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Research Article

Gene expression, cell cycle arrest and MAPK signalling regulation in Caco-2 cells exposed to ellagic acid and its metabolites, urolithins

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Novel gene expression profiles and cellular functions modulated in Caco-2 cells in response to the dietary polyphenol, ellagic acid (EA), and its colonic metabolites, urolithin-A (3,8-dihydroxy-6H-dibenzo[*b,d*] pyran-6-one) and urolithin-B (3-hydroxy-6H-dibenzo[*b,d*] pyran-6-one) have been identified. Exposure of cells to EA and urolithins arrested cell growth at the S- and G_2/M-phases. Transcriptional profiling using microarray and functional analysis revealed changes in the expression levels of MAPK signalling genes such as, growth factor receptors (*FGFR2*, *EGFR*), oncogenes (*K-Ras*, *c-Myc*), and tumour suppressors (*DUSP6*, *Fos*) and of genes involved in cell cycle (*CCNB1*, *CCNB1IP1*). Results suggest that EA and urolithin-A and -B, at concentrations achievable in the lumen from the diet, might contribute to colon cancer prevention by modulating the expression of multiple genes in epithelial cells lining the colon. Some of these genes are involved in key cellular processes associated with cancer development and are currently being investigated as potential chemopreventive targets.

Keywords: Colon cancer / Ellagic acid / Gene expression / G_2/M arrest / Urolithins

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1 Introduction

Colorectal cancer (CRC) is one of the most frequent causes of death in westernized countries [1]. CRC development and progression is the result of genetic and environmental factors such as lifestyle and diet [2]. Cancerous cells are characterized by an abnormal proliferation partially caused by mutations and/or deregulated expression of key regulatory genes: oncogenes (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue (*K-Ras*) or v-myc myelocytomatosis viral oncogene homologue (avian) (*c-Myc*) involved in the Ras-mitogen activated protein kinase (MAPK) signalling pathway that modulates cell growth and survival), tumour suppressors (adenomatosis polyposis coli (*APC*), catenin (cadherin-associated protein), β1, 88 kDa (*CTNNB1*) or tumour protein p53 (Li-Fraumeni syndrome) (*p53*) affecting cell differentiation, cell proliferation, cell cycle and apoptosis) and DNA mismatch repair genes [3]. CRC is characterized by an unknown genetic heterogeneity with new genes and pathways involved in tumourigenesis continually emerging. The modern human diet regularly supplies a mixture of cancer promoters such as food contaminants [4] and cancer inhibitors which may exert an important modulating role in the development of CRC. Epidemiology has shown an inverse correlation between plant food intake and mortality by cancer. Some human prospective studies indicate that the consumption of vegetable and fruit might protect against CRC [5, 6]. Fibre, vitamins, minerals,

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Abbreviations: CCNB1, cyclin B1; **CCNB1IP1,** cyclin B1 interacting protein; **CD44,** CD44 molecule (Indian blood group); **CDK,** cyclin dependent kinase; **c-Myc,** v-myc myelocytomatosis viral oncogene homologue (avian); **CRC,** colorectal cancer; **DUSP6,** dual specificity phosphatase 6; **EA,** ellagic acid; **EGFR,** epidermal growth factor receptor; **ERK,** extracellular signal-regulated protein kinase; **ETs,** ellagitannins; **FBS,** foetal bovine serum; **FGFR2,** fibroblast growth factor receptor 2; **Fos,** V-fos FBJ murine osteosarcoma viral oncogene homologue; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **GO,** gene ontology; **HRP,** horseradish peroxidase; **IPA,** ingenuity pathways analysis; **K-Ras,** v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue; **MAPK,** mitogen activated protein kinase; **MAP4K4,** mitogen-activated protein kinase 4; **p53,** tumour protein p53 (Li-Fraumeni syndrome); **PI,** propidium iodide

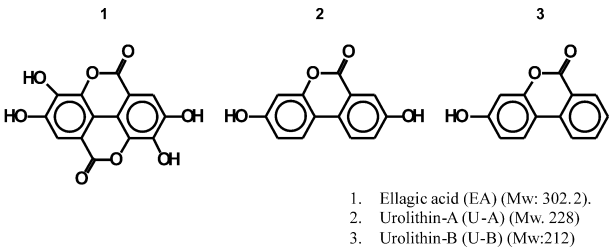

Figure 1. Chemical structures of: (1) EA, (2) urolithin-A and (3) urolithin-B.

folates, polyphenols or a combination of these nutrients, may play a role in the anticancer properties of plant foods [7]. Extensive research has focused on the anticancer properties of polyphenols, reported in many *in vitro* and animal studies [8] although clinical trials are still scarce and inconclusive [9]. Since bioavailability of polyphenols is low [10] and substantial levels of these compounds may remain in the lumen and suffer metabolic changes by the colon microflora, CRC development may be affected or modulated by dietary polyphenols and their colonic metabolites.

Ellagitannins (ETs) are polyphenols abundant in berries, walnuts and pomegranates [11]. ETs are hydrolysed in the gut to ellagic acid (EA) which is metabolized by the colon microflora to form the urolithins-A (3,8-dihydroxy-6H-dibenzo[*b,d*] pyran-6-one) and -B (3-hydroxy-6H-dibenzo[*b,d*] pyran-6-one) (Fig. 1) [12 – 14]. EA and urolithins can accumulate in the intestine and prostate [15, 16]. ETs, EA and urolithin-A exhibit cancer chemopreventive activities in various cell and animal models [16 – 19] but the antiproliferative activity of EA metabolites in colon cells has not been studied, and the molecular mechanisms involved have not been thoroughly investigated. Because of the poor absorption of EA, its conversion to urolithins, and the accumulation of both EA and urolithins in the intestine, studies on the cancer inhibitory effects of these compounds are most relevant in models of the intestinal tract. In this work, the antiproliferative effects of EA and/or urolithin-A and -B in sub-confluent homogeneously undifferentiated Caco-2 cells as a model of colon cancer cells were examined. Changes in gene expression and associated biological functions induced by the treatments were also investigated using microarray and appropriate bioinformatic tools. This was done as an attempt to link gene expression and functional analysis results with the phenotypic response of the cells and to establish potential molecular mechanisms of action of EA and its colonic metabolites in the cancerous human colon cells.

2 Materials and methods

2.1 Materials

EA (95% purity) was purchased from Sigma – Aldrich (Steinheim, Germany) and further purified to almost 100% purity by using previously described methods [20]. Urolithin-A (95% purity) and urolithin-B (98% purity) were chemically synthesized by Kylolab S.A. (Murcia, Spain). The specific inhibitor of MEK-1/2, PD98059, was obtained from Sigma – Aldrich (Madrid, Spain).

2.2 Cell culture

The human colon cancer cell line Caco-2 was obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Cells were grown in minimal essential medium (MEM) supplemented with 10% v/v foetal bovine serum (FBS), 1% v/v nonessential amino acids, 1% v/v L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Invitrogen S.A., Barcelona, Spain) at a final pH 7.2 – 7.4 and maintained at 37°C under a 5% CO_2/95% air atmosphere at constant humidity. Caco-2 cells were seeded at 10^4 cells cm^{-2} on six-well plates (Nunc, Roskilde, Denmark), allowed to adhere for 48 h and treated on day 3 after seeding as follows: (i) 10 μM EA (based on previous results, [18]); (ii) 40 μM urolithin-A (based on maximum solubility in the culture media); (iii) 40 μM urolithin-B (based on maximum solubility in the culture media) and (iv) a mixture of 40 μM urolithin-A, 40 μM urolithin-B and 10 μM EA (Mix). All the compounds were dissolved in DMSO (< 0.5% in the culture medium) and filtered (0.2 μm) prior to addition to the culture media. Control cells were treated with DMSO. Cells were incubated with each of the compounds for up to 96 h.

2.3 Cell proliferation and viability

At the end of each day of treatment, trypsinized cells (2.5 g/L trypsin, 0.2 g/L EDTA) were suspended in culture medium, counted using a haemocytometer and viability measured using Trypan blue dye exclusion [21]. Results of proliferation and viability in treated cells are presented in comparison to control cells. Data are presented as mean values ± SD from three independent experiments (n = 2 plates *per* experiment).

2.4 Cell cycle analysis

Cells (2×10^5) were collected after each day of treatment, fixed in ice-cold ethanol/PBS (70:30) for 30 min at 4°C, further resuspended in PBS containing 100 μg/mL RNase and 40 μg/mL propidium iodide (PI), and incubated at 37°C for 30 min [21]. DNA content (20 000 events) was analysed using an FACScan flow cytometry instrument equipped with FACStation running Cell Quest software (Becton Dickinson, Madrid, Spain). The CV was always less than 10%. Data are presented as mean values ± SD of at least three independent experiments (n = 3 plates *per* experiment). Where indicated, comparisons between mean values from control and treated samples were carried out with the

two tailed unpaired student's *t*-test. A p-value < 0.05 was considered statistically significant.

2.5 Determination of apoptosis

Floating and adherent cells (1×10^6) were collected after each day of treatment and were washed twice with PBS with 2% FBS and resuspended in 100 μL of PBS with 2% FBS. Cells were incubated with 10 μL/mL of fluorescein-di-acetate (FDA) (1 μM) (Sigma, Madrid, Spain) and 12.5 μL/mL of PI (400 μg/mL) (Orpegen Pharma, Heidelberg, Germany) in PBS with 2% FBS for 30 min at 4°C in the darkness [22]. After incubation, the cells were diluted in PBS with 2% FBS to give a final volume of 200 μL prior to analysis. Samples were measured using an FACStation running Cell Quest software (Becton Dickinson). Apoptosis analyses were done in triplicate.

2.6 RNA extraction

After 72 h of exposure to EA and/or to the urolithins, control and treated cells were lysed in the plates and total RNA was isolated using an RNeasy mini kit (Qiagen, Barcelona, Spain). RNA concentration and purity were checked using the Nanodrop spectrophotometer system (ND-1000 3.3 Nanodrop Technologies). The integrity of the ribosomal RNA was further checked using agarose gel electrophoresis (1%). Only samples with a ratio (Abs 260/280 nm) between 1.8 and 2.1 were used for microarray experiments. Treatments, extractions and hybridizations were done in triplicate.

2.7 Microarray analysis

Five microgram of total RNA were used for double-stranded cDNA synthesis and generation of biotin-labelled cRNA, following the manufacturer's one-cycle protocol (Affymetrix, High Wycombe, UK). The quality and size of fragmented biotin-labelled cRNAs was assessed with the Agilent Bioanalyzer 2100 prior to hybridization onto Human Genome U133 Plus 2.0 chips (http://www.affymetrix.com/products/arrays/specific/hgu133plus.affx). Fluorescence intensity was captured with a laser GeneChip® Scanner 3000 (Affymetrix). Affymetrix Gene Chip Operative software with the Expression Default Settings was used for data acquisition and quantification. To remove systematic bias resulting from experimental artefacts, microarray original data (CEL files) were normalized using the robust multi-array average (RMA) method which shows consistent good performance [23]. To detect genes that show significant differences between sample classes, normalized data from control and treated cells were imported into the T-Rex tool of the GEPAS software (Gene Expression Pattern Analysis Suite; http://gepas.bioinfo.cipf.es/) for statistical differential gene expression analysis using an unpaired *t*-test [24]. Because of the multiple testing situation of microarrays (several thousand of genes tested in parallel), the false discovery rate (FDR) by Benjamini and Hochberg [25] is applied to control the expected proportion of false positives among the declared significant results.

To identify the biological meaning of the microarray's results, the Babelomics software [26] (http://babelomics.bioinfo.cipf.es/cgi-bin/tools.cgi) that includes several tools for functional annotation was used: (i) Fatigo + Search extracts functional relevant terms from gene ontology (GO) for a given list of genes. It determines significant enrichment of functional characteristics in one list of genes with respect to a reference one (usually the rest of genes involved in the experiment). Only the hierarchy 'biological process' level 7 was used in this analysis; (ii) FatiScan detects blocks of functionally related genes with significant but modest coordinate behaviour, across the complete list of probes displayed in the microchip ranked by the *t* statistics for differential expression between treated and control cells. It uses a segment test (two-tailed Fisher's exact test; 30 partitions; threshold p-value, 0.05; human repository, GO biological process; only deepest significant terms) to select significantly over-represented functional terms associated with genes most induced and most repressed in treated cells *versus* control cells. Data sets containing the significantly regulated genes and their corresponding fold-changes were also uploaded into the ingenuity pathways analysis (IPA) software (http://www.ingenuity.com) in order to identify significantly altered biological networks and genes potentially involved in specific functions and pathways. This web-delivered application uses a knowledge base derived from the literature to relate gene products with each other based on their interaction and function.

2.8 RT-PCR

Changes in the expression of ten selected genes responding to EA and/or urolithins were further assessed by one-step quantitative RT-PCR (Taqman system, Applied Biosystems, ABI, Madrid, Spain). RT-PCR reactions were performed in aliquots of the same RNA sample used for microarray hybridizations and in new RNA samples prepared from independent experiments. Each experiment consisted of triplicate RNA samples extracted from three separate plates. Primers and probes for the genes were selected from Assays-on-demand (ABI) and are as follows: epidermal growth factor receptor (*EGFR*), Hs00193306_m1; fibroblast growth factor receptor 2 (*FGFR2*), Hs00240796_m1; *K-Ras*, Hs00364282_m1; dual specificity phosphatase 6 (*DUSP6*), Hs00169257_m1; *c-Myc*, Hs00153408_m1; V-fos FBJ murine osteosarcoma viral oncogene homologue (*Fos*), Hs00170630_m1; CD44 molecule (Indian blood group) (*CD44*), Hs00174139_m1; cyclin B1 (*CCNB1*), Hs00259126_m1; cyclin B1 interacting protein (*CCNB1IP1*), Hs00603841_m1; mitogen-activated protein kinase 4 (*MAP4K4*), Hs00377415_m1 (_m indicates an

assay whose probe spans an exon junction and will not detect genomic DNA). The one-step real-time RT-PCRs were run on the ABI 7500 system following manufacturer's conditions, using a total reaction volume of 25 μL in a MicroAmp Optical 96-well plate covered by optical adhesive covers and using Taqman Universal Master Mix (ABI). All assays for a particular gene were undertaken at the same time under identical conditions and in triplicate. The expression levels of target genes were normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Hs99999905_m1) utilizing a standard curve method for quantification.

2.9 Western blot analysis

Western blot analyses were performed as previously described [18]. Following 24, 48 and 72 h of exposure to the mixture of EA and urolithins, cells were washed twice with PBS and lysed with ice-cold RIPA buffer containing protease inhibitors. Lysates were centrifuged at 15 000 × *g* for 15 min at 4°C, and protein concentration was determined by the Bradford's method. Equal amounts of protein (30 μg *per* lane) were separated on a 12% SDS-PAGE and transferred to polyvinylidine diflouride membranes (Amersham Biosciences, Buckinghamshire, UK) by electroblotting. The membranes were washed and incubated for 2 h with the primary antibodies, anti-CCNB1 (BD Biosciences, Madrid, Spain) or anti-c-Myc (Sigma) at 1:1000, followed by 1 h with anti-mouse (CCNB1) or anti-rabbit (c-Myc) horseradish peroxidase (HRP)-linked secondary antibody (Sigma) at 1:5000. Membranes were developed with ECL Plus (Amersham Biosciences) according to the manufacturer's instructions and exposed to X-ray film. GAPDH antibody (Abcam, Cambridge, UK) was used to control for protein loading. Western blot analyses were done in triplicate.

2.10 Cell-based extracellular signal-regulated protein kinase (ERK)1/2 phosphorylation ELISA assay

Changes in the relative amount of ERK1/2 phosphorylation in treated Caco-2 cells were determined using a RayBio Cell-Based ERK1/2 ELISA kit (RayBiotech, Norcross, GA, USA) following the manufacturer's protocol. Briefly, 10 000 Caco-2 cells were seeded into each well of a 96-well culture plate. After 48 h of culture, cells were exposed to the mixture of EA and urolithins for 72 h with or without pretreatment with the specific inhibitor of MEK-1/2, PD98059 (25 or 50 μM, 1 or 12 h pretreatment). After the treatments, the culture medium was discarded and cells were washed and fixed for 20 min at room temperature followed by a 60 min blocking step. Anti-phospho-ERK1/2 (Thr202/Tyr204) antibody or Anti-ERK1/2 antibody were added to the corresponding wells and incubated for 2 h at room temperature. The cells were then washed and incubated with HRP-conjugated anti-mouse IgG for a further 1 h. After washing, the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was added and incubated for 30 min at room temperature. Colour develops in proportion to the amount of protein. Finally, a stop solution (sulphuric acid) changes the colour from blue to yellow, and the intensity of the colour is measured at 450 nm. Treatments were all done in triplicate.

3 Results

3.1 Effects of EA and urolithins on cell proliferation and cell cycle

Results on cell proliferation and viability are presented in Table 1. Proliferation was slightly inhibited, although not significant, in a time-dependent fashion by each individual compound. However, proliferation of cells was substantially reduced after exposure to the mixture of EA and urolithins. The percentage of treated cells compared to control was 56% on day 1 and 12% by day 4. The viability of cells treated with each individual compound was constant during the experiment and similar to that of control cells (∼90%). The viability of cells treated with the mixture of EA and urolithins was at ∼77% and was also constant during the 4 days treatment. There were not significant differences in the proportion of apoptotic cells between control and any treated group, and thus the slight decrease in viability was probably due to necrosis.

Inhibition of proliferation was further examined by measuring cell cycle distribution. On day 1 of treatment, control cells were distributed as follows: 46.2 ± 1.6% in G_0/G_1 phase, 36.9 ± 2.0% in S phase and 16.8 ± 1.4% in G_2/M phase. Within the 4 days of the experiment, the proportion of control cells in G_0/G_1 phase increased to 72.3 ± 0.6% ($p < 0.01$) whereas cells in S and G_2/M phases decreased to 21.9 ± 1.4% ($p < 0.01$) and to 5.8 ± 1.0% ($p < 0.05$), respectively (Fig. 2a). Caco-2 cells spontaneously undergo cell cycle arrest, with cells accumulating in the G_0/G_1 phase as a function of time under normal culture conditions [21]. A similar trend was observed in cells treated with urolithin-A, urolithin-B or EA (Figs. 2b–d, respectively), indicating that there were not any detectable effects of each individual compound on the cell cycle distribution. However, following treatment with the mixture of EA and urolithins there was an increase of cells in phase S after first day of treatment from 36.9 ± 2.0% in control cells to 44.6 ± 7.5% in the treated group ($p < 0.05$) (Fig. 2e). This increase was maintained during the 4 days of treatment and was accompanied by a decrease of cells in G_0/G_1 phase. From day 2 of treatment, cells also started to accumulate in the G_2/M phase (from 12.8 ± 0.3% in control cells to 27.6 ± 2.2% in the treated cells; $p < 0.01$). The G_2/M arrest was also maintained throughout the rest of the experiment. Significant

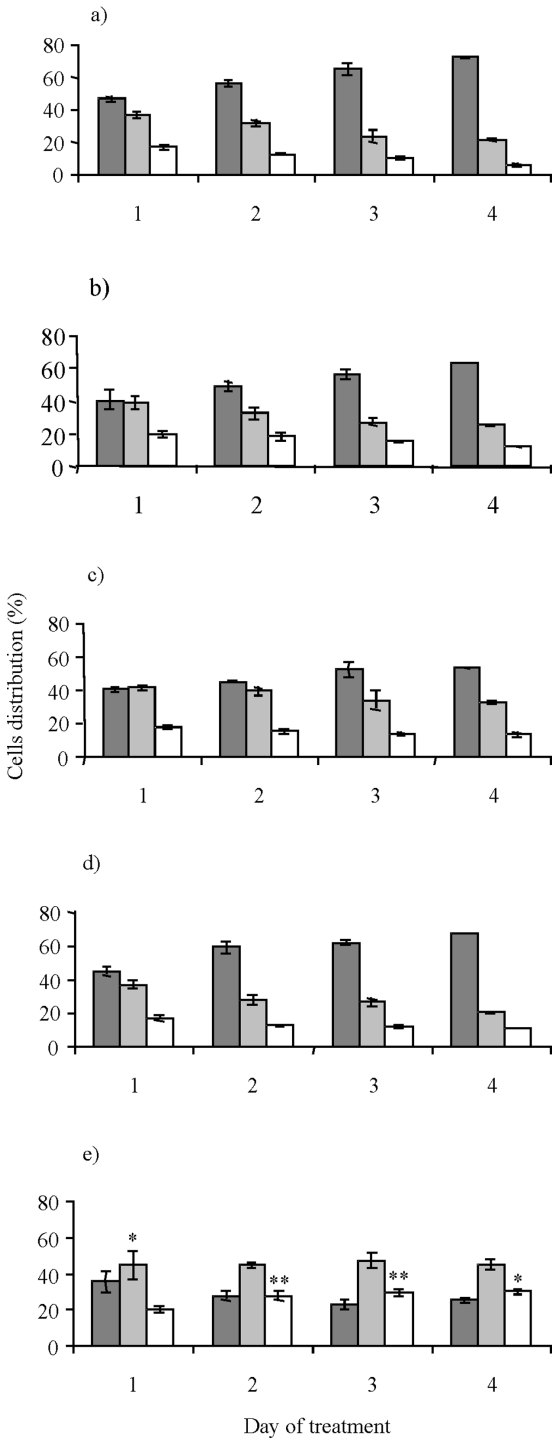

Figure 2. Changes in Caco-2 cell cycle distribution exposed to: (a) DMSO (control); (b) 40 μM U-A; (c) 40 μM U-B; (d) 10 μM EA; (e) 10 μM EA, 40 μM U-A and 40 μM U-B (Mix) after 1, 2, 3 and 4 days of treatment. The percentage of cells in each cycle phase was determined by flow cytometry after incubating the cells with RNase (100 μg/mL) and PI (40 μg/mL) at 37°C for 30 min. Data are presented as mean values ± SD ($n = 3$; significant differences between control and treated cells, *: $p < 0.05$; **: $p < 0.01$). ■ G_0/G_1 phase; ■ S phase; □ G_2/M phase.

differences were seen between control and treated cells on day 3 ($p < 0.01$) and day 4 ($p < 0.05$). These results indicated that the cytotoxicity exerted in Caco-2 cells by the mixture of EA and urolithins was due to some cell death and to inhibition of cell cycle progression.

3.2 Gene expression analyses

Between 1 and 3% of the probes represented in the chip (54120 total probe sets) exhibited significant altered levels of expression in response to each treatment. This relatively low % of changes has been reported in other studies looking at transcription profiling of treated cancer cells [21] and in studies looking at genes differentially expressed between normal and tumour tissues [27]. Selected ratios (treated/control) were mostly between 1.5- and 3.0-fold although some genes exhibited inductions of almost 30-fold and downregulations below 10-fold. A complete listing of selected genes with corresponding *p*-values and expression levels (ratio treated/control) can be seen in Supporting Information Tables 1 – 4.

GO biological annotations search using the Fatigo + Search tool revealed that treatment with EA and/or urolithins had affected a range of functions: transcription, cell death, protein metabolism and transport, nucleic acid processing, protein kinase signalling pathway and cell cycle mitosis (Supporting Information Table 5). Further analysis using the FatiScan tool indicated that regulation of transcription was generally overrepresented in genes most expressed in Caco-2 cells treated with EA, urolithin-A or the mixture of EA and urolithins (Supporting Information Table 6). No significant terms were found for treatment with urolithin-B using this analysis. Some of the functional terms, G-protein signalling, cell adhesion and ion transport found to be overrepresented among genes most induced in EA-treated cells, were also overrepresented in genes most repressed in cells treated with urolithin-A. Conversely, those functions overrepresented among genes most induced by the urolithin-A (cell cycle, protein metabolism and transport, RNA processing and DNA repair) were overrepresented in genes most repressed by EA. In addition, IPA analysis revealed that modulated genes were primarily associated with cancer related cell functions: cell signalling, cell cycle, cell growth, cell death, cell assembly and organization and DNA repair (Supporting Information Table 7). IPA also identified changes in genes associated with the metabolism of amino acids and small bio-molecules, and in genes involved in cardiovascular and haematological function as well as in immune response. A summary of the main cancer related functions found by the various bioinformatic tools is presented in Supporting Information Tables 8a and b. In general, results revealed that exposure of Caco-2 cells to EA and/or to its metabolites, urolithins, modulates the expression of genes related to transcription and nucleic acid processing, protein and amino acid metabolism, small mol-

Table 1. Inhibition of cell proliferation and cell viability in Caco-2 cells after treatment

	Control	Urolithin-A (40 μM)	Urolithin-B (40 μM)	EA (10 μM)	Mix: EA (10 μM) + urolithin-A (40 μM) + urolithin-B (40 μM)
24 h	–	69.7 ± 1.9[a)]	102.5 ± 13.5	77.0 ± 14.3	55.9 ± 3.8
	90.0 ± 1.4	87.4 ± 4.9[b)]	83.0 ± 13.6	82.6 ± 8.4	75.6 ± 12.4
48 h	–	43.5 ± 10.7	77.1 ± 12.1	81.4 ± 8.4	31.9 ± 6.4
	89.8 ± 7.4	81.0 ± 6.9	87.0 ± 5.9	91.9 ± 4.6	76.5 ± 4.6
72 h	–	60.3 ± 11.5	76.3 ± 0.6	64.5 ± 5.6	19.6 ± 6.3
	92.0 ± 1.5	86.8 ± 4.1	91.9 ± 1.0	92.0 ± 4.9	79.1 ± 7.5
96 h	–	54.0 ± 13.7	67.7 ± 2.2	74.3 ± 5.8	12.0 ± 5.3
	91.2 ± 2.6	87.6 ± 3.8	91.9 ± 0.1	92.8 ± 1.3	76.3 ± 8.1

a) % of proliferative cells against control.
b) % of viability; values are presented as mean values ± SD from three independent experiments (n = 2 plates *per* experiment).

ecule metabolism, cell signalling, cell cycle and proliferation, cell death and cell organization. Since the main phenotypic outcome of the experiments was inhibition of cell proliferation and cell cycle arrest in cells treated with the mixture of EA and urolithins, genes with altered expression involved in cell cycle regulation and in signalling pathways that affect cell proliferation and cell cycle were further examined.

Genes with significant altered expression in cells treated with the mixture of EA and urolithins that were associated with cell cycle regulation are listed in Tables 2a and b. It includes several cyclins, cyclin dependent kinases (CDKs) and other cell cycle regulators that have been annotated in relation to the S or G_2/M phases and may be implicated in the observed growth arrest. Functional analysis also indicated that kinase signalling pathways may be involved in the response of Caco-2 cells to EA and/or urolithins. The relative expression changes (ratio treated/control) of some genes that are well recognized members of this signalling pathways were confirmed by RT-PCR (Table 3). The analysis detected lower mRNA levels of *FGFR2* in cells exposed to EA and/or the urolithins and lower mRNA levels of *EGFR* in cells treated with the urolithin-B. The mRNA levels of *K-Ras* were also lower after exposure to the mixture of EA and urolithins and to urolithin-B alone. Exposure of Caco-2 cells to EA or to urolithin-B decreased the expression levels of the *MAP4K4* gene. *DUSP6* transcripts were significantly higher in cells treated with the mixture of EA and urolithins or with each individual urolithin. Caco-2 cells treated with EA and/or urolithins also exhibited higher levels of *c-Myc* and *Fos* mRNA. The expression of CD44, a cell surface receptor for hyaluronan implicated in the regulation of tumour growth and metastasis and a target of the *K-Ras* signalling pathway [28], was also induced in cells treated with the mixture of EA and urolithins. In addition, the downregulation of CCNB1 and the upregulation of CCNB1IP1 in Caco-2 cells treated with the mixture of these molecules were also confirmed by RT-PCR.

To further validate the results, changes at the protein level in two of the selected genes with altered expression were also assessed (Fig. 3). The levels of c-Myc were undetectable in control cells and were noticeably induced in cells treated with the mixture of EA and urolithins after 48 and 72 h of treatment. The decrease in CCNB1 protein concentration in cells treated with the mixture of molecules was also confirmed and was more marked after 48 and 72 h of treatment.

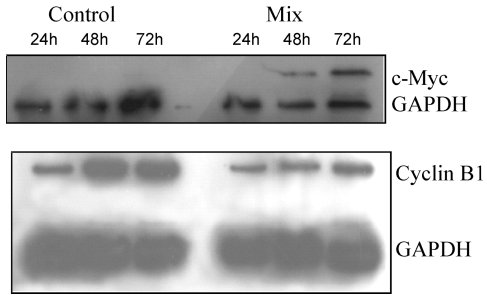

Figure 3. Differences in the protein levels of c-Myc and CCNB1 between Caco-2 cells exposed to a mixture (Mix) of 10 μM EA, 40 μM U-A and 40 μM U-B and control cells (at 24, 48 and 72 h). Analyses were performed in whole cell lysates (30 μg of protein), after SDS-PAGE separation (12% SDS polyacrylamide gel) and electroblotting using specific antibodies against the respective proteins. GAPDH was included for control of protein loading.

3.3 Effect of the mixture of EA and urolithins on the phosphorylation status of ERK signalling in Caco-2 cells

Microarrays and RT-PCR results presented here showed that in Caco-2 cells, EA and urolithins induced significant changes in the expression levels of several genes that are part of the ERK1/2 signalling pathway (Table 3 and Fig. 4) which is indicative of deregulation of the pathway. To determine the state of activation of this pathway, both total forms and the phosphorylated forms of ERK1/2 in cells treated with the mixture of EA and urolithins were measured using a cell-based ELISA assay (Fig. 5). Treatment of cells with PD98059 slightly suppressed the phosphorylation of ERK1/2 in Caco-2 cells. These results were equally observed at 25 or 50 μM concentrations of inhibitor and at

Table 2. Downregulated and upregulated probes in Caco-2 cells after exposure to the mixture of EA and urolithins (Mix) and associated to cell cycle regulation by IPA functional analysis

Affymetrix probe	Gene symbol	Gene name	Mix	EA	U-B	U-A
Downregulated						
202834_at	*AGT*	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	0.5	n.c.	↓	↓
210962_s_at	*AKAP9*	A kinase (PRKA) anchor protein (yotiao) 9	0.5	n.c.	↓↓	0.8
209425_at, 209426_s_at, 209424_s_at	*AMACR*	α-Methylacyl-CoA racemase	0.4	↓↓↓↓	↓↓↓	↓↓↓↓
210334_x_at	*BIRC5*	Baculoviral IAP repeat-containing 5 (survivin)	0.8	0.4	↑↑↑	↑↑↑
209591_s_at	*BMP7*	Bone morphogenetic protein 7 (osteogenic protein 1)	0.4	n.c.	0.6	↓↓
214710_s_at	*CCNB1*	Cyclin B1	0.7	n.c.	↑↑	↑↓
201700_at	*CCND3*	Cyclin D3	0.6	n.c.	↓	n.c.
224848_at	*CDK6*	Cyclin-dependent kinase 6	0.7	0.5	n.c.	n.c.
213348_at, 213182_x_at	*CDKN1C*	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	0.5	n.c.	↓↓↓↓	↓↓↓↓↓↓
204039_at	*CEBPA*	CCAAT/enhancer binding protein (C/EBP), alpha	0.5	n.c.	↓	n.c.
216836_s_at	*ERBB2*	v-erb-b2 erythroblastic leukaemia viral oncogene homologue 2, neuro/glioblastoma derived oncogene homologue (avian)	0.6	↓	n.c.	n.c.
210891_s_at, 201065_s_at	*GTF2I* /// *GTF2IP1* /// LOC649791	General transcription factor II, i /// general transcription factor II, i, pseudogene 1 /// similar to general transcription factor II, i isoform 1	0.6	n.c.	↓↓	n.c.
201163_s_at, 201162_at	*IGFBP7*	Insulin-like growth factor binding protein 7	0.2	n.c.	0.3	↓↓
214352_s_at, 204009_s_at	*KRAS*	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue	0.5	n.c.	0.7	↓↓
204880_at	*MGMT*	*O*-6-methylguanine-DNA methyltransferase	0.5	0.6	↓	0.7
209757_s_at	*MYCN*	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	0.4	n.c.	n.c.	↓↓↓
207700_s_at	*NCOA3*	Nuclear receptor coactivator 3	0.7	↓↓↓↓↓	↓↓↓↓↓	n.c.
203625_x_at	*SKP2*	S-phase kinase-associated protein 2 (p45)	0.8	0.2	↑↑↓	n.c.
201010_s_at	*TXNIP*	Thioredoxin interacting protein	0.4	↓↓↓	n.c.	↓↓↓
Upregulated						
237571_at	*APP*	Amyloid b (A4) precursor protein (peptidase nexin-II, Alzheimer disease)	1.9	n.c.	n.c.	n.c.
202672_s_at	*ATF3*	Activating transcription factor 3	4.5	n.c.	n.c.	↑↑
201170_s_at	*BHLHB2*	Basic helix-loop-helix domain containing, class B, 2	3.7	↑↑	↑↑	n.c.
217988_at	*CCNB1IP1*	Cyclin B1 interacting protein 1	2.5	n.c.	n.c.	1.4
225824_at	*CCNK*	Cyclin K	1.2	n.c.	n.c.	n.c.
204490_s_at, 204489_s_at, 212063_at	*CD44*	CD44 molecule (Indian blood group)	2.4	n.c.	2.4	1.7
225081_s_at	*CDCA7L*	Cell division cycle associated 7-like	1.8	n.c.	↑	↑
201938_at	*CDK2AP1*	CDK2-associated protein 1	1.2	↑	n.c.	↑
211297_s_at	*CDK7*	Cyclin-dependent kinase 7 (MO15 homologue, Xenopus laevis, cdk-activating kinase)	1.7	n.c.	n.c.	1.3
1553112_s_at	*CDK8*	Cyclin-dependent kinase 8	1.4	n.c.	n.c.	n.c.
219831_at	*CDKL3*	Cyclin-dependent kinase-like 3	1.8	n.c.	n.c.	n.c.
212501_at	*CEBPB*	CCAAT/enhancer binding protein (C/EBP), b	2.1	n.c.	↓	↑
209383_at	*DDIT3*	DNA-damage-inducible transcript 3	2.8	n.c.	↓	↑
2028_s_at	*E2F1*	E2F transcription factor 1	1.3	n.c.	↑↑	↑↑
218995_s_at	*EDN1*	Endothelin 1	2.1	↑↑	↑↑	↑
205767_at	*EREG*	Epiregulin	4.2	n.c.	↑↑	↑
211719_x_at, 216442_x_at, 210495_x_at, 212464_s_at	*FN1*	Fibronectin 1 /// fibronectin 1	1.2	↓↑↑	↑↑	↑↑↑↑
210002_at	*GATA6*	GATA binding protein 6	1.4	n.c.	n.c.	n.c.
221577_x_at	*GDF15*	Growth differentiation factor 15	2.4	n.c.	n.c.	↑
201667_at	*GJA1*	Gap junction protein, alpha 1, 43 kDa (connexin 43)	2.5	↑	↑	2.1
208937_s_at	*ID1*	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	2.0	n.c.	↑	↑

Table 2. Continued

Affymetrix probe	Gene symbol	Gene name	Mix	EA	U-B	U-A
230348_at	*LATS2*	LATS, large tumour suppressor, homologue 2 (Drosophila)	1.4	n.c.	↓↓	n.c.
202520_s_at	*MLH1*	mutL homologue 1, colon cancer, nonpolyposis type 2 (E. coli)	1.4	n.c.	n.c.	↑
202431_s_at	*MYC*	v-myc myelocytomatosis viral oncogene homologue (avian)	4.2	1.6	↑↑↑	1.5
217863_at	*PIAS1*	Protein inhibitor of activated STAT, 1	1.6	n.c.	↓↓↓↓	n.c.
209193_at	*PIM1*	pim-1 oncogene /// pim-1 oncogene	1.4	n.c.	n.c.	↑
211012_s_at	*PML* /// *LOC161527* /// *LOC652671*	Promyelocytic leukaemia /// hypothetical protein LOC161527 /// similar to promyelocytic leukaemia protein isoform 9	1.3	n.c.	n.c.	n.c.
225291_at	*PNPT1*	Polyribonucleotide nucleotidyltransferase 1	1.6	n.c.	↑	↑
208799_at	*PSMB5*	proteasome (prosome, macropain) subunit, β type, 5	1.3	n.c.	↑	n.c.
233254_x_at	*PTEN*	Phosphatase and tensin homologue (mutated in multiple advanced cancers 1)	1.2	n.c.	↓↓↓↓	↓↓
1554997_a_at, 204748_at	*PTGS2*	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	4.3	n.c.	n.c.	↑↑
218723_s_at	*RGC32*	Response gene to complement 32	5.7	n.c.	n.c.	
218878_s_at	*SIRT1*	Sirtuin (silent mating type information regulation 2 homologue) 1 (S. cerevisiae)	1.6	n.c.	n.c.	n.c.
202936_s_at	*SOX9*	SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)	2.0	↑↑	2.2	↑↑
204011_at	*SPRY2*	Sprouty homologue 2 (Drosophila)	2.4	↑	↑	↑
207332_s_at	*TFRC*	Transferrin receptor (p90, CD71)	1.4	n.c.	↑↑↑	n.c.
212171_x_at, 211527_x_at, 210512_s_at	*VEGF*	Vascular endothelial growth factor	3.0	n.c.	n.c.	1.6

Results are presented as relative expression (ratio treated/control). n.c.: no change, ↓: Downregulated but not significant (the number of arrows indicate the number of probes for a particular gene that exhibited the same change). ↑: Upregulated but not significant (the number of arrows indicate the number of probes for a particular gene that exhibited the same change).

1 or 12 h of preincubation times. After treatment of cells with the mixture of EA and urolithins, the activated form p-ERK1/2 was noticeably decreased (ratio p-ERK1/2/ERK1/2 was reduced by 30%). Preincubation with the inhibitor did not have any further effect on the levels of the phosphorylated forms in cells treated with the mixture of molecules. The effect of the ERK1/2 inhibitor alone or prior to treatment with the mixture of EA and urolithins on Caco-2 cells cycle distribution was also examined (Fig. 6). PD98059 alone did not have a significant effect on the cell cycle phases at any of the times/doses tested. However, in all the cases the inhibitor significantly attenuated the EA + urolithins-induced S arrest (the S population was decreased by 8%) concomitant with a small increase in the G_1 population. The proportion of cells at the G_2/M phase was not affected by the inhibitor.

4 Discussion

Many plant polyphenols exhibit a substantial inhibitory activity against the growth of colon cancer cells *in vitro* and against colon carcinogenesis in animal models, which suggests that regular consumption of these compounds with the diet may have a preventive role against CRC development [29]. The antiproliferative effects of polyphenols appear to be mediated through the modulation of multiple cellular processes that affect the homeostasis between cell survival and cell death. The molecular mechanisms, transcription changes and protein level changes involved in the cellular response to polyphenols are beginning to emerge [30] but are not yet completely understood. In this study, transcriptional changes and cell functions altered by the dietary polyphenol EA and its derived colon metabolites, urolithins, in colon cancer Caco-2 cells were investigated using microarray technology and bioinformatic tools.

In performing this study, selection of the most appropriate concentrations of EA and urolithins to treat the colon cancer cells was a concern. The growth inhibitory effects of EA on colon cancer cells have been previously reported at concentrations ≥100 μM [17]. Under the culture conditions used in the present study, EA maximum solubility was ~30 μM. In a recent study [18], it was shown that EA inhib-

Table 3. Relative changes in expression (ratio treated/control cells) of genes involved in kinase signalling pathways, transcription and cell cycle in Caco-2 cells after exposure to EA (10 μM) and/or urolithins -A and -B (40 μM) for 72 h as determined by Affymetrix microarray *versus* RT-PCR

	Mix			EA			U-B				U-A		
Gene	Affyx	RT-PCR[a)]	RT-PCR[b)]	Affyx	RT-PCR[a)]	RT-PCR[b)]	Affyx	RT-PCR[a)]	RT-PCR[b)]	RT-PCR[c)]	Affyx	RT-PCR[a)]	RT-PCR[b)]
Kinase signalling pathway													
EGFR	n.c.	n.d.	n.d.	n.c.	n.d.	n.d.	0.4	0.7 ± 0.6	0.5 ± 0.1	0.7 ± 0.2	n.c.	n.d.	n.d.
FGFR2	0.5	0.5 ± 0.2	0.6 ± 0.2	0.6	0.7 ± 0.1	0.6 ± 0.2	0.5	n.d.	0.5 ± 0.1	0.4 ± 0.2	0.6	0.6 ± 0.1	0.7 ± 0.1
K-Ras	0.6	0.5 ± 0.1	0.4 ± 0.1	n.c.	n.d.	n.d.	0.6	n.d.	0.6 ± 0.1	0.5 ± 0.2	n.c.	n.d.	n.d.
MAP4K4	0.6	0.9 ± 0.2	0.9 ± 0.1	0.8*)	0.7 ± 0.1	0.5 ± 0.1	0.6*)	n.d.	0.5 ± 0.1	0.4 ± 0.1	n.c.	n.d.	n.d.
DUSP6	2.3	5.3 ± 2.3	2.3 ± 0.5	0.6	1.9 ± 0.1	1.0 ± 0.2	5.9	2.2 ± 0.7	2.1 ± 0.2	7.9 ± 2.3	2.4	3.5 ± 0.9	3.4 ± 0.9
c-Myc	4.2	3.5 ± 0.6	5.4 ± 1.2	1.6	1.6 ± 0.3	1.4 ± 0.3	3.8	4.9 ± 2.4	3.5 ± 0.8	2.1 ± 0.5	1.5	1.9 ± 0.3	3.0 ± 0.5
Fos	n.c.	n.d.	n.d.	n.c.	n.d.	n.d.	6.4	1.5 ± 0.5	2.4 ± 0.3	2.7 ± 1.3	1.6	1.4 ± 0.2	2.3 ± 0.2
CD44	3.2	3.4 ± 0.5	2.3 ± 0.3	n.c.	n.d.	n.d.	2.3	n.d.	1.5 ± 0.2	1.4 ± 0.3	2.1	1.2 ± 0.1	1.7 ± 0.3
Cell cycle													
CCNB1	0.6	0.5 ± 0.1	0.4 ± 0.1	n.c.	n.d.	n.d.	n.c.	n.d.	n.d.	n.d.	n.c.	n.d.	n.d.
CCNB1IP1	2.5	1.5 ± 0.4	1.8 ± 0.4	n.c.	n.d.	n.d.	n.c.	n.d.	n.d.	n.d.	1.4	n.d.	n.d.

n.c.: no change; n.d.: not determined.
a) Performed in the same RNA sample used for microarrays.
b) Performed in RNA samples from other independent experiments.
*) Not significant.

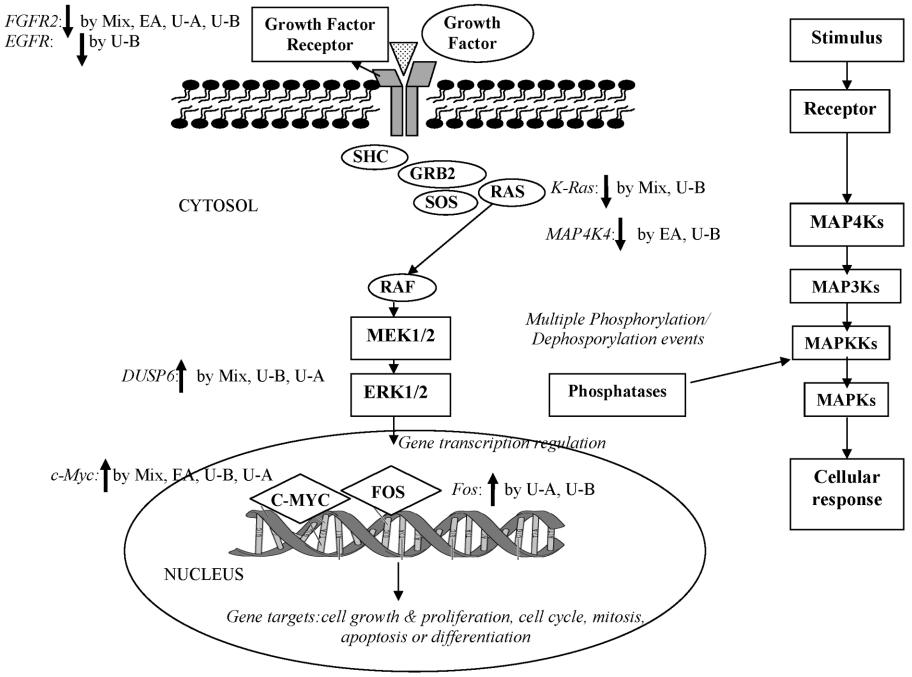

Figure 4. Schematic representation of the tiered MAPK (ERKs) signalling cascade indicating specific genes with significant differences in mRNA levels between Caco-2 cells treated with 10 μM EA, 40 μM U-A, 40 μM U-B or the mixture of the three compounds (Mix) and control cells (black arrows indicate ↑ up- or ↓ downregulation by EA and/or urolithins). Relative expression values (ratio treated/control) for each gene are specified in Table 3.

ited Caco-2 cells proliferation at 1, 10 and 30 μM concentrations. At 30 μM, however, inhibition of proliferation was very drastic, >70% inhibition of proliferation after 72 h exposure, which was not suitable for gene expression analysis. Therefore, a 10 μM concentration of EA was chosen to treat the cells. The 40 μM concentration of urolithins selected was based on solubility. Concentrations of EA and urolithins achievable in the intestine from dietary intake were also considered. The total content of polyphenols excreted in ileostomy bags in humans can be used as an estimate of colonic availability of orally consumed polyphenols and was reported to be ~92 μmols [31]. In this context, 10

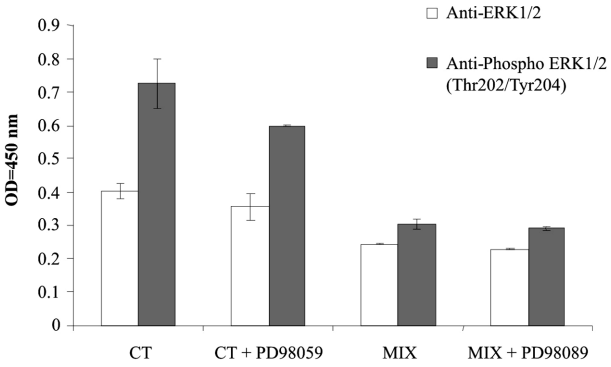

Figure 5. Comparison of the ERK1/2 pathway state of activation between control Caco-2 cells (CT) and Caco-2 cells treated with the mixture of 10 μM EA, 40 μM U-A and 40 μM U-B (Mix) for 72 h, alone and in cells preincubated with the MEK1/2 specific inhibitor PD98059 (25 μM, 1 h). Changes in the relative amount of ERK1/2 phosphorylation were determined using a cell-based ELISA kit in 96-well culture plates. After treatments, fixed cells were blocked and incubated with specific antibodies: anti-phospho ERK1/2 (Thr202/Tyr204) or anti-ERK1/2 followed by incubation with HRP-conjugated anti-mouse IgG. Intensity of colour (450 nm) was developed by the addition of the substrate TMB and the stop solution (sulphuric acid). Data are presented as mean values ± SD of triplicate experiments.

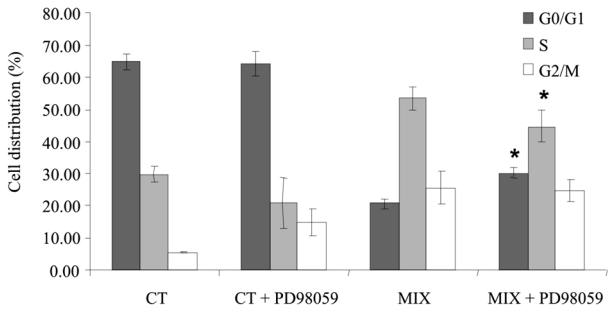

Figure 6. Effect of the MEK1/2 specific inhibitor, PD98059 (25 μM, 1 h), alone or prior to treatment with the mixture (Mix) of 10 μM EA, 40 μM U-A and 40 μM U-B for 72 h on Caco-2 cell cycle distribution. The percentage of cells in each cycle phase was determined by flow cytometry after incubating the cells with RNase (100 μg/mL) and PI (40 μg/mL) at 37°C for 30 min. Data are presented as mean values ± SD ($n = 3$; significant differences between control and treated cells, *: $p < 0.05$).

or 40 μM of EA or urolithins, respectively seemed appropriate concentrations for the study.

Results indicate that regulators of cell cycle appear to be modulated by EA and urolithins in Caco-2 cells. Cell cycle is regulated by an intricate network of cyclin/CDK complexes. Amongst the 'G_2 cyclins', CCNB1 forms a complex with CDK1 and is involved in the control of the G_2/M transition and mitosis [32]. The data presented here show that the G_2/M arrest observed in Caco-2 cells exposed to the mixture of EA and urolithins is associated with the downregulation of CCNB1, both at the mRNA and protein levels. These results are in agreement with a previous report where it was shown that EA reduced the levels of CCNB1 protein in Caco-2 cells in a dose- and time-dependent manner [18]. Other natural compounds induce G_2/M arrest in cancer cells. Genistein induces G_2/M arrest associated with the downregulation of CCNB1 and upregulation of p21 in prostate human cancer cells [33] whereas, in leukaemia cells, diosgenin causes G_2/M arrest by decreasing the levels of CCNB1 and p21 and increasing the levels of CDK1 [34]. The antibiotic lidamycin also induces G_2/M arrest in p53 wild-type MCF-7 cells associated with an increase of p53 and p21 and a decrease of CDK1 and CCNB1. In a p53 mutant MCF-7 cell line, the G_2/M arrest induced by lidamycin was correlated only with a reduction of CCNB1 mRNA and protein levels [35]. It does appear that depending on the compound and type of cells, the molecular changes associated with the G_2/M arrest may differ and multiple pathways can regulate the process and converge on the downregulation of CCNB1. Many natural compounds, including EA and its derivatives urolithins, can inhibit cancer cell growth through this mechanism. Interestingly, CCNB1IP1, a novel RING finger protein that functions in the progression through G_2/M, inhibits the mitotic entry and reduces cyclin B levels in *Xenopus* [36], was found to be upregulated in Caco-2 cells after treatment with EA and urolithins and may be involved in the observed arrest. The cell cycle regulators, CDKs, are themselves regulated by other proteins such as the members of the KIP family including p21, p27 and p57 [32]. It has been reported that the knockdown of *p57* gene expression alters mitotic progression yielding an increase of ana-telophase cells. To the contrary, there is a reduction of G_2/M cells upon overexpression of *p57* [37]. The expression levels of cyclin-dependent kinase inhibitor 1C (p57, Kip2) (*CDKN1C*) (*p57*) were downregulated in Caco-2 cells treated with the mixture of compounds which may be associated with the arrest of cells in the G_2/M phase. Other interesting proteins, like CDK2 associated protein (CDK2AP1) that is involved in cell proliferation and promotes cell cycle arrest [38] and chromosome 13 ORF (RGC32) that suppresses cell growth by inducing G_2/M arrest [39] were also induced after the treatment and may also be involved in the cells response to EA and urolithins.

Results also pointed out that kinase signalling pathways (MAPKs) may be involved in the response of Caco-2 cells to EA and urolithins. MAPKs can be activated by different extracellular signals acting through various receptors. Transduction of signals is usually initiated by activation of small G proteins (*e.g.* Ras) followed by sequential phosphorylation and activation of cascades composed of three to five tiers (MAP4Ks, MAP3Ks, MAPKKs, MAPKs). Phosphatases serve as negative regulators of the kinase signalling. Activated MAPKs can be translocated to another cell compartment (*e.g.* the nucleus), where they can activate their target proteins such as transcription factors which are

implicated in the regulation of fundamental cellular processes including gene transcription, cell cycle progression and mitosis, cell growth and proliferation, differentiation or apoptosis [40] (Fig. 4). Amongst the MAPKs families, the ERKs pathway responds primarily to growth factors through various receptors including fibroblast growth factor receptor (FGFR) and EGFR [41]. The downstream propagation involves the proto-oncogene *Ras* and is controlled by phosphatases such as the DUSP6 [42]. Modulation of the ERK1/2 signalling pathway influences many cell events including regulation of immediate early genes such as *c-Myc* and *Fos*, which are implicated in cell cycle progression and cell growth and proliferation [43]. Activation of the ERK signalling pathway is generally linked to growth stimulating actions. However, there are studies that indicate that both ERK activation [44, 45] and deactivation [46, 47] is associated with growth inhibition and cell arrest. It appears that the response can be molecule-, cell type- and time-dependant. Results presented here indicate that treatment of Caco-2 cells with the mixture of EA and urolithins deactivates the ERK signalling pathway and suggest an inhibitor-like influence of these molecules on the pathway.

MAPKs have an important role in the G_2/M transition. Recently, it has been shown that changes in the expression of *c-Myc*, *Fos* and *DUSPs* were associated with G_2 cell cycle arrest and re-entry into mitosis [48]. However the role of MAPKs signalling pathways on the G_2/M arrest is not yet well understood. Both activation and inhibition of the ERK pathway have been associated with the G_2/M delay [44 – 47], and preincubation of cells with ERKs specific inhibitors not always abolishes the G_2/M arrest [49, 50] implying that progression through the G_2/M phase is a complex process and that other pathways may be implicated. It has been shown that the PI3K/Akt may also contribute to the G_2/M transition [48, 49]. Further studies are needed to determine whether the response of Caco-2 cells to EA and urolithins involves the modulation of additional pathways. It is of note that amongst the genes with significant expression changes induced by the mixture of EA and urolithins, several members of phosphoinositide 3-kinase (PI3K) family are included.

Deregulation of some of the proteins involved in the MAPK pathways (*e.g. EGFR*, *K-Ras* or *c-Myc*) occurs frequently in various tumours including tumours in CRC and become targets for directed cancer therapy [51]. Signalling pathways are very intricate and modulation of single targets is not always effective due to alterations/mutations in other proteins of the pathway or because of alternate pathways. There is now increasing evidence that therapy against combined molecular targets will improve treatment of cancer. Data presented here show that EA and urolithins altered simultaneously the expression levels of several of these cancer target genes, *FGFR2*, *EGFR*, *K-Ras*, *MAP4K4*, *DUSP6*, *c-Myc* and *Fos*, and inhibited the growth of colon cancer Caco-2 cells. These results suggest that regular consumption of dietary ETs and EA, that yields EA and derived urolithins in the colon, may contribute to CRC prevention by effectively inducing changes in the expression of multiple targets in the colon epithelia and regulating whole signalling pathways and fundamental cell processes such as cell growth and cell cycle. To date, there is only one report on the effects of EA in human patients with cancer [52]. In this study, oral administration of EA to chemotherapy patients with refractory prostate cancer led to a better tumour response, higher survival rates, and decreased toxic effects. At present, there are no clinical trials on CRC prevention with EA or their metabolites. These molecules are, however, promising anticancer agents and thus, more animal and human studies are needed to validate their usefulness.

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5 References

[1] Jemal, A., Murray, T., Ward, E., Samuels, A., *et al*., Cancer Statistics CA, *Cancer J. Clin.* 2005, *55*, 10 – 30.

[2] Rodrigo, L., Riestra, S., Diet and colon cancer, *Rev. Esp. Enferm. Dig.* 2007, *99*, 183 – 189.

[3] Kellof, G. J., Lippman, S. M., Dannenberg, A. J., Sigman, C. C., *et al*., Progress in chemoprevention drug development. The promise of molecular biomarkers for prevention of intraepithelial neoplasia and cancer- a plan to move forward, *Clin. Cancer Res.* 2006, *12*, 3661 – 3697.

[4] Abnet, C. C., Carcinogenic food contaminants, *Cancer Invest.* 2007, *25*, 189 – 196.

[5] Marques-Vidal, P., Ravasco, P., Camilo, M. E., Foodstuffs and colorectal cancer risk: A review, *Clin. Nutr.* 2006, *25*, 14 – 36.

[6] Koushik, A., Hunter, D. J., Spiegelman, D., Beeson, W. L., *et al*., Fruits, vegetables, and colon cancer risk in a pooled analysis of 14 cohort studies, *J. Natl. Cancer Inst.* 2007, *99*, 1471 – 1483.

[7] Ferguson, L. R., Philpott, M., Karunasinghe, N., Dietary cancer and prevention using antimutagens, *Toxicology* 2004, *198*, 147 – 159.

[8] Rudolf, E., Andelová, H., Cervinka, M., Polyphenolic compounds in chemoprevention of colon cancer-targets and signalling pathways, *Anticancer Agents Med. Chem.* 2007, *7*, 559 – 575.

[9] Thomasset, S. C., Berry, D. P., Garcea, G., Marczylo, T., *et al*., Dietary polyphenolic phytochemicals-promising cancer chemopreventive agents in humans? A review of their clinical properties, *Int. J. Cancer* 2006, *120*, 451 – 458.

[10] Manach, C., Williamson, G., Morand, C., Scalbert, A., Rémésy, C., Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies, *Am. J. Clin. Nutr.* 2005, *81*, 230S – 242S.

[11] Clifford, M. N., Scalbert, A., Ellagitannins- nature, occurrence and dietary burden, *J. Sci. Food Agric.* 2000, *80*, 118 – 125.

[12] Cerdá, B., Llorach, R., Cerón, J. J., Espín, J. C., Tomás-Barberán, F. A., Evaluation of the bioavailability and metabolism in the rat of punicalagin, and antioxidant polyphenol from pomegranate juice, *Eur. J. Nutr.* 2003, *42*, 18 – 28.

[13] Cerdá, B., Periago, P., Espín, J. C., Tomás-Barberán, F. A., Identification of urolithin A as a metabolite produced by human colon microflora from ellagic acid and related compounds, *J. Agric. Food Chem.* 2005, *53*, 5571 – 5579.

[14] Cerdá, B., Tomás-Barberán, F. A., Espín, J. C., Metabolism of antioxidant and chemopreventive ellagitannins from strawberries, raspberries, walnuts, and oak-aged wine in humans: Identification of biomarkers and individual variability, *J. Agric. Food Chem.* 2005, *53*, 227 – 235.

[15] Whitley, A. C., Sweet, D. H., Walle, T., Site-specific accumulation of the cancer preventive dietary polyphenol ellagic acid in epithelial cells of the aerodigestive tract, *J. Pharm. Pharmacol.* 2006, *9*, 1201 – 1209.

[16] Seeram, N. P., Aronson, W. J., Zhang, Y., Henning, S. M., *et al*., Pomegranate ellagitannin-derived metabolites inhibit prostate cancer growth and localize to the mouse prostate gland, *J. Agric. Food Chem.* 2007, *55*, 7732 – 7737.

[17] Seeram, N. P., Adams, L. S., Henning, S. M., Niu, Y., *et al*., In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice, *J. Nutr. Biochem.* 2005, *16*, 360 – 367.

[18] Larrosa, M., Tomás-Barberán, F. A., Espín, J. C., The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway, *J. Nutr. Biochem.* 2006, *17*, 611 – 625.

[19] Päivärinta, E., Pajari, A. M., Törrönen, R., Mutanen, M., Ellagic acid and natural sources of ellagitannins as possible chemopreventive agents against intestinal tumorigenesis in the Min mouse, *Nutr. Cancer* 2006, *1*, 79 – 83.

[20] Castonguay, A., Gali, H. U., Perchellet, E. M., Gao, X. M., *et al*., Antitumorigenic antipromoting activities of ellagic acid, ellagitannins and oligomeric anthocyanin and procyanidin, *Int. J. Oncol.* 1997, *10*, 367 – 373.

[21] Bermúdez-Soto, M. J., Larrosa, M., Garcia-Cantalejo, J. M., Espín, J. C., *et al*., Up-regulation of tumour suppressor carcinoembryonic antigen-related cell adhesion molecule 1 in human colon cancer Caco-2 cells following repetitive exposure to dietary levels of a polyphenol-rich chokeberry juice, *J. Nutr. Biochem.* 2007, *4*, 259 – 271.

[22] Jakubikova, J., Bao, Y., Sedlak, J., Isothiocyanates induce cell cycle arrest, apoptosis and mitochondrial potential depolarization in HL-60 and multidrug-resistant cell lines, *Anticancer Res.* 2005, *25*, 3375 – 3386.

[23] Irizarry, R. A., Hobbs, B., Collin, F., Beazer-Barclay, Y. D., *et al*., Exploration, normalization, and summaries of high density oligonucleotide array probe level data, *Biostatics* 2003, *4*, 249 – 264.

[24] Montaner, D., Tárraga, J., Huerta-Cepas, J., Burguet, J., *et al*., Next station in microarrays data analysis: GEPAS, *Nucleic Acids Res.* 2006, *34*, W486 – W491.

[25] Benjamini, Y., Hochberg, Y., Controlling the false discovery rate: A practical and powerful approach to multiple testing, *J. R. Stat. Soc.* 1995, *57*, 289 – 300.

[26] Al-Shahrour, F., Minguez, P., Vaquerizas, J. M., Conde, L., Dopazo, J., Babelomics: A suite of web tools for functional annotation and analysis of groups of genes in high-throughput experiments, *Nucleic Acids Res.* 2005, *33*, W460 – W464.

[27] Cardoso, J., Boer, J., Morreau, H., Fodde, R., Expression and genomic profiling of colorectal cancer, *Biochim. Biophys. Acta* 2007, *1775*, 103 – 137.

[28] Germann, A., Dihlmann, S., Gergenhahn, M., von Knebel-Doeberitz, M., Koesters, R., Expression profiling of CC531 colon carcinoma cells reveals similar regulation of β-catenin target genes by both butyrate and aspirin, *Int. J. Cancer* 2003, *106*, 187 – 197.

[29] Rudolf, E., Andelová, H., Cervinka, M., Polyphenolic compounds in chemoprevention of colon cancer – targets and signalling pathways, *Anticancer Agents Med. Chem.* 2007, *7*, 559 – 75.

[30] Ramos, S., Cancer chemoprevention and chemotherapy: Dietary polyphenols and signalling pathways, *Mol. Nutr. Food Res.* 2008, *52*, 507 – 526.

[31] Kahle, K., Kraus, M., Scheppach, W., Ackermann, M., *et al*., Studies on apple and blueberry fruit constituents: Do the polyphenols reach the colon after ingestion? *Mol. Nutr. Food Res.* 2006, *50*, 418 – 423.

[32] Viallard, J. F., Lacombe, F., Belloc, F., Pellegrin, J. L., Reiffers, J., Mécanismes moléculaires contrôlant le cycle cellulaire: Aspects fondamentaux et implications en cancérologie, *Cancer Radiother.* 2001, *5*, 109 – 129.

[33] Choi, Y. H., Lee, W. H., Park, K. Y., Zhang, L., p53-independent induction of p21 (WAF1/CIP1), reduction of cyclin B1 and G_2/M arrest by the isoflavone genistein in human prostate carcinoma cells, *Jpn. J. Cancer Res.* 2000, *2*, 164 – 173.

[34] Liu, M. J., Wang, A., Ju, Y., Wong, R. N., Wu, Q. Y., Diosgenin induces cell cycle arrest and apoptosis in human leukaemia k562 cells with the disruption of Ca^{2+} homeostasis, *Cancer Chemother. Pharmacol.* 2005, *1*, 79 – 90.

[35] Liu, X., He, H., Feng, Y., Zhang, M., *et al*., Difference of cell cycle arrest induced by lidamycin in human breast cancer cells, *Anticancer Drugs* 2006, *2*, 173 – 179.

[36] Toby, G. G., Gherraby, W., Coleman, T. R., Golemis, E. A., A novel RING finger protein, human enhancer of invasion 10, alters mitotic progression through regulation of cyclin B levels, *Mol. Cell. Biol.* 2003, *23*, 2109 – 2122.

[37] Ma, Y., Cress, W. D., Transcriptional upregulation of p57(Kip2) by the cyclin dependent kinase inhibitor BMS-387032 is E2F dependent and serves as a negative feedback loop limiting cytotoxicity, *Oncogene* 2007, *26*, 3532 – 3540.

[38] Yuan, Z., Gaba, A. G., Kent, T. S., Bennett, A., *et al*., Modulation of CDK2-AP1 (p12(DOC-1)) expression in human colorectal cancer, *Oncogene* 2005, *22*, 3657 – 3668.

[39] Saigusa, K., Imoto, I., Tanikawa, C., Aoyagi, M., *et al*., RGC32, a novel p53-inducible gene, is located on centrosomes during mitosis and results in G_2/M arrest, *Oncogene* 2007, *8*, 1110 – 1121.

[40] Cowan, K. J., Storey, K. B., Mitogen-activated protein kinases: New signalling pathways functioning in cellular responses to environmental stress, *J. Exp. Biol.* 2003, *206*, 1107 – 1115.

[41] Shaul, Y. D., Seger, R., The MEK/ERK cascade: From signalling specificity to diverse functions, *Biochim. Biophys. Acta* 2007, *1773*, 1213 – 1226.

[42] Camps, M., Nichols, A., Gillieron, C., Antonsson, B., *et al*., Catalytic activation of the phosphatase MKP-3 by ERK2 mitogen-activated protein kinase, *Science* 1998, *282*, 1262 – 1265.

[43] McCubrey, J. A., Steelman, L. S., Chappell, W. H., Abrams, S. L., *et al*., Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation-n and drug resistance, *Biochim. Biophys. Acta* 2007, *1773*, 1263 – 1284.

[44] Li, Z., Li, J., Mo, B., Hu, C. *et al*., Genistein induces G_2/M cell cycle arrest via stable activation of ERK1/2 pathway in MDA-MB-231 breast cancer cells, *Cell Biol. Toxicol.* 2008, *24*, 401 – 409.

[45] Lee, S.-J., Cho, Y.-H., Park, K., Kim, E.-J., *et al*., Magnolol elicits activation of the extracellular signal-regulated kinase pathway by inducing p27KIP1-mediated G2/M-phase cell cycle arrest in human urinary bladder cancer 5637 cells, *Biochem. Pharmacol.* 2008, *75*, 2289 – 2300.

[46] Lee, Y.-M., Ting, C.-M., Cheng, Y.-K., Fan, T.-P., *et al*., Mechanisms of 2-methoxyestradiol-induced apoptosis and G2/M cell-cyle arrest of nasopharyngeal carcinoma cells, *Cancer Lett.* 2008, *268*, 295 – 307.

[47] Kim, D. S., Lee, H. K., Park, S. H., Lee, S., *et al*., Terrein inhibits keratinocyte proliferation via ERK inactivation and G2/M cell cycle arrest, *Exp. Dermatol.* 2008, *17*, 312 – 317.

[48] Foijer, F., Simonis, M., van Vliet, M., Wessels, L., *et al*., Oncogenic pathways impinging on the G2-restriction point, *Oncogene* 2008, *27*, 1142 – 1154.

[49] Jakubíkova, J., Sedlák, J., Mithen, R., Bao, Y., Role of PI3K/AKT and MEK/ERK signalling pathways in sulphorophane- and erucin-induced phase II enzymes and MRP2 transcription, G_2/M arrest and cell death in Caco-2 cells, *Biochem. Pharmacol.* 2005, *69*, 1543 – 1552.

[50] Bacus, S. S., Gudkov, A. V., Lowe, M., Lyass, L., *et al*., Taxol-induced apoptosis depends on MAP kinase pathways (ERK and p38) and is independent of p53, *Oncogene* 2001, *20*, 147 – 155.

[51] Roberts, P. J., Der, C. J., Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer, *Oncogene* 2007, *26*, 3291 – 3310.

[52] Falsaperla, M., Morgia, G., Tartarone, A., Ardito, R., Romano, G., Support ellagic acid therapy in patients with hormone refractory prostate cancer (HRPC) on standard chemotherapy using vinorelbine and estramustine phosphate, *Eur. Urol.* 2005, *4*, 449 – 454.

