Research Article

Hydroxytyrosol inhibits the proliferation of human colon adenocarcinoma cells through inhibition of ERK1/2 and cyclin D1

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Extra virgin olive oil is rich in phenolic compounds which are believed to exert beneficial effects against many pathological processes, including the development of colon cancer. We show that one of the major polyphenolic constituents of extra virgin olive oil, hydroxytyrosol (HT), exerts strong antiproliferative effects against human colon adenocarcinoma cells *via* its ability to induce a cell cycle block in G2/M. These antiproliferative effects were preceded by a strong inhibition of extracellular signal-regulated kinase (ERK)1/2 phosphorylation and a downstream reduction of cyclin D1 expression, rather than by inhibition of p38 activity and cyclooxygenase-2 (COX-2) expression. These findings are of particular relevance due to the high colonic concentration of HT compared to the other olive oil polyphenols and may help explain the inverse link between colon cancer and olive oil consumption.

Keywords: Colon cancer / Hydroxytyrosol / MAPK kinase / Olive oil / Polyphenol

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1 Introduction

The regular consumption of extra virgin olive oil is believed to protect against a variety of pathological processes, including the development of cancer, in particular colon cancer [1]. Its ability to inhibit colon cancer development has been demonstrated in cultured large intestinal cancer cells [2], in animals [3] and in humans [4]. Its ability to inhibit colorectal neoplastic processes [4] are thought to be mediated, in part, by phenolic compounds present in olive oil, such as hydroxytyrosol (HT) [5], lignans [6] and secoiridoids [7]. In fact, treatment of human colon adenocarcinoma cells with olive oil phenolics inhibits initiation, promotion and metastasis of the colon carcinogenesis process [8, 9]. Although extra virgin olive oil is rich in a variety of phenolic compounds, HT has been the subject of most investigations, primarily because it is the most bioavailable. However, the biological properties of any olive oil polyphenol will depend on the extent to which it is absorbed or metabolized in the gastrointestinal tract. Previously, we have shown that whilst HT levels are relatively low in olive oil, they may increase following the gastric hydrolysis and colonic fermentation of secoiridoids present in olive oil [10], resulting in colonic concentrations in the high μM. For these reasons, HT is likely to be a major candidate for the biological activity exerted by olive oil polyphenols on adenocarcinoma cells *in vivo* [10]. Indeed, the anticancer properties of HT have previously been demonstrated in HL60 leukaemia cells [11 – 13], melanoma cells [14] and colon cancer cell lines [5, 13].

The cellular mechanisms by which olive oil polyphenols exert anticancer effects is thought to be linked to their ability to interact with the mitogen activated protein kinase (MAPK) pathway and cyclooxygenase-2 expression (COX-2). The overexpression of COX-2 in colorectal cancer cells has a strong association with colorectal neoplasias *via* the

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Abbreviations: COX-2, cyclooxygenase-2; **ERK,** extracellular signal-regulated kinases; **HT,** hydroxytyrosol; **HVA,** homovanillyl alcohol; **MAPK,** mitogen activated protein kinase; **SRB,** sulphorhodamine B

promotion of cell survival, cell growth, migration, invasion and angiogenesis [15]. The MAPKs, extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase (JNK) and p38 are viewed as attractive candidates for anticancer therapies, based on their central role in regulating COX-2, cell cycle progression and thus the growth of cells from a broad spectrum of human cancers [16]. Recently, we reported that an olive oil phenolic extract, containing only 6.3% of free HT, exerted a strong inhibitory effect on the growth of colon adenocarcinoma cells through the inhibition of p38/cAMP response element-binding (CREB) signalling, a decrease in COX-2 expression and the stimulation of a G2/M phase cell cycle block [17]. The aim of the present work was to ascertain the contribution of HT and its intestinal metabolite homovanillyl alcohol (HVA) to the antiproliferative effects of olive oil polyphenols on epithelial colorectal adenocarcinoma cells and to investigate the cellular mechanism of action.

2 Materials and methods

2.1 Materials

HT was obtained from Cayman Chemical Company (IDS, Boldon, UK). Caco-2 cells were from ECACC (Salisbury, Wiltshire, UK). Cell culture media and supplements were purchased from Cambrex (Wokingham, UK). HVA, sulphorhodamine B (SRB) assay kit, 5-bromo-2′-deoxyuridine (BrdU), RNAse, secondary horseradish peroxidase-conjugated goat antirabbit Ab, secondary rabbit antimouse FITC Ab, Ponceau Red solution and propidium iodide were from Sigma (Poole, Dorset, UK). Primary antibodies used were: anti-ACTIVE MAPK (ERK 1/2) Ab, anti-total ERK1/2 MAP kinase Ab (Upstate, Dundee, UK); anticyclin D1 Ab, antiphospho-p38 MAP kinase (Thr^{180}/Tyr^{182}) Ab, anti-total-p38 MAP kinase Ab and anti-COX-2 Ab (New England Biolabs, Hertfordshire, UK). Prestained molecular-mass markers were purchased from BioRad (Hemel, Hempstead, UK). Antibromodeoxyuridine Ab was from Becton-Dickinson (Cowley, Oxford, UK). ECL reagents, horseradish peroxidase (HRP) chemiluminescent reagent/luminol and hydrogen peroxide were from Millipore (Watford, UK), whilst Hyperfilm-ECL and nitrocellulose membrane were from Amersham Biosciences (Chalfont, St. Giles, UK). All other reagents used were from Sigma.

2.2 Cell culture and proliferation

Human colon adenocarcinoma cells (Caco-2) were cultured in DMEM, supplemented with 20% heat-inactivated bovine serum, 2 mM L-glutamine, 1% nonessential amino acids, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were seeded at low confluence in 24-well plates (2.5×10^4 *per* well) and were exposed to HVA and HT (5.0 – 200 μM) or vehicle (methanol 2%), 8 h after seeding. Methanol did not exert significant cytotoxicity below 2% v/v, and its toxicity above this defined the highest concentration of HT used. Following 24, 48, 72 and 96 h, cells were fixed and cell biomass was determined using the SRB assay, as previously described [17]. Cells were fixed by the addition of 125 μL ice-cold TCA (10% final concentration; 4°C; 1 h). After fixing, media was removed, cells were washed and total biomass was determined using SRB (250 μL of 0.4% SRB; 0.5 h). Unincorporated dye was discarded by washing with 1% acetic acid, whilst cell incorporated dye was solubilized using Tris Base (10 mM, pH 10.5). Dye incorporation, reflecting cell biomass, was measured at 492 nm, using a GENios microplate reader (TECAN, Reading, UK).

2.3 Cell cycle analysis

For cell cycle analysis, cells were seeded on six-well plates (1×10^5 cells/well) and grown for 3 days (~70% confluent) prior to treatment with HT (5.0 – 162.5 μM; 24 h) or vehicle (methanol 2%). The cells were analysed using a double staining procedure with PI and BrdU to ascertain the cell cycle phase distribution, as detailed previously [17]. Samples were analysed by flow cytometry using a FACS Calibur benchtop flow cytometer (Becton-Dickinson) equipped with a 15 mW blue argon laser source (excitation wavelength: 488 nm). The percentages of the cells in G0/G1, S and G2/M phases were assessed using the FL3-A channel (675 nm), and the fluorescence shift was fixed at 200 for the G0/G1 population, at 400 for the G2/M and the S phase in between using CellFIT Cell-Cycle Analysis Version 2.0.2 software (Becton-Dickinson).

2.4 Western immunoblotting

Caco-2 cells (1×10^5 cells/well) were grown on six-well plates (80% confluent) and were exposed to either HT (5.0 – 162.5 μM) or to vehicle (methanol 2%) for 15 min for p38 and ERK1/2 phosphorylation or for 24 h for cyclin D1 and COX-2 expression. The Bradford assay was performed to assess protein loading and Western immunoblotting was performed as described previously [18]. Briefly, samples containing 20 μg of protein were run on 9% SDS-acrylamide gel and proteins were transferred to nitrocellulose membranes by semidry electroblotting (1.5 mA/cm^2). Blots were incubated with antiphospho-p38 MAP kinase (Thr^{180}/Tyr^{182}) Ab (1:1000 dilution), antitotal-p38 MAP kinase Ab (1:1000 dilution), anti-COX-2 Ab (1:1000 dilution) anti-ACTIVE MAPK (ERK 1/2) Ab (1:2000 dilution), antitotal ERK1/2 (1:2000 dilution) and anticyclin D1 Ab (1:1000 dilution) in TTBS containing 1% w/v skimmed milk powder. Blots were exposed to Hyperfilm-ECL (30 s – 2 min) in an autoradiographic cassette and developed, before protein bands were quantified using Quantity One software (Bio-Rad). For p38 and ERK1/2 analyses, the relative band intensity of the phosphorylated form was divided by the relative

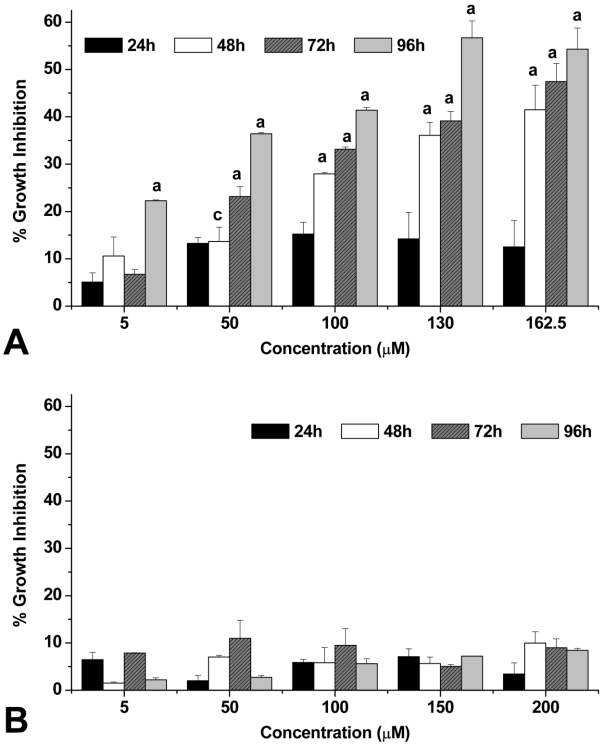

Figure 1. Growth inhibition induced by HT (A) and HVA (B). Caco-2 cells were exposed to HT (5 – 162.5 μM) and HVA (5 – 200 μM) for 24, 48, 72 and 96 h before SRB assays were conducted. Data are means of three separate experiments, each performed in quadruplicate ($n = 12$), and presented as mean ± SD. a = $p < 0.001$; c = $p < 0.05$ represent a significant increase in growth inhibition relative to vehicle treated cells.

band intensity of the total form to obtain the ratio phospho/total.

2.5 Statistical analysis

Data are expressed as means ± SD. The statistical evaluation of the results was performed by one-way analysis of variance (ANOVA) followed by a Student – Newman – Keuls *post hoc t*-test using GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA, USA). Significant changes are indicated as follows: a = $p < 0.001$; b = $p < 0.01$; c = $p < 0.05$.

3 Results

3.1 Assessment of cell proliferation

We studied the ability of HT and HVA treatment to elicit antiproliferative effects towards the human colonic cancer cell line, Caco-2 (Fig. 1). A significant growth inhibition was observed following exposure to HT, as indicated by reductions in cell biomass determined by the SRB assay (Fig. 1A). Significant growth inhibition was observed at all concentrations tested (5 – 162.5 μM) at 96 h and from 50 μM at 48 and 72 h. In contrast, the proliferation of Caco-2 cells was not significantly inhibited when the cells were exposed to HVA (Fig. 1B) at any concentration tested (5 – 200 μM) and at any time of incubation (24 – 96 h).

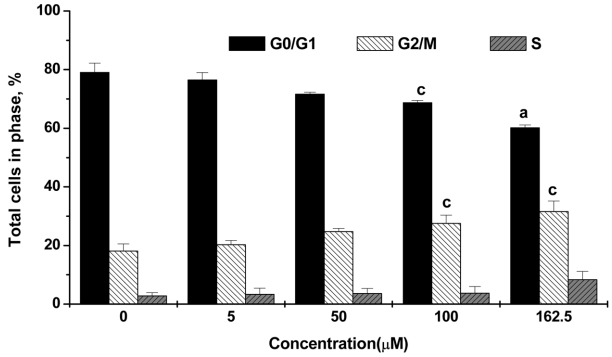

Figure 2. Effects of HT on cell cycle phase distribution. Subconfluent Caco-2 cells were exposed to vehicle (methanol) or HT (5 – 162.5 μM) for 24 h. Cells were trypsinized, pelleted and collected prior to fixing/digestion with RNase. Cellular DNA was stained with PI and the distribution of cells in G0/G1, S and G2/M phase was analysed by flow cytometry. Results are obtained from three independent experiments and presented as means ± SD. a = $p < 0.001$ and c = $p < 0.05$ represent a significant decrease in cells in G0/G1 relative to vehicle treated cells, and a significant increase in cells in G2/M relative to vehicle treated cells.

3.2 Assessment of cell cycle distribution

Untreated subconfluent cells revealed a consistent cell cycle phase distribution of cells in G2/M (18.13 ± 2.40%), G0/G1 (79.07 ± 3.15%) and S (2.80 ± 1.07%) phases (Fig. 2). Treatment with HT at low concentrations (5 and 50 μM) had no significant effect on cell cycle distribution, whilst higher concentrations (100 and 162.5 μM) significantly blocked the cell cycle at the G2/M phase (31.54 ± 3.64% for 162.5 μM; $p < 0.001$), with a concomitant reduction of the number of cells in G0/G1 phase (60.13 ± 0.95 for 162.5 μM; $p < 0.05$), and did not significantly alter S phase (8.32 ± 2.82 for 162.5 μM) (Fig. 2).

3.3 p38 activity and COX-2 expression

The phosphorylation state of p38, was probed using a phospho-specific antibody that recognizes the dually phosphorylated motif Thr^{180}-Pro-Tyr^{182} within activated p38. Treatment with HT (5.0 – 162.5 μM; 15 min) did not induce any significant modification of the phosphorylation state of p38 and parallel immunoblots with an antibody that detects total levels (nonphosphorylated and phosphorylated p38) indicated that there were no significant changes in total p38 (Fig. 3A). As such, the ratio of phospho p38 to total p38, reflecting its activation, did not show a significant differ-

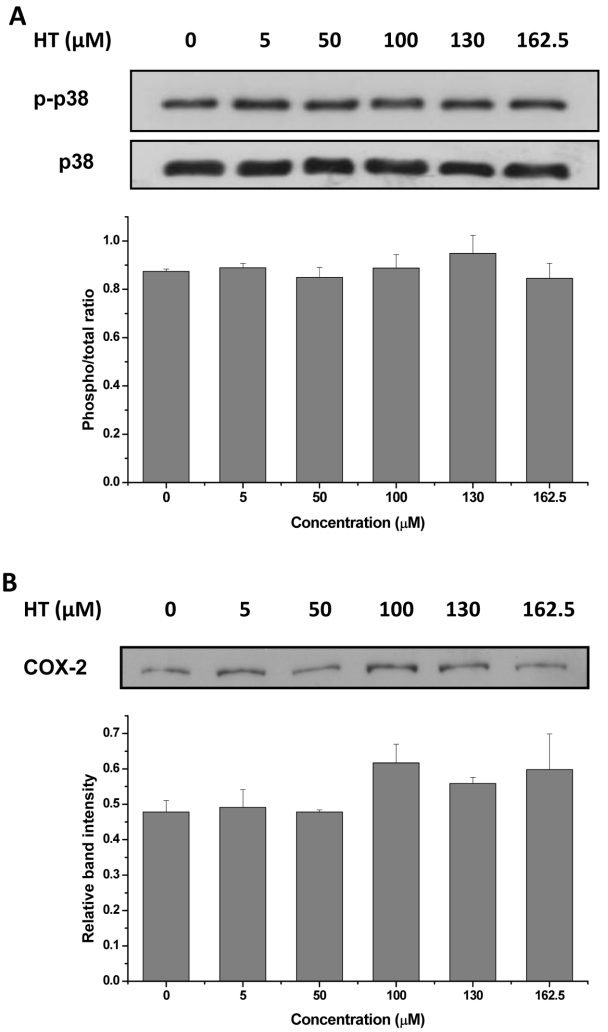

Figure 3. Effects of HT exposure on p38 activity and COX-2 levels. Crude lysates (20 μg) prepared from Caco-2 cells exposed to vehicle (methanol) or HT (5 – 162.5 μM) for 15 min (p38) or 24 h (COX-2) were immunoblotted with antibodies that specifically recognize phosphorylated and total p38 (panel A) and COX-2 (panel B). Data obtained from experiments were analysed using BioRad Quantity One 1-D Analysis software. Results are obtained from four independent experiments and presented as means ± SD.

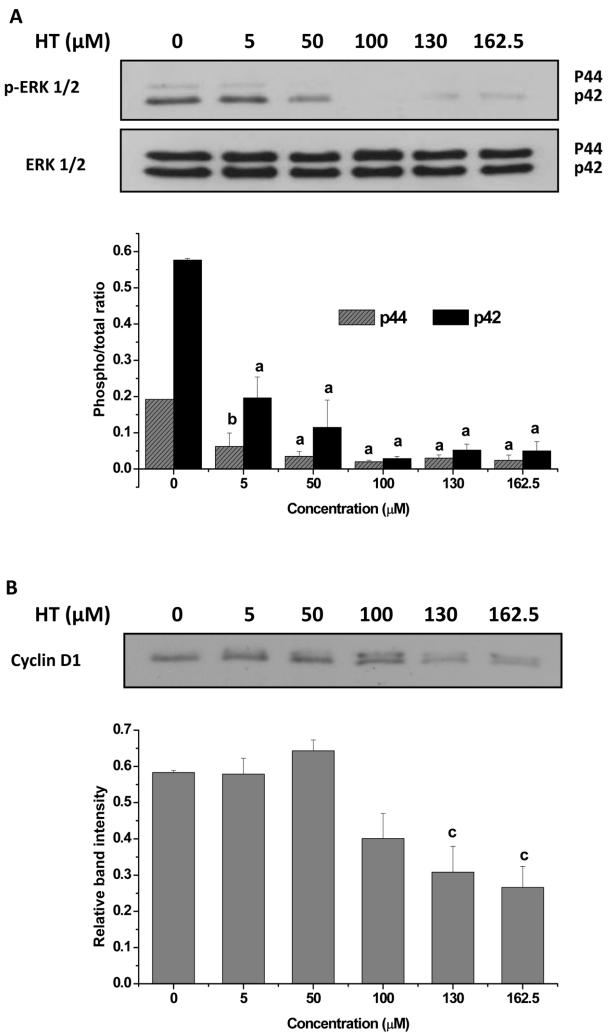

Figure 4. Effects of HT exposure on ERK1/2 activity and cyclin D1 levels. Crude lysates (20 μg) prepared from Caco-2 cells exposed to vehicle (methanol) or HT (5 – 162.5 μM) for 15 min (ERK 1/2) or 24 h (cyclin D1) were immunoblotted with antibodies that specifically recognize phosphorylated and total ERK 1/2 (panel A) and cyclin D1 (panel B). Data obtained from experiments were analysed using BioRad Quantity One 1-D Analysis software. Results are obtained from four independent experiments and presented as means ± SD. a = $p < 0.001$; b = $p < 0.01$; c = $p < 0.05$ represent significant decrease relative to vehicle treated cells.

ence compared to basal levels. Similarly, the level of COX-2 expression did not significantly change ($p > 0.05$) following exposure of cells to HT (5.0 – 162.5 μM; 24 h) (Fig. 3B).

3.4 ERK1/2 activity and cyclin D1 expression

To establish a possible signalling pathway responsible for the observed cell growth inhibition and cell cycle arrest induced by HT treatment, we also looked at the early changes (15 min) in the phosphorylation state of extracellular signal-related kinase 1 and 2 (ERK 1/2). We observed a strong inhibition of ERK 1/2 activation (Fig. 4A) in cells exposed to HT at all concentrations tested (5 – 162.5 μM), with no changes in total levels of ERK. Consequently, a statistically significant reduction in the ratio of phospho to total ERK1/2 was observed at all concentrations. These changes in the ERK activation state were paralleled by significant reductions (HT 130 and 162.5 μM; 24 h) in the levels of cyclin D1 (Fig. 4B).

4 Discussion

Extra virgin olive oil consumption occupies a central position in the Mediterranean diet and its regular ingestion has been proposed to provide protection against the development of numerous diseases, most notably cancer [1]. Along with macronutrients, such as a high proportion of MUFAs, its habitual intake delivers relatively high quantities of polyphenols, in particular HT [5], lignans [6] and secoiridoids [7] to the intestinal epithelium. These phenolic compounds, which are present in high amounts in olive oil, compared to other oils, are thought to contribute to the anticancer properties of this dietary oil [4].

Extra virgin olive oil polyphenols have been suggested to possess anticancer effects against colorectal carcinogenesis, by inhibiting different stages (initiation, promotion, metastasis) of the colon carcinogenetic process [8] or by an induction of apoptosis [19]. The cellular mechanisms by which olive oil polyphenols exert these anticancer effects are still poorly understood, although evidence suggested that they may induce differentiation and inhibit cell cycle progression (G0/G1 phase block) by reducing cyclin D3 and CDK6 levels, and increasing the levels of the CDK inhibitors p21 and p27 [20]. We indicate that a phenolic extract derived from olive oil also exerts strong antiproliferative effects, *via* a mechanism involving a cell cycle block in G2/M phase, a rapid inhibition of p38 and CREB phosphorylation and a downstream reduction in COX-2 expression [17]. Here, we show that HT, the major un-conjugated phenolic in olive oil, but not one of its gastrointestinal-derived metabolites, HVA, is also capable of exerting a strong antiproliferative effect on colon adenocarcinoma cells through the ability to induce a cell cycle block at G2/M. However, the mechanism by which HT exerts these effects appears to be mediated by a rapid inhibition of ERK1/2 phosphorylation and a downstream inhibition of cyclin D1 levels rather than by the inhibition of p38 phosphorylation/activation and/or COX-2 expression [17]. Thus, it seems likely that the COX-2 and p38 modulation observed following exposure to the whole olive oil phenolic extract are due to components other that HT in the extract. Indeed, previous studies have indicated that the olive oil phenol tyrosol and oleocanthal (the dialdehydic form of (–)-deacetoxy-ligstroside aglycone) are capable of inhibiting COX-2 [21, 22].

A wide range of phenolic compounds have been reported to modulate the ERK1/2 pathway, including epicatechin [23], quercetin [18], kaempferol [24], apigenin [25] and luteolin [26]. Furthermore, specific polyphenols have been shown to inhibit the progression of adenoma to adenocarcinoma *in vivo via* a down-regulation of ERK1/2 activity [27]. The MAPK signalling pathway is known to be involved in the promotion of cellular proliferation, and is generally found to be up-regulated in human cancer cells [16]. In the context of human colon tumour formation, it is well documented that ERK1/2 are constitutively activated in cell lines derived from colorectal cancers, suggesting that the activation of the MEK-ERK pathway may be linked to the proliferation of colonic epithelial cells and to their progression into the states of uncontrolled growth associated with colorectal tumour formation [28]. Therefore, the inhibition of ERK1/2 by dietary agents, such as HT, highlights their potential to act as therapeutic agents for the treatment of colonic cancer progression *via* their potential to switch off the ability of colonic cells to undergo rapid division.

The inhibition of ERK1/2 phosphorylation has been shown to impact on the levels and activity of cell cycle proteins, in particular cyclins [29, 30]. MAPK cascades drive specific cell cycle responses to extracellular stimuli, at least in part, through the modulation of cyclin D1 expression [29, 30]. Indeed, the expression of cyclin D1 has been shown to be positively regulated by MEK – ERK signalling, indicating that ERK activation is required for cyclin D1 expression [29]. We show that in addition to inhibiting ERK1/2 phosphorylation, HT was also able to reduce cellular cyclin D1 levels, in a similar manner to that previously reported for the stilbene resveratrol [31]. These results are in agreement with a previous study which indicated that the antiproliferative activity of the flavanols epicatechin, dimer B2 and their di-nitroso derivatives were mediated by a rapid inhibition of ERK1/2 phosphorylation and the downstream inhibition of cyclin D1 [32]. Whilst the inhibition of cyclin D1 is commonly associated with a block of the cell cycle in G1, many studies have also reported that ERK1/2 activity can affect progression through both G1 and G2 phases of the cell cycle [33]. For example, ERK1/2 has been shown to be required for the control of G2/M transition of the cell cycle in human fibroblasts [33] and the differential roles of ERK1 and ERK2 (and MEK1 and MEK2) in the control of the cell cycle have been described [30].

The antiproliferative effects of HT on colon adenocarcinoma cells may be of particular relevance as the large intestinal epithelium is likely to be exposed to higher concentrations of this phenolic, compared to others present in olive oil. This is due to the specific metabolism of olive oil polyphenols in the gastrointestinal tract, where the gastric hydrolysis of secoiridoids, and bacterial-induced metabolism of oleuropein in the colon, leads to high levels of tyrosol and HT in the large intestine [10]. This high concentration of HT in the large intestine and its ability to inhibit the proliferation of human colon adenocarcinoma cells *via* ERK1/2 and cyclin D1 may help explain the inverse link between colon cancer and olive oil consumption.

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The authors have declared no conflict of interest.

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