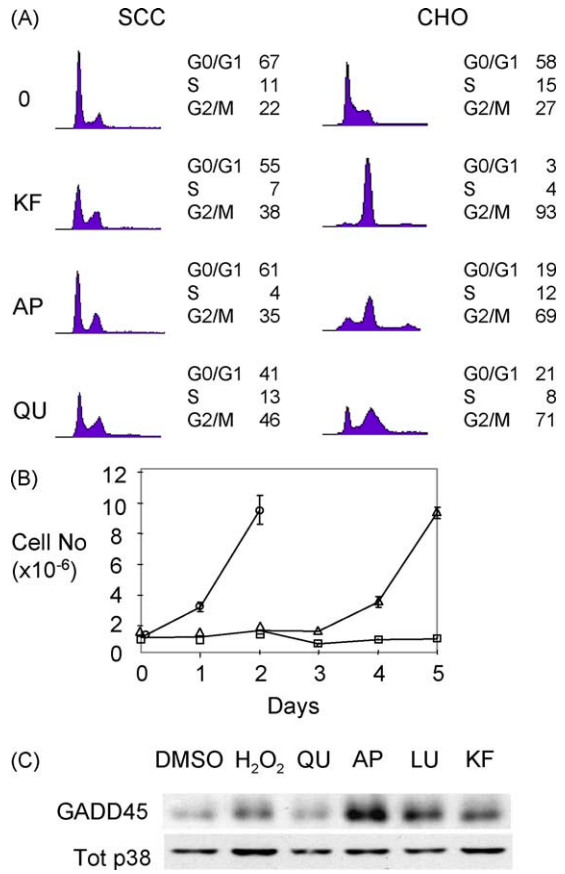

Fig. 4. Induction of growth/cell cycle arrest and GADD45 by FVs. (A) Effects on cell cycle profiles of SCC and CHO cells after treatment with twice the IC_{50} concentrations (Table 1) of QU, AP and KF. Cells in active growth were treated with FV for 48 hr, then fixed and the DNA content determined by flow cytometric analysis as described in Section 2, analysing 20,000 events per sample. The percentage of cells in G_0/G_1, S and G_2/M are shown. The data shown represent one of three independent experiments. (B) CHO cells were left untreated (circles) or treated with the twice the IC_{50} concentration of AP (squares) and the increase in cell number determined over the subsequent 5 days. After 2 days of treatment with AP, the cultures were washed and re-fed either with medium with the same concentration of AP (squares) or without AP (triangles). (C) SCCs were treated with 1 mM H_2O_2 (as positive control), or twice the IC_{50} concentrations of QU, AP, LU or KF for 18 hr and the levels of GADD45 determined by Western blotting, or, as a loading control, total p38 kinase by re-probing the same blot.

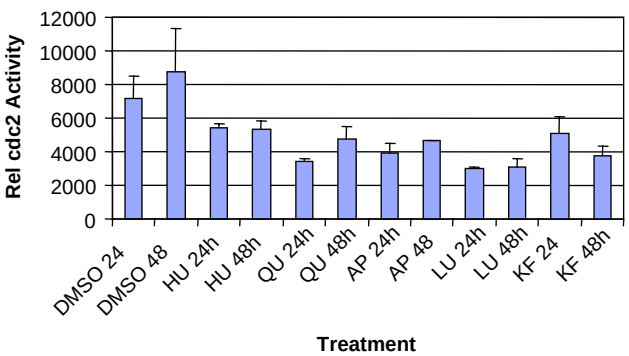

Fig. 5. Effect of FVs on cdc2 kinase activity. The data represent the average of two experiments. Cells were treated with 2× the IC_{50} concentration of FV for 24 or 48 hr, extracts prepared and analysed for cdc2 activity as described in Section 2.7. Hydroxyurea (2 mM) was included as a positive control.

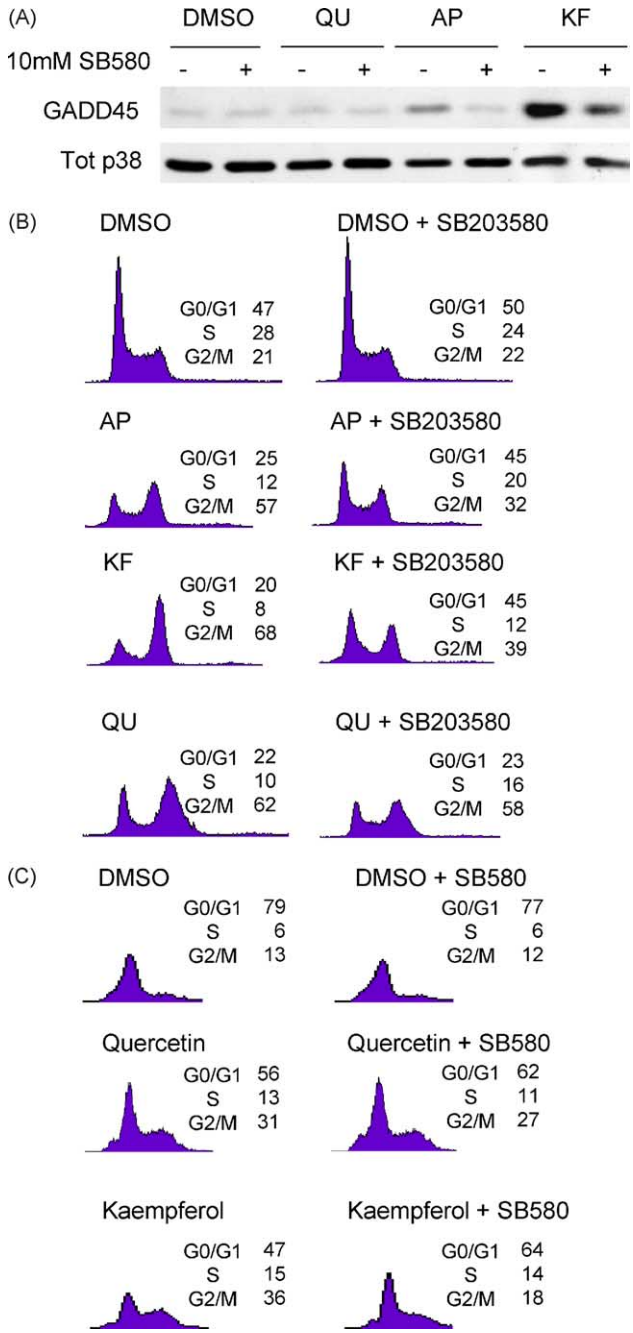

Fig. 6. Reversal of the cell cycle effects of FVs by the p38 inhibitor, SB203580. (A) SCC were pretreated with 10 μM SB203580 for 1 hr before treatment with twice the IC_{50} concentration of the indicated FV for 18 hr. Cell lysates were prepared and the levels of GADD45 determined by Western blotting. As a loading control, the level of total p38 kinase was determined by re-probing the same blot (B, C). CHO (B) or SCC (C) cells were treated with FV and/or SB203580 as described in (A), and the effect on cell cycle profiles determined as described in Fig. 3.

only AP and KF (but not QU) induced ATR (Fig. 7). The ATR (and possibly ATM) kinases seem to be functionally important since pretreatment of SCC with caffeine, a well established inhibitor of ATM and ATR [75–77], attenuated the growth inhibition/G2 arrest by KF and, to a lesser extent, AP (Fig. 8A and B) and this was associated with a reduction in induction of GADD45 and p38 kinase

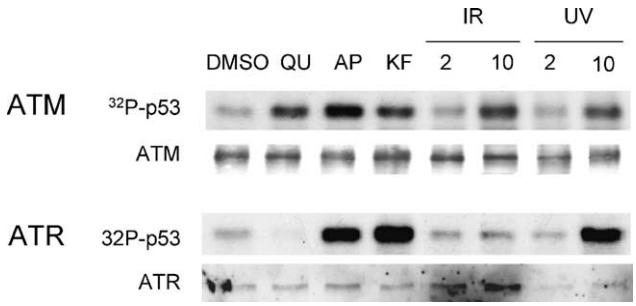

Fig. 7. Activation of ATM and ATR by FVs. SCCs were treated with twice the IC_{50} concentrations of FVs for 18 hr, or with 2 or 10 Gray Co^{60} irradiation or 2 or 10 J/m^2 UV radiation. Cell lysates were prepared and immunoprecipitated with anti-ATM or anti-ATR antibodies and then kinase assays performed using p53 as substrate. Immunoprecipates were also immunoblotted using ATM or ATR antibodies as loading controls.

(Fig. 9A). GADD45 can be induced by various mechanisms, including the p53 pathway which is activated by phosphorylation on serine 15 in response to DNA damage and various other signals that induce G2 cell cycle arrest [70]. To test whether any of the FVs induced the p53 pathway, we used normal oral mucosa (NE) cells for these experiments in order to monitor wild-type p53 responses to FVs: NE cells were treated with twice the IC_{50} concentrations of QU, AP, KF or LU for 18 hr and the level of p53 protein and the degree of phosphorylation of p53 at serine 15 determined. These experiments showed clearly that treatment with AP, KF and LU significantly increased the p53 level and induced p53 phosphorylation on serine 15: moreover, these changes were reversed by pretreatment of cells with caffeine (Fig. 9B). In contrast, QU did not induce p53 or serine 15 p53 phosphorylation (Fig. 9B) and caffeine had only a marginal effect on the extent of growth arrest and inhibition of proliferation induced by QU (Fig. 8A and B). This suggests that induction of the ATR-p38-GADD45 pathway may responsible for the G2 arrest by AP, LU and KF, but the signalling pathway(s) affected by QU is(are) clearly different.

3.8. Involvement of lipoxygenase pathway(s) in the growth inhibitory effect of QU

Preliminary experiments showed that both NEs and SCCs were relatively sensitive to the LOX inhibitors NDGA and REV5901 but not to cycloxygenase inhibitors, such as piroxicam at concentrations at which other cell lines have been shown to be sensitive [78] (Fig. 10). Subsequent RT/PCR analysis, using primers specific for

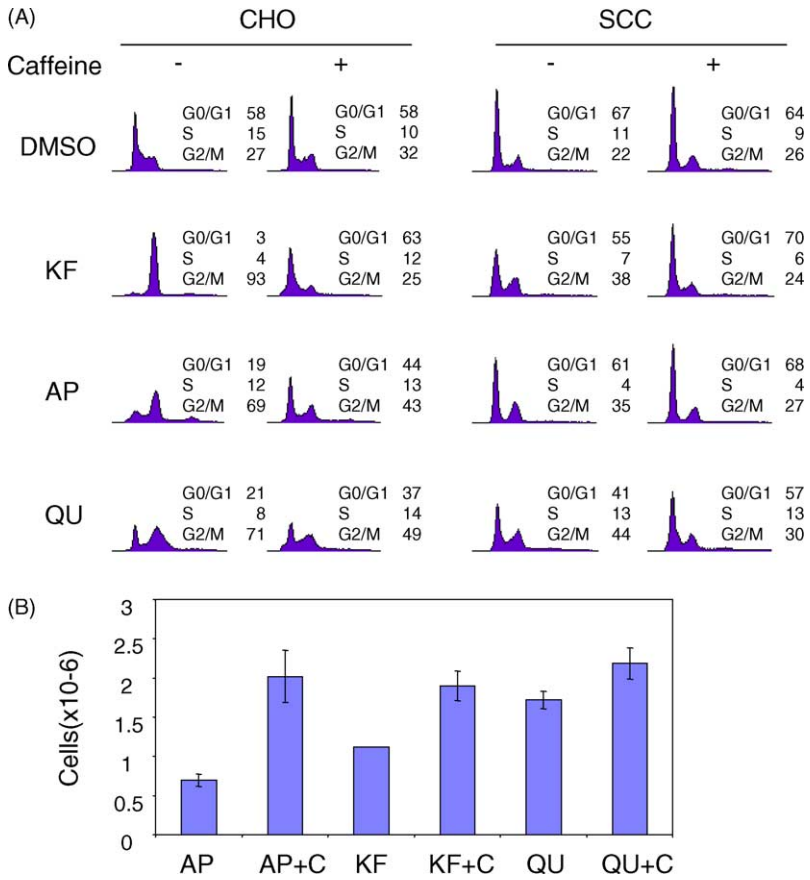

Fig. 8. Reversal of the growth-inhibitory and biochemical effects of FVs by caffeine. Cells were treated with 2 mM caffeine for 4 hr prior to addition of twice the IC_{50} concentrations of FVs. (A) Cell cycle profiles by FACS analysis after treatment of CHO cells or SCCs with FVs for 2 days. (B) CHO cell growth after treatment with FVs for 2 days with or without 2 mM caffeine.

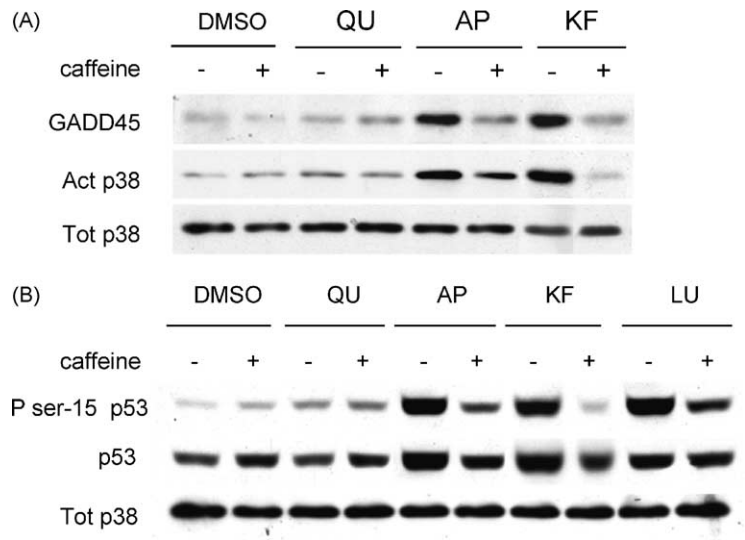

Fig. 9. (A) Expression of GADD45, activated or total p38 levels after treatment of SCCs with FVs and/or caffeine, measured by Western blotting. (B) NEs were treated with twice the IC_{50} concentrations of the indicated FVs for 18 hr and the levels of p53 and serine 15 phosphorylated p53 measured by Western blotting. The blots were then stripped and then re-probed with an anti-p38 kinase antibody as a loading control. In some cases, cells were pretreated with 2 mM caffeine for 4 hr prior to addition of FV. This result was obtained in two separate experiments.

the individual LOX mRNAs (2 forms of 15S-LOX, 12S-LOX, 12R-LOX, 5S-LOX) showed that both the 12- and 15-LOX mRNAs were expressed in SCC, albeit at relatively low levels compared to the levels of 5- and 12-LOX mRNAs in white blood cells (Fig. 11). To test whether LOX pathway(s) might be causally involved in mediating the biological effects of any of the four FVs, we investigated whether their cell cycle effects could be reduced by treatment of cells with specific LOX metabolites, such as 5, 12 or 15 hydroxyeicosatetraenoic acids (HETEs).

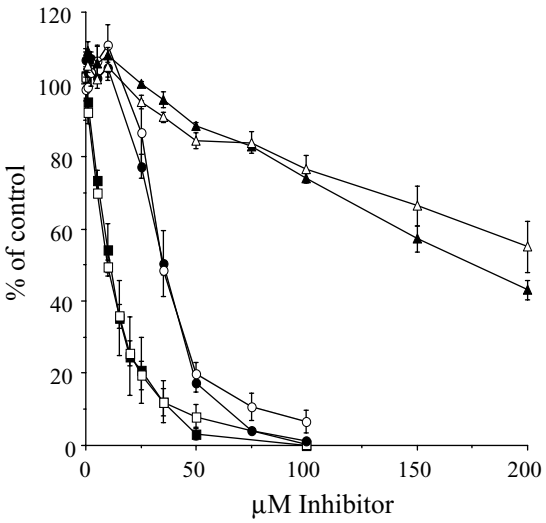

Fig. 10. Effects of LOX (NDGA and REV5901) and COX (piroxicam) inhibitors on SCC proliferation. Proliferation was determined as described in Section 2 and dose–inhibition curves determined, using five replicates for each dose, and standard errors calculated. Dose–response curves are shown as the averages for two tumour cultures (solid symbols) or three normal cultures (open symbols) for REV5901 (squares), NDGA (circles) and piroxicam (triangles).

Cells were fed daily with fresh medium or medium containing 1.5 μM HETE with varying concentrations of FV and the effect on cell proliferation measured after 3 days. The results (Fig. 12) showed clearly that the presence of 15 HETE substantially reduced the extent of growth inhibition by QU but not by the other dietary FVs: similar results were obtained with 12 HETE, but 5 HETE was less effective (data not shown). This implies that the 12 and 15 LOX pathways may be involved in some way in the mediating the growth inhibitory effects of QU, but not KF, AP or LU.

4. Discussion

Previous work in the literature, primarily employing purified enzymes or cell extracts, has demonstrated some selectivity in the ability of various FVs to inhibit specific serine/threonine and tyrosine kinases or LOX/COX signalling pathways (see Section 1 for references).

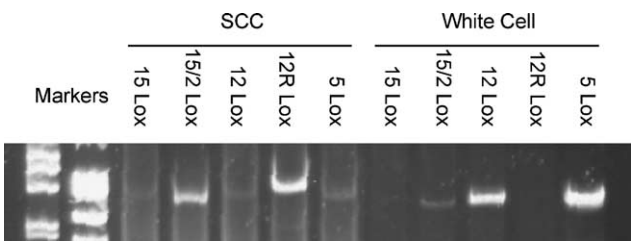

Fig. 11. Expression of LOX mRNAs in SCCs. The mRNAs encoding the various LOX isoforms were detected in total RNA isolated from SCC or human WBCs by RT/PCR using primers specific for mRNAs encoding various forms of LOX, as indicated (note 10× the amount of RNA was used for the SCC analysis compared to the WBCs). The two left hand lanes are molecular weight markers.

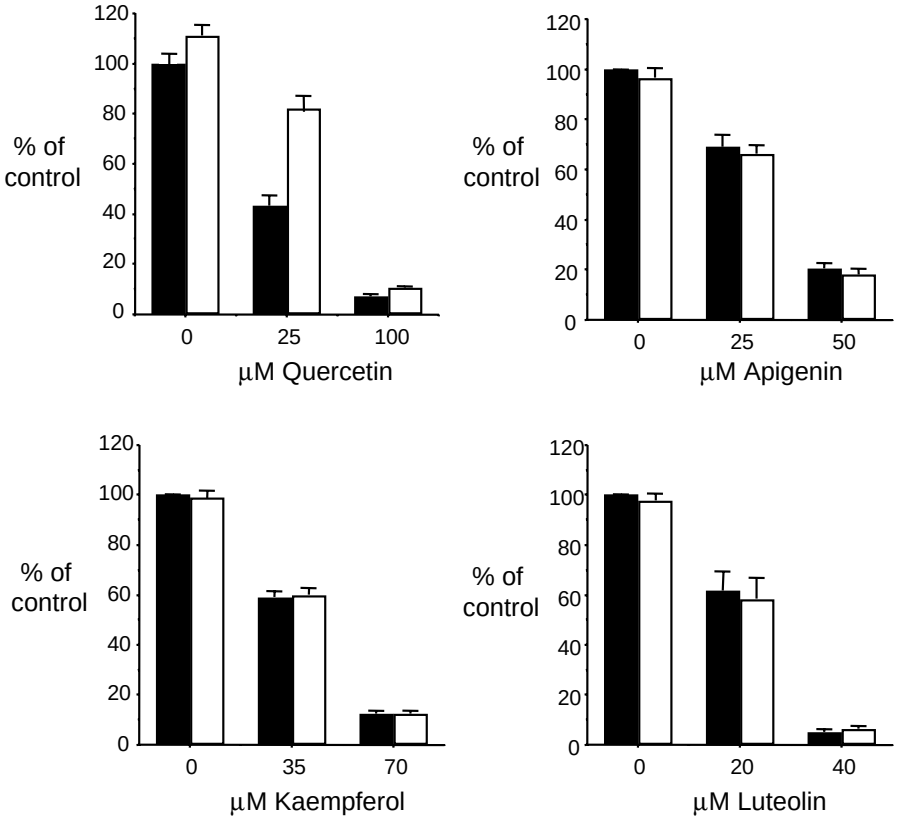

Fig. 12. Effect of the LOX metabolite, 15 HETE on the growth inhibitory effects of FVs on SCC. Cells were treated with two concentrations of FVs or DMSO at the same concentration as present in the FV cultures and fed daily with fresh medium (black boxes) or 1.5 μM 15 HETE (white boxes). The results are expressed as a percentage of untreated cells.

However, there is no information on whether FVs activate the important classes of kinases involved in so-called stress responses to environmental chemicals or drugs or radiation. Furthermore, there is little data available concerning the effects of FVs on these signalling pathways in intact cells or *in vivo*. The present study therefore concentrated on elucidating the effects of dietary FVs on intact cells that are as relevant as possible to the *in vivo* situation, using primary cultures of epithelial cells freshly isolated from oral biopsies.

Perhaps the most interesting/surprising finding to emerge from this work is the fact that QU has completely different effects on signal transduction pathways to the other major dietary FVs, AP, LU and KF. Despite evidence that AP, KF or LU can inhibit tyrosine kinase and serine–threonine kinases in cell extracts [29–32], we found no evidence in our oral cancer model that they inhibit the activation of ERKs 1&2 by EGF: indeed, they all strongly activate ERKs 1&2. In contrast, QU did not activate ERKs 1&2, nor did it inhibit the activation of ERKs 1&2 by EGF (Fig. 1). A previous study did demonstrate inhibition of MAP kinase pathway signalling by AP in Ras-transformed NIH 3T3 cells [42], using similar concentrations of AP (25 μM) and treatment times (up to 24 hr). The explanation for the differences in these results is not clear, except possibly the fact that human oral SCCs do not have *ras* mutations. QU had some inhibitory effect on activation of the AKT pathway, in contrast to KF and AP (Fig. 2). However, the functional significance of this effect of QU on AKT activation is questionable since overexpression of either a constitutively active or a membrane-targeted version of AKT in cells did not prevent growth inhibition by QU (data not shown). Under the conditions used, these AKT constructs were active as measured by the expected down regulation of $p27^{KIP1}$ in the transfected cells [79] (data not shown). In other, cell free, studies, QU, KF and LU all have similar IC_{50} concentrations against PI-3-K, an upstream activator of AKT (reviewed in [80]).

The main novel conclusion from the present study using human oral epithelial cells is the importance of activation of stress response pathways by AP, LU and KF. All these three FVs activate the ATM/ATR, wild-type p53 and p38 stress kinase pathways resulting in induction of GADD45 and a G2/M cell cycle arrest that also involves inhibition of cdc2 kinase activity (Figs. 3–9). This is not a toxic response *per se*, since the cell cycle arrest by these FVs is reversible. In contrast, QU causes G2-arrest by a different mechanism that appears to involve interference with LOX pathways (Figs. 10–12). Although AP, KF and LU can activate wild-type p53, this is clearly not required for G2 growth arrest in oral SCCs since virtually all our SCC cultures have p53 mutations [57,81]. Other work in the literature has also

found that some FVs induce a G2 arrest but this is not invariably the case: for example, whereas KF and AP induced a G2 arrest in two melanoma cell lines, QU and LU induced a G1 cell cycle arrest [47]; furthermore, whereas AP induced a G2 arrest in mouse keratinocyte cell lines [44] and in human carcinoma cell lines [49], it induced a G1 arrest in diploid fibroblasts [43]. The reasons for these differences are presently unknown but may reflect the relative importance of different signalling pathways in different cell types. As in our human oral keratinocyte model, in human colon cancer cell lines [49] and in mouse keratinocytes [48], the G2 arrest induced by AP is also associated with inhibition of cdc2 activity and in keratinocytes, at least, this occurs in a $p21^{waf1}$-indpendent manner [48].

The role of ATM induction in G2 cell cycle arrest has been well established for various DNA damaging agents including X- or γ-radiation, whereas the ATM-related kinase, ATR, has been implicated in the G2 cell cycle arrest induced by UV or hydroxyurea (reviewed in [70,73,74]). The p38 kinase pathway is also well established as a mediator of cell cycle checkpoint responses to environmental agents and stresses [69]. Induction of ATM/ATR has been shown to result in phosphorylation of different targets according to the phase of the cell cycle, for example p53 in G1 and possibly also G2 [82,83] and alternative downstream 'checkpoint' kinases, Chk2/CDS1 or Chk1 in S/G2, depending on whether ATM or ATR is involved [84–89]. Chk1 and Chk2 inhibit the cell cycle kinase cdc2 by inactivating cdc25, the phosphatase that normally activates cdc2; Chk2 (and possibly Chk1) also stabilises p53 resulting in induction of GADD45, which in turn dissociates cdc2 from cyclin B1 [70].

Our data also demonstrate that the p38 stress kinase is selectively induced by AP, LU and KF, whereas JNK is not. Moreover, p38 kinase induction is dependent on ATM/ATR (as judged by the inhibition of p38 kinase induction by caffeine). The JNK and p38 stress kinase pathways have been implicated in the action of a variety of 'stress' agents, including irradiation and UV and are closely linked to the cell-cycle checkpoint pathways (reviewed in [69]). In particular, p38 kinase is required for UV- and γ-irradiation-induced G2 arrest in mammalian cells in an ATM/ATR-dependent manner [90,91]. However, as far as we are aware, our data implicating ATM/ATR and p38 kinase in the mechanism of action of flavonoids are completely novel, although the isoflavonoid, genistein, has recently been also shown to activate p53 and Chk2 in an ATM-dependent manner [92,93] and this accounts for the involvement of p21(WAF1) in genistein action [94].

Like the other dietary FVs, QU induces a G2 growth arrest but the mechanism is clearly different since this is not associated with induction of GADD45 or p38. QU did not affect any of the other MAP kinase pathways tested (ERKs 1&2, ERK5 or JNK). Moreover, although QU attenuated the activation of AKT to some extent, it is unclear whether this is of any functional significance since expression of constitutively-activated or membrane-targeted versions of AKT in cells did not reverse the G2-arrest induced by QU (data not shown). Furthermore, although QU activated ATM (but not ATR) in SCCs, caffeine, an inhibitor of ATM/ATR, did not prevent induction of the G2 arrest by QU to the same extent as with AP, LU and KF (Fig. 8), suggesting that activation of ATM is not primarily responsible for the growth arrest by QU. This may be reflected in the fact that QU did not activate p53, in contrast to the other FVs (Fig. 9B).

A clue as to how QU acts may lie in our finding that the QU-induced growth arrest can be selectively reversed by LOX metabolites, whereas they had no effect of growth arrest induced by AP, LU and KF. RT/PCR analysis confirmed that human oral epithelial cells express 12- and 15-LOX mRNAs but at such low levels that it was not possible to prove directly that LOX activity was inhibited by treatment of cells with QU (although this was attempted in collaboration with Professor B. Thiele and Professor H. Kuhn, Humboldt University, Berlin). However, it cannot be excluded that the reversal of these effects of QU by LOX metabolites may due to an effect on an unrelated pathway that remains to be elucidated. In cell-free studies, one report found QU to be a more efficient inhibitor of mammalian LOXs than KP [36] but others suggests QU and KP are similar but more effective than LU or AP [37,38].

There are a variety of complex issues affecting the extent to which these effects on signal transduction pathways may be relevant *in vivo* at the FV levels naturally consumed in human diets or (potentially) given during chemotherapy. One important issue is that the *in vivo* absorption and metabolism of FVs (consumed as a mixture of glycoside derivatives, rather than as aglycones) is complex [11,95–98] (recently reviewed in [23]) and so it is not known what the active species responsible for the *in vivo* biological effects of FVs actually are, what their *in vivo* concentrations might be, and whether they are produced by epithelial cells *in vitro*. Nevertheless, with these caveats, our work has shown that, in human epithelial cells at least, the main dietary FVs divide into two groups in terms of mechanisms of action, i.e. QU and the remainder (AP, KF, LU). We know that glycoside derivatives of dietary FVs, such as rutin, do not show these effects (data not shown), perhaps because they are not transported efficiently into cells *in vitro* and/or they require to be metabolised. The peak plasma level of QU after consumption of FV-rich food is about 2 μM [8,99,100] and the level of all the other major dietary FVs together may reach 1 μM (given that QU is about 70% of total FV intake [1–4]). However, plasma concentrations of 5 μM can be obtained in humans after consumption of pure QU-glucosides [101]. Due to the limitations in sensitivities of the assays for signalling pathways available, the effects of FVs demonstrated in this report are only readily detectable at FV concentrations perhaps 5–10 times the likely *in vivo* level, at least from

natural diets. However, this does not exclude the possibility that minor changes in the signal transduction pathways demonstrated could have significant biological consequences at *in vivo* levels. Certainly, supplementation of animal diets with up to 5% QU, which is very much higher than the level in human diets, substantially reduces the growth of chemical-induced cancers without any apparent signs of ill health [13,14,16]. In terms of possible benefits for reducing heart disease, very low oral doses of QU reduce the elevated blood pressure and other vascular changes in a spontaneously hypertensive rat model without any effects on normotensive rats [102]. Although the changes in signalling pathways identified in this report in response to dietary FVs are most readily detectable at pharmacological levels, less dramatic changes occur at lower levels and may therefore underlie some of these physiological responses affecting cancer progression or heart disease.

Acknowledgments

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