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The Pro-Apoptotic Effect of Quercetin in Cancer Cell Lines Requires ERβ-Dependent Signals

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Quercetin has potentially beneficial effects on disease prevention, including cancer. An intriguing issue regarding the mechanisms of action of quercetin is the ability of this drug to modulate estrogen receptor (ER) activities. In a previous study, we demonstrated that quercetin elicited apoptosis through an ERα-dependent mechanism. However, the contribution of ERβ in quercetin-induced apoptosis remains elusive. Here, we report that quercetin, at nutritionally relevant concentrations, mimicked the 17β-estradiol (E2)-induced apoptotic effect in both ERβ1-transfected HeLa and in ERβ1-containing DLD-1 colon cancer cell lines by inducing the activation of p38. p38 activation is responsible for pro-apoptotic activation of caspase-3 and the cleavage of poly(ADP-ribose) polymerase. Notably, no inactivation or downregulation of the survival kinases (i.e., AKT and ERK1/2) or the antiapoptotic protein Bcl-2 was observed after quercetin stimulation. On the contrary, quercetin acted similarly to E2 by increasing the levels of the oncosuppressor protein PTEN and by impeding ERβ-dependent cyclin D1 promoter activity, which subsequently resulted in the transcription of the estrogen-responsive element remaining unchanged. As a whole, these data indicate that quercetin mimics the E2 effects in the presence of ERβ1, thus maintaining its anti-carcinogenic potential. In addition, the quercetin pro-apoptotic action in the presence of ERα may render it as a dual-sided protective agent against E2-related cancer in the reduction of tumour growth in organs that express ERα and/or ERβ.

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Cancers of the breast (Bai and Gust, 2009), prostate (Fitzpatrick et al., 2009), and colon (Janakiram et al., 2009) are well known to involve female steroid hormone signalling. In particular, there are major sex differences in colorectal cancer; it is more common in men than in women, where the difference is more striking between pre-menopausal women and age-matched men (Wong et al., 2005). These differences suggest that the sex steroid hormone, 17β-estradiol (E2), could elicit protective effects against this disease. Estrogen receptors (ERα and ERβ) are differentially expressed in the colon. ERα is minimally expressed in normal colon mucosa and colon cancer cells (Campbell-Thompson et al., 2001), whereas ERβ is the predominant subtype expressed in the human colon (Foley et al., 2000; Campbell-Thompson et al., 2001; Konstantinopoulos et al., 2003). Thus, the E2 protective effect on colon cancer should be mediated by specific signal transduction pathways activated by ERβ (Acconcia et al., 2005; Galluzzo et al., 2007). ERβ is significantly decreased in colonic tumours compared with normal mucosa (Konstantinopoulos et al., 2003). The loss of ERβ leads to hyperproliferation, loss of differentiation, and decreased apoptosis in the epithelium of the colon, suggesting a pivotal role for ERβ in the organisation and architectural maintenance of the colon and its potential role in the regulation of colon tumour growth (Foley et al., 2000; Campbell-Thompson et al., 2001; Wada-Hiraike et al., 2006; Warner and Gustafsson, 2010). These data establish the rationale for the potential use of ERβ-specific agonists as a new type of pharmacotherapy against colon cancer (Warner and Gustafsson, 2010; Nilsson and Gustafsson, 2011).

Flavonoids are a large diverse group of phytochemicals with a concentration in the gut (especially in colorectal mucosa) that could be higher than in tissues (e.g., liver, brain, veins) depending on flavonoid bioavailability (Halliwell et al., 2000). Research into the anti-carcinogenic potential of flavonoids with animal and cellular model systems supports a protective role against the progression of colorectal cancer (Williams et al., 2004; Daskiewicz et al., 2005; Surh et al., 2005; Kyle et al., 2010). Besides other flavonoids, quercetin [IUPAC name: 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one], present in apples, onions, and other vegetables, has been reported to protect against important diseases, including lung and colon cancers (De Stefani et al., 1999; van Erk et al., 2005; Dihal et al., 2006). In contrast, a dualistic influence of quercetin on cell proliferation has also been found (van der Woude et al., 2003, 2005). The contrasting data from these studies could be a result of the high quercetin concentrations used in (i.e., ≥25 μM), which could trigger different signal transduction pathways in the target cells (e.g., antioxidative, anti-mutagenic, receptor-mediated signals; Murakami et al., 2008). However, we recently demonstrated that besides antioxidant properties, quercetin, at nutritionally relevant concentrations, induces cancer cell death through an ERα-dependent mechanism, which involves p38 kinase and caspase-3 activation (Galluzzo et al., 2009). Currently, the efficacy and the mechanisms of nutritionally relevant concentrations of the aglycone, quercetin, in activating apoptosis in ERβ1-positive cancer cells are still unknown. Recently, it has been demonstrated that in combination with other flavonoids (final concentration of mixture = 25 μM) quercetin-modulated ERβ activities and reduced prostate cancer cell proliferation (Kumar et al., 2010). In the colon cancer cells, Caco-2, which contain low levels of ERβ, 5 or 50 μM of quercetin downregulated cell cycle gene

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expression and cell proliferation resulting in cell cycle arrest (van Erk et al., 2005). However, upon ingestion, the amount of quercetin in the human plasma is in the high nanomolar (0.1–0.2 μM) or low micromolar range (0.5–1.5 μM; Boots et al., 2008) due to its rapid metabolism (Manach et al., 2005; Murota and Terao, 2005). As a consequence, the reported anti-proliferative effect of quercetin could depend on its metabolites (Shirai et al., 2006). The first description of a direct effect of nutritionally relevant quercetin concentrations (i.e., from 0.1 nM to 1 μM) in the presence of ERβ has been reported on human colonic smooth muscle contraction (Hogan et al., 2010), but no correlation with cell proliferation has been observed.

In this study, we evaluated the effects of quercetin on growth and apoptosis in a colon cancer cell line, DLD-1, which only expresses the ERβ1 subtype (Galluzzo et al., 2007). In addition, HeLa cells that are devoid of ERs, but were transiently transfected with an empty vector or the ERβ1 expression vector, were used to discriminate between ERβ-dependent and ERβ-independent effects of quercetin. ERβ-dependent extra-nuclear (i.e., membrane-initiated and cytosolic) and nuclear signalling pathways involved in quercetin effects have also been investigated.

Materials and Methods

Reagents

17β-estradiol, quercetin, L-glutamine, gentamicin, trypsin, penicillin, DMEM (with and without phenol red), charcoal-stripped fetal calf serum, and GenElute plasmid maxiprep kit were purchased from Sigma–Aldrich (St. Louis, MO). The p38 inhibitor, SB 203580, was purchased from Calbiochem (San Diego, CA), and the ER inhibitor, fulvestrant, (ICI 182,780, ICI) was obtained from Tocris (Ballwin, MO). Lipofectamine reagent was obtained from GIBCO-BRL Life-technology (Gaithersburg, MD). A luciferase kit was obtained from Promega (Madison, WI). A Bradford Protein Assay was obtained from BIO-RAD Laboratories (Hercules, CA). Polyclonal anti-phospho-AKT, anti-p38, and anti-phospho-p38 antibodies were obtained from Cell Signaling Technology (Danvers, MA); polyclonal anti-ERK and monoclonal anti-phospho-ERK, anti-AKT, anti-caspase-3, anti-poly(ADP-ribose) polymerase (PARP), anti-Bcl-2, anti-phosphatase and tensin homolog deleted on chromosome 10 (-PTEN), anti-α-actin, and all secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). ECL chemiluminescence reagent for Western blotting was obtained from Amersham Bioscience (Uppsala, Sweden).

All other products were obtained from Sigma–Aldrich. Analytical or reagent grade products, without further purification, were used.

Plasmids, transfection, and luciferase assay

Gene reporter plasmids pC3-luciferase (pC3) and pXP2-D1-2966-luciferase (pD1), expression vectors for pCR3.1-β-galactosidase, human pCNX2-ERβ1, and an empty vector, pCMV5, were used. Plasmids were purified for transfection using a plasmid preparation kit according to the manufacturer's instructions. A luciferase dose–response curve showed that the maximum luciferase activity was attained when 1 μg of plasmid was transfected together with 1 μg of pCR3.1-β-galactosidase to normalise for transfection efficiency (~55–65%). HeLa cells were grown to ~70% confluence and then transfected with different expression vectors using Lipofectamine Reagent according to the manufacturer's instructions. Six hours after transfection, the medium was changed, and 24 h thereafter cells were stimulated with E2 or quercetin for 6 h. The cell lysis procedure, as well as the subsequent measurement of luciferase gene expression, was performed using a luciferase kit according to the manufacturer's instructions with an EC & G Berthold luminometer (Bad Wildbad, Germany).

Cell culture and cell cycle analysis

HeLa and DLD-1 cells were routinely grown in air containing 5% CO_2 in modified, phenol red-free, DMEM and RPMI medium, respectively, containing 10% (v/v) charcoal-stripped fetal calf serum, L-glutamine (2 mmol/L), gentamicin (10 mg/ml), and penicillin (100 U/ml). Cells were passaged every 2 days using 0.5 ml trypsin (0.05% v/v), and the media was exchanged every 2 days. Thirty hours after treatment, cells were harvested with trypsin, centrifuged, stained with trypan blue solution, and counted in a haemocytometer (improved Neubauer chamber) in quadruplicate. HeLa cells were transfected with human pCNX2-ERβ1 or pCMV5 expression vectors and 24 h later were stimulated with different concentrations of E2 or quercetin. After stimulation, cells were fixed with 1 ml of ice-cold 70% ethanol and subsequently stained with 2 mg/ml DAPI/PBS solution. The fluorescence of the DNA was measured with a DAKO Galaxyflow cytometer equipped with an HBO mercury lamp, and the percentage of cells present in sub-G1 phase was calculated using FloMax© Software.

Electrophoresis and immunoblotting

Sub-confluent cells (~80%) were stimulated with either vehicle (DMSO:PBS, 1:1, v:v), E2 (10^{-8} M in DMSO:PBS, 1:1, v:v) or quercetin (10^{-6} M in DMSO:PBS, 1:1, v:v). When indicated, 10^{-6} M of ER inhibitor, ICI 182,780, dissolved in vehicle, or 5×10^{-6} M of p38 inhibitor, SB 203580, dissolved in vehicle, were added 30 or 15 min before treatment with quercetin. HeLa cells (transfected with pCNX2-ERβ1 or pCMV5 expression vectors) and DLD-1 cells were lysed and solubilised in 0.125 M Tris–HCl (pH 6.8) containing 10% SDS (w/v), 1 mM phenylmethylsulfonyl fluoride and 5 μg/ml leupeptin and boiled for 2 min. Proteins were quantified using the Bradford Protein Assay. A total of 20 μg of solubilised proteins were resolved using SDS–PAGE (from 7% to 10%) at 100 V for 1 h. The proteins were then electrophoretically transferred to nitrocellulose overnight at 30 V at 4 °C. The nitrocellulose was treated with 3% BSA (v/v) in 138 mM NaCl, 26.8 mM KCl, 25 mM Tris–HCl (pH 8.0), 0.05% Tween-20 (v/v), 0.1% BSA (w/v), and then probed at 4 °C overnight with one of the following antibodies: anti-phospho-ERK, anti-phospho-AKT, anti-phospho-p38, anti-caspase-3, anti-Bcl-2, anti-PTEN, or anti-PARP antibodies (1 μg/ml). The nitrocellulose was treated with specific secondary antibodies at room temperature for 1 h. The nitrocellulose was stripped by Restore Western Blot Stripping Buffer (Pierce Chemical Company, Rockford, IL) for 10 min at room temperature and then probed with either anti-ERK, anti-AKT, or anti-p38 (1 μg/ml) antibodies. Probing the membrane with anti-α-actin antibody (1 μg/ml) was used to normalise the sample loading. Antibody reaction was visualised with the chemiluminescence reagent for Western blotting. Densitometric analyses were performed using ImageJ software for Windows.

Statistical analysis

A statistical analysis was performed with Student's *t*-test with the GraphPad INSTAT3 software system for Windows. In all cases, probability (*P*) values below 0.05 were considered significant.

Results

Nutritionally relevant quercetin concentrations affect cancer cell growth in presence of ERβ

High concentrations of quercetin (10^{-4} and 10^{-3} M) decreased the number of ERβ1-devoid (ERβ−) HeLa cells (Fig. 1A). On the contrary, in ERβ1-transfected (ERβ+) HeLa cells, the quercetin effect was dose-dependent because nutritionally relevant quercetin concentrations significantly decreased the cell number by about 25% up to 10^{-7} M and by about 30% at 10^{-6} M (Fig. 1B). As observed with quercetin, high E2 concentration (10^{-3} M) reduced cell number in both ERβ− and in ERβ+ HeLa cells (Fig. 1A,B), and as expected (Galluzzo et al.,

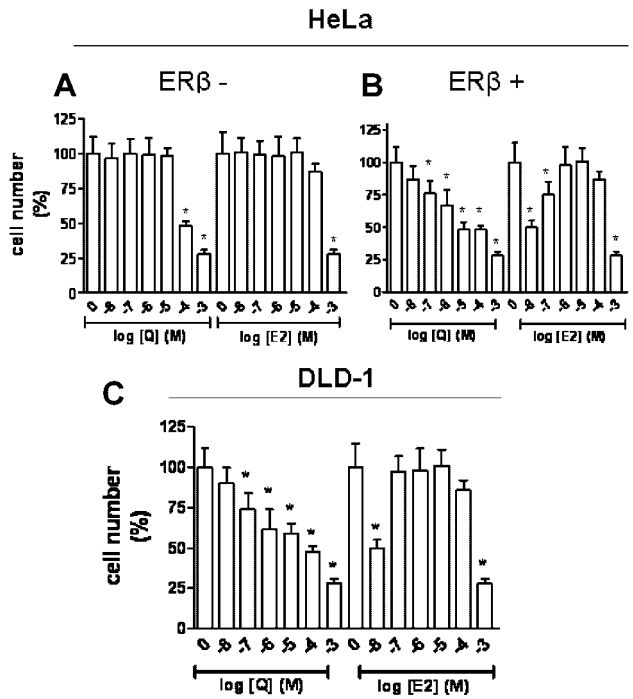

Fig. 1. Quercetin effect on cancer cell growth. HeLa cells transfected with empty vector (ERβ−) (panel A) or with ERβ expression vector (ERβ+) (panel B) and DLD-1 cells (panel C) were grown for 30 h in the presence of different concentrations of quercetin (Q) or 17β-estradiol (E2). Cells were harvested and counted as described in the Materials and Methods section. The data represent the mean ± SD of six independent experiments carried out in duplicate. *$P < 0.001$, calculated with the Student's *t*-test, compared with non-stimulated control values (0).

2007), E2 decreased ERβ-transfected (ERβ+) HeLa cell number also at 10^{-8} M. Notably, similar results were obtained in DLD-1 colon cancer cells (Fig. 1C) which express only ERβ1 (Galluzzo et al., 2007; Caiazza et al., 2007). Thus, in the following experiments, concentrations of 10^{-8} M for E2 and 10^{-6} M for quercetin were used. These concentrations correspond to saturating conditions reported both for quercetin and for E2 binding to ERβ (Kuiper et al., 1997; Bolli et al., 2010).

Flow cytometry analyses of ERβ− and ERβ+ HeLa cells showed (Fig. 2) that 30 h of quercetin treatment (10^{-6} M), like E2 (10^{-8} M), increased the number of cells in the sub-G1 region of the cell cycle only in the presence of ERβ, thus strongly suggesting the presence of DNA fragmentation.

Quercetin:ERβ complex activates a pro-apoptotic cascade

To link the effect of quercetin reported in Figure 2 to the induction of an apoptotic cascade, the presence of the active subunit of caspase-3 (i.e., the caspase-3 17-kDa band) and the cleavage of caspase-3 substrate (i.e., PARP) were analysed. The caspase-3 17-kDa band was detected only in ERβ+ HeLa and in DLD-1 cells treated for 24 h with either E2 or quercetin (Fig. 3A,B). To confirm that the quercetin-induced appearance of the caspase-3 17-kDa band was associated with an increase in caspase-3 activity, the cleavage of the caspase-3 substrate, the DNA repair enzyme PARP was analysed. Quercetin treatment, like E2, induced the cleavage of 116-kDa PARP into its inactive 85-kDa fragment (Fig. 3A,B) in both ERβ+ HeLa and DLD-1 cells, whereas no effects were observed in ERβ− HeLa cells. In addition, the cell pre-treatment with the pure ER inhibitor, ICI 182,780, completely prevented the quercetin-induced caspase-3 activation and PARP cleavage (Fig. 3A,B), strongly suggesting the ERβ-dependence of this quercetin effect. As previously reported, the E2-induced caspase-3-dependent apoptotic cascade in DLD-1 cells requires the activation of the p38 kinase (Galluzzo et al., 2007; Caiazza et al., 2007). To evaluate the possible involvement of p38 in quercetin-induced apoptosis, ERβ+ HeLa cells were pre-treated with the specific p38 inhibitor, SB 203580, which completely prevented both E2- and quercetin-induced caspase-3 activation as well as PARP cleavage (Fig. 3C,D).

Action mechanism of quercetin:ERβ complex

These results prompted us to verify the quercetin ability to directly activate p38 kinase in ERβ-expressing cells. Quercetin, as well as E2, induced p38 phosphorylation after 15 min of stimulation in ERβ+ HeLa (Fig. 4A) and DLD-1 cells (Fig. 4B). This effect required ERβ because it is absent in ERβ− HeLa cells and it was prevented by DLD-1 cell treatment with ICI 182,780 (Fig. 4A–C).

We next determined the putative impact of quercetin on two other signal transduction effector kinases that are important for cell survival: the serine/threonine protein kinase AKT and the extracellular regulated kinase (ERK). Quercetin did not induce AKT or ERK phosphorylation in both ERβ+ HeLa and DLD-1 cell lines at any of the stimulation times tested (data not shown and Fig. 4D,E). Notably, 1 μM quercetin did not decrease the basal, constitutive, phosphorylation status of both kinases or the expression level of total ERK and AKT (phosphorylated and non-phosphorylated) in either in ERβ+ (Fig. 4D,E) or in ERβ− HeLa cells (data not shown). Finally, AKT phosphorylation is negatively regulated by the phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a phosphatase that dephosphorylates the 3′-position to reverse the reactions catalysed by phosphatidylinositol 3-kinase (PI3K; Marino et al., 2003). As shown in Figure 4D–F, 24 h of stimulation with quercetin or E2 induced the accumulation of protein PTEN in the cell lines. Parallel to the inability to activate AKT and increase the level of PTEN, no accumulation of the anti-apoptotic protein Bcl-2 occurred when ERβ+ HeLa and DLD-1 cells were treated for 24 h with quercetin (Fig. 4D,E).

ERβ, like other members of the nuclear receptor superfamily, is a ligand-activated transcription factor. Gene transcription can be activated by E2 via direct binding of the receptor to the estrogen-responsive element (ERE) present in the promoter of responsive genes or indirectly via receptor association with other transcription factors (Ascenzi et al., 2006; Paech et al., 1997; Saville et al., 2000). Thus, the effect of quercetin on the activation of an ERE containing complement 3 (pC3) as well as that of an ERE-devoid gene promoter (cyclin D1, pD1) were evaluated. Quercetin increased the activity of the ERE containing the pC3 promoter up to the activity levels seen in E2-treated ERβ+ HeLa cells (Fig. 5), whereas no promoter activity was detected when these cells were co-transfected with the ERE-devoid of the pD1 promoter (Fig. 5).

Discussion

Quercetin treatment causes cell cycle arrest and apoptosis in different cell types including prostate, glioblastoma, breast, and colon cancer cell lines (Kuo, 1996; Kuo et al., 2004; Lee et al., 2006; Vijayababu et al., 2006; Kim et al., 2008; Mense et al., 2008; Murakami et al., 2008; Galluzzo et al., 2009; Kumar et al., 2010). Several mechanisms have been described for these quercetin effects, including DNA strand breakage, inhibition of enzymes and kinases associated with survival signal transduction pathways (e.g., PI3 kinase, protein kinase C, ERK), and anti- or

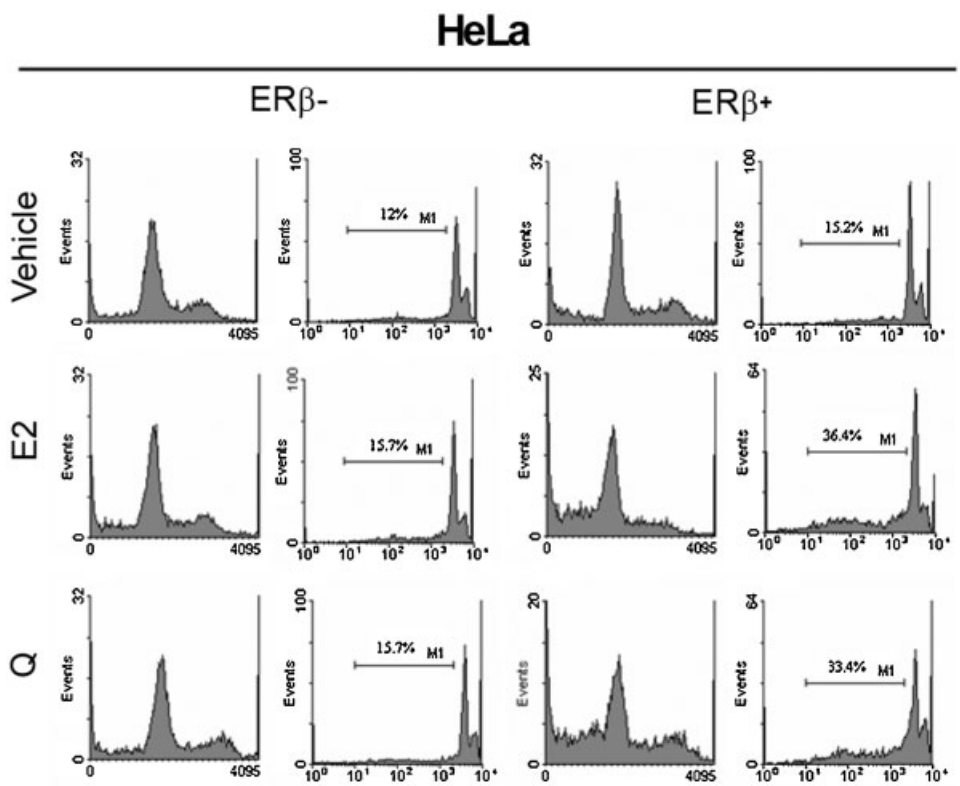

Fig. 2. Quercetin effect on HeLa cell distribution in the cell cycle. HeLa cells transfected with empty vector (ERβ−), or with ERβ expression vector (ERβ+) were grown for 30 h in the presence of quercetin (Q, 10^{-6} M), 17β-estradiol (E2, 10^{-8} M), or vehicle (DMSO:PBS, 1:1, v:v). The cell cycle distribution was evaluated by cytofluorimetric analysis. The numbers reported in left panes indicate the percentage of cells present in sub-G1 phase of the cell cycle. Graphs are examples of four indpendent experiments carried out in duplicate.

pro-oxidant activity (Matter et al., 1992; Kim et al., 2004; Vijayababu et al., 2006; Stevenson and Hurst, 2007). Unfortunately, due to the high quercetin concentrations used in these studies (i.e., either $\geq 25\ \mu M$ or $\geq 100\ \mu M$), the useful chemo-preventive effect of quercetin on cancer cells is uncertain. Although it is possible that quercetin metabolites release the bioactive aglycone via β-glucuronidase, which has high activity in the tumour micro-environment (Yue et al., 2002; O'Leary et al., 2003), quercetin aglycone is present in plasma at very low concentrations (Manach et al., 2005). However, in a population-based case-control study, an inverse association between colorectal cancer risk and non-tea flavonol (principally quercetin) intake has been recently reported strongly pointing to quercetin as a potential candidate for both cancer prevention and treatment (Kyle et al., 2010). These results prompt further investigation to ascertain the contribution of the mechanism(s) of action of quercetin in cancer cells.

We previously demonstrated that in addition to its strong antioxidant properties, quercetin induced cancer cell death through an ERα-dependent mechanism involving p38 kinase and caspase-3 activation (Galluzzo et al., 2009). The present study was undertaken to examine the involvement of the other ER isotype (i.e., ERβ1) in the well-known growth inhibition and cell death effects of quercetin at nutritional relevant concentrations.

At least five splice variant isoforms of the ERβ gene product (ERβ1-ERβ5) have been described (Moore et al., 1998; Wong et al., 2005): the 530-amino acid human ERβ isoform is currently regarded as the wild-type ERβ; ERβ2 (also called ERβcx; Ogawa et al., 1998) is identical to the ERβ long form, except that 26 unique amino acid residues replace the C-terminal of ligand binding domain (LBD). Additionally, two truncated isoforms have been identified and named ERβ4 and ERβ5. Several additional ERβ isoforms have been reported, although the full-length sequences have not been determined (Ascenzi et al., 2006; Heldring et al., 2007). ERβ1, ERβ2, and ERβ5 have been demonstrated in normal colorectal mucosa to be expressed at higher levels than ERα (Foley et al., 2000; Campbell-Thompson et al., 2001). ERβ1 is essential for ERβ-induced transcription initiation of the ERE, whereas the other ERβ isoforms have no innate transcriptional activity, but they play an enhancing role when dimerised with ERβ1. ERβ1 is the only fully functional ERβ, which preferentially hetero-dimerises with other ERβ isoforms, particularly ERβ4 and ERβ5, forming "variable dimer partners" under the stimulation of estrogens (Leung et al., 2006). Furthermore, ERβ2, which is unable to bind ligands or coactivators and has no transcriptional activity in reporter assays, shows preferential hetero-dimerisation with ERα rather than with ERβ. ERβ2 inhibits ERα DNA binding and has a dominant-negative effect on ligand-dependent ERβ reporter gene activity (Ogawa et al., 1998). These data suggest that the ERβ isoforms could differentially modulate E2 action (Ascenzi et al., 2006) and make elucidating the physiological role of this receptor more difficult. To examine the quercetin effects in the presence of ERβ, we have selected two experimental models. The first model is the colon cancer cell line, DLD-1 cells, which expresses only the ERβ1 isoform (Caiazza et al., 2007; Galluzzo et al., 2007), and the second model is the

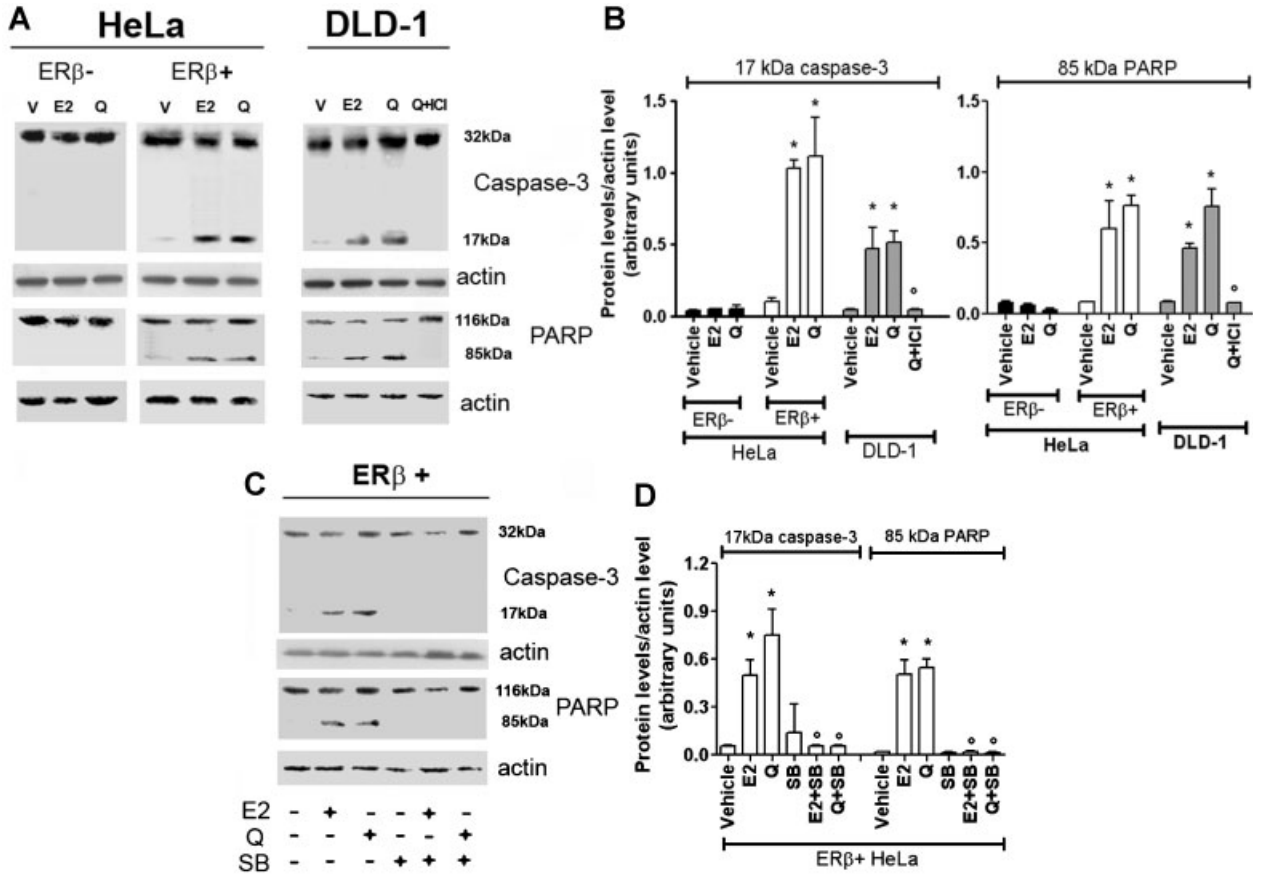

Fig. 3. Quercetin effect on the activation of the pro-apoptotic cascade. A representative example of three independent Western blot analyses of caspase-3 activation and PARP cleavage after 24 h of vehicle (V, DMSO:PBS, 1:1, v:v), 17β-estradiol (E2, 10^{-8} M), and quercetin (Q, 10^{-6} M) stimulation in HeLa cells transfected with empty vector (ERβ−) or with ERβ expression vector (ERβ+) and DLD-1 cells is shown in panel A. In panel B, the densitometric analyses are reported. Data are mean ± SD of three different experiments. $P < 0.001$ was calculated with the Student's *t*-test compared with (*) vehicle- or (°) Q-treated samples. The p38 inhibitor SB 203580 (SB, 5×10^{-6} M) or anti-estrogen ICI 182,780 (ICI, 10^{-6} M) were added 15 min before E2 or quercetin in ERβ+ HeLa and DLD-1 cells (panel C). In panel D, the densitometric analyses are reported. Data are mean ± SD of three different experiments. $P < 0.001$ was calculated with the Student's *t*-test compared with (*) vehicle- or (°) E2- and Q treated samples.

ERβ-devoid HeLa cells that can be rendered E2-responsive after the transient transfection of the ERβ1 expression vector.

Quercetin, even at 0.1 or 1 μM concentrations, decreased the cell number triggering a pro-apoptotic cascade that culminates in caspase-3 activation and protein PARP cleavage, a caspase-3 substrate. These effects, obtained at low quercetin concentrations, were comparable to those obtained at physiological E2 concentrations (i.e., 10 nM), which requires the presence ERβ. In contrast, quercetin at a higher concentration than one of nutritional relevance (i.e., 100 μM) triggered ERβ-independent effects. Notably, 1 mM quercetin, like E2, reduced cell proliferation both in ERβ- and ERβ+ cell lines, suggesting that a cytotoxic effect occurred.

One of the main aims of this article was to understand the signalling events leading to the potential chemotherapeutic activity of quercetin in cancer cell lines. Like 10 nM E2, 1 h of 1 μM quercetin induced p38 phosphorylation only in DLD-1 and in ERβ+ HeLa cells. Moreover, pre-treatment with the pure anti-estrogen, ICI 182,780, completely prevented quercetin effects in the DLD-1 colon cancer cell line. Intriguingly, at the same concentration (1 μM), quercetin did not affect either the phosphorylation status or the levels of the survival kinase AKT, while it increased PTEN levels, as seen in E2 treatment. It is well known that the tumour suppressor PTEN dephosphorylates PI3K, preventing AKT recruitment to the plasma membrane for activation through phosphorylation. In several cell lines, p38 is one of the main kinases involved in PTEN upregulation via activating transcription factor-2 (ATF-2), which binds to ATF sites in the PTEN promoter (Shen et al., 2006). In addition, Bcl-2 transcription is downregulated by PTEN in the prostate cancer cell line (Huang et al., 2001). Similarly in DLD-1 cells, the rapid (15 min) quercetin:ERβ-induced p38 activation, like E2, seems to control PTEN levels which in turn could impede AKT activation and Bcl-2 accumulation, thus driving cells into apoptosis (Chiacchiera and Simone, 2008; Wagner and Nebreda, 2009; Kim and Choi, 2010). The parallel absence of ERK phosphorylation upon quercetin stimulation further guarantees that cell survival pathways are not operative in these cells. Finally, the quercetin inability (like E2) to modify AKT and ERK phosphorylation status impedes the indirect transcription from the cyclin D1 promoter, which is strongly dependent on these kinases for activity and is important for cell cycle progression (Marino et al., 2002, 2003).

However, the activity of the ERE-containing promoter pC3 is enhanced by 1 μM quercetin stimulation, independent of ERβ,

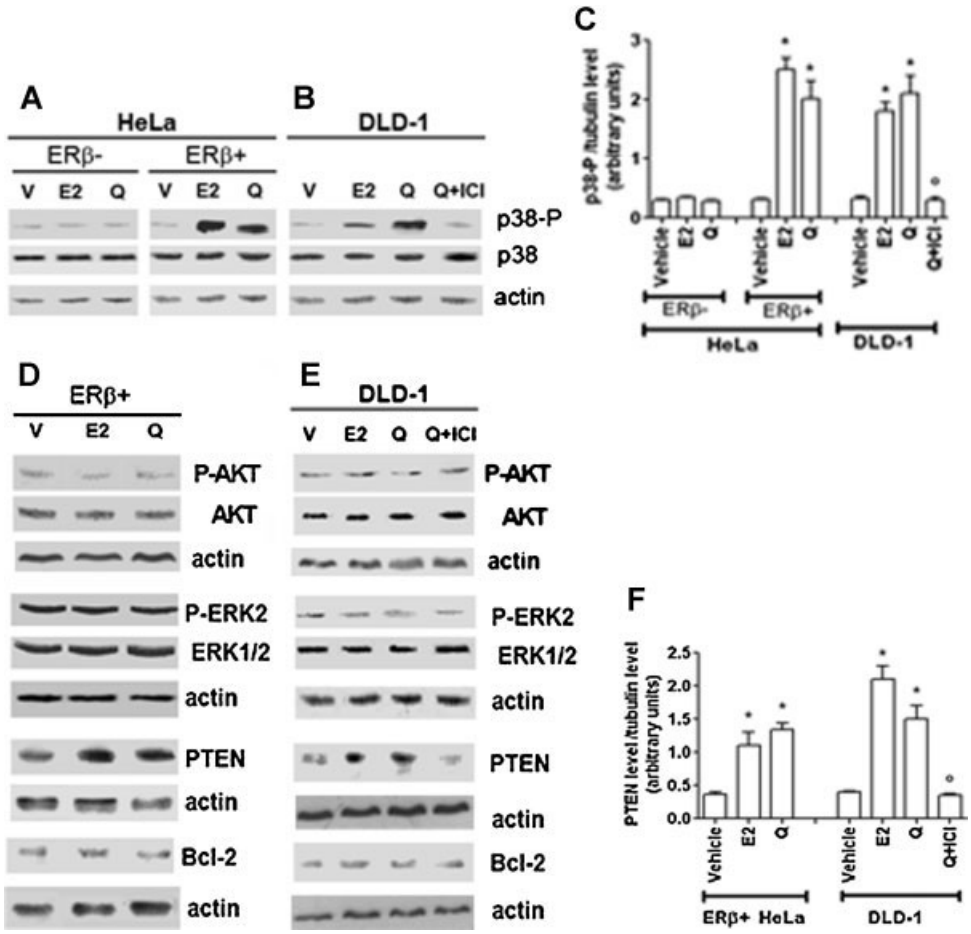

Fig. 4. Quercetin effect on kinase activation, PTEN and Bcl-2 protein level. A representative example of three independent Western blot analyses of phosphorylated and total p38 levels after 15 min of vehicle (V, DMSO:PBS 1:1, v:v), 17β-estradiol (E2, 10^{-8} M), and quercetin (Q, 10^{-6} M) stimulation in HeLa cells transfected with empty vector (ERβ−) or with ERβ expression vector (ERβ+) and DLD-1 cells is shown in panels A and B respectively. In panel C, the densitometric analyses are reported. Data are mean ± SD of three different experiments. $P < 0.001$ was calculated with the Student's t-test compared with (*) vehicle- or (°) Q-treated samples. The same compound concentrations were used to evaluate phosphorylated and total AKT and ERK1/2 levels (1 h of stimulation), PTEN levels (24 h of stimulation), and Bcl-2 levels (24 h of stimulation) (panels D and E). Anti-estrogen ICI 182,780 (ICI, 10^{-6} M) was added 15 min before E2 or Q in DLD-1 cells. In panel F, the densitometric analyses of PTEN levels are reported. Data are mean ± SD of three different experiments. $P < 0.001$ was calculated with the Student's t-test compared with (*) vehicle- or (°) Q-treated samples.

above the level that can be maximally induced by E2. This phenomenon of super-induction has been observed in a cell-context specific fashion for several dietary polyphenols, including resveratrol, genistein, diphenyl esters, daidzein and others (Gehm et al., 1997; Kuiper et al., 1998; Legler et al., 1999; Totta et al., 2005). The underlying mechanism remains obscure. Resveratrol and some flavonoids have been recently characterised as antagonist of the aromatic-hydrocarbon receptor that subsequently can interfere with oestrogenic signalling at the DNA level (Lee and Safe, 2000; Singh et al., 2000). Additionally, the binding of quercetin to ERβ might induce conformational changes in the receptor structure that result in a greater recruitment of transcriptional co-activators to the C3 promoter than E2. As a whole, these results further prove that extra-nuclear and genomic signals synergise with each other to guarantee the pro-apoptotic ERβ-dependent effects of E2 and quercetin.

Finally, it has been reported that quercetin (from 8.5 to 25 μM) decreases human colon smooth muscle contractility via ERβ-dependent p38 activation (Hogan et al., 2010). This effect could be related to an alteration in gut transit time similar to that underling the protective role of fibre against the development of colon cancer (Burkitt et al., 1972). This result along with the population-based case-control study before mentioned (Kyle et al., 2010) and others reported here strongly suggest that nutritionally relevant quercetin concentrations positively impact colon functions by modulating ERβ-dependent signals. In particular, the rapid actions of the quercetin:ERβ complex in the colon (i.e., activation of p38 kinase; Hogan et al., 2010 and present results) indicate not only that quercetin could mimic the protective effect of E2 but also suggest that these mechanisms could also be operative in males. Taking into the account that quercetin binds to ERα as an E2 antagonist and induces apoptosis even in the presence of this receptor isotype (Galluzzo et al., 2009); quercetin could be considered as a dual-sided agent against E2-related cancer by reducing tumour growth in organs that express both ERα and ERβ. Thus, present findings provide impetus to investigate further what ER-based effects may be exerted by quercetin on a variety of tissues.

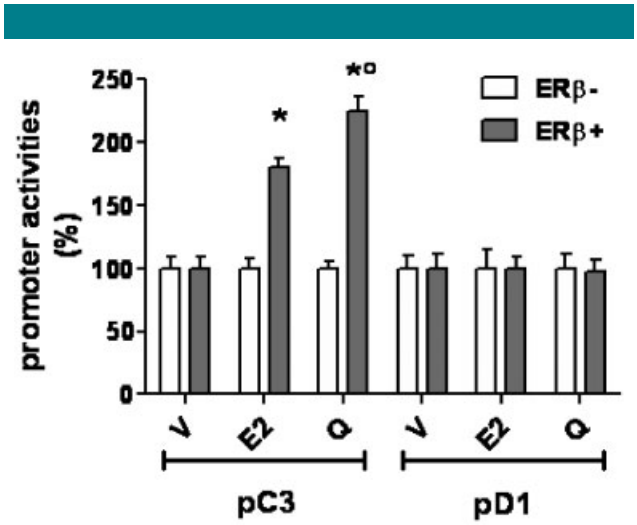

Fig. 5. Quercetin effect on C3 and cyclin D1 promoter activities. HeLa cells transfected with empty vector (ERβ−) or with ERβ expression vector (ERβ+) were co-transfected with pC3-luciferase (pC3) or pD1-luciferase (pD1) reporter plasmids. After 24 h, cells were treated for 24 h with vehicle (V, DMSO:PBS 1:1, v:v), 10^{-8} M E2 or 10^{-6} M quercetin, and the activity of luciferase was evaluated. The data are represented as the mean of six different experiments ± SD. $P > 0.001$ was calculated with the Student's *t*-test with respect to samples treated with vehicle (V) (*) or E2 (°).

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Literature Cited

Acconcia F, Totta P, Ogawa S, Cardillo I, Inoue S, Leone S, Trentalance A, Muramatsu M, Marino M. 2005. Survival versus apoptotic 17β-estradiol effect: Role of ERα and ERβ activated non-genomic signaling. J Cell Physiol 203:193–201.

Ascenzi P, Bocedi A, Marino M. 2006. Structure–function relationship of estrogen receptor α and β: Impact on human health. Mol Aspects Med 27:299–402.

Bai Z, Gust R. 2009. Breast cancer, estrogen receptor and ligands. Arch. Pharm (Weinheim) 342:133–149.

Bolli A, Bulzomi P, Galluzzo P, Acconcia F, Marino M. 2010. Bisphenol A impairs estradiol-induced protective effects against DLD-1 colon cancer cell growth. IUBMB Life 62:684–687.

Boots AW, Haenen GR, Bast A. 2008. Health effects of quercetin: From antioxidant to nutraceutical. Eur J Pharmacol 585:325–337.

Burkitt DP, Walker AR, Painter NS. 1972. Effect of dietary fibre on stools and the transit-times, and its role in the causation of disease. Lancet 2:1408–1412.

Caiazza F, Galluzzo P, Lorenzetti S, Marino M. 2007. 17β-estradiol induces ERβ up-regulation via p38/MAPK activation in colon cancer cells. Biochem Biophys Res Commun 359:102–107.

Campbell-Thompson M, Lynch IJ, Bhardwaj B. 2001. Expression of estrogen receptor (ER) subtypes and ERβ isoforms in colon cancer. Cancer Res 61:632–640.

Chiacchiera F, Simone C. 2008. Signal-dependent regulation of gene expression as a target for cancer treatment: Inhibiting p38α in colorectal tumors. Cancer Lett 265:16–26.

Daskiewicz JB, Depeint F, Viornery L, Bayet C, Comte-Sarrazin G, Comte G, Gee JM, Johnson IT, Ndjoko K, Hostettmann K, Barron D. 2005. Effects of flavonoids on cell proliferation and caspase activation in a colonic cell line HT 29, SAR study. J Med Chem 48:2790–2804.

De Stefani E, Boffetta P, Deneo-Pellegrini H, Mendilaharsu M, Carzoglio JC, Ronco A, Olivera L. 1999. Dietary antioxidants and lung cancer risk: A case-control study in Uruguay. Nutr Cancer 34:100–110.

Dihal AA, de Boer VC, van der Woude H, Tilburgs C, Bruijntjes JP, Alink GM, Rietjens IM, Woutersen RA, Stierum RH. 2006. Quercetin, but not its glycosidated conjugate rutin, inhibits azoxymethane-induced colorectal carcinogenesis in F344 rats. J Nutr 136:2862–2867.

Fitzpatrick JM, Schulman C, Zlotta AR, Schröder FH. 2009. Prostate cancer: A serious disease suitable for prevention. Br J Urol Int 103:864–870.

Foley EF, Jazaeri AA, Shupnik MA, Jazaeri O, Rice LW. 2000. Selective loss of estrogen receptor β in malignant human colon. Cancer Res 60:245–248.

Galluzzo P, Caiazza F, Moreno S, Marino M. 2007. Role of ERβ palmitoylation in the inhibition of human colon cancer cell proliferation. Endocr Relat Cancer 14:153–167.

Galluzzo P, Martini C, Bulzomi P, Leone S, Bolli A, Pallottini V, Marino M. 2009. Quercetin-induced apoptotic cascade in cancer cells: Antioxidant versus estrogen receptor α-dependent mechanisms. Mol Nutr Food Res 53:699–708.

Gehm BD, McAndrews JM, Chien PY, Jameson JL. 1997. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proc Natl Acad Sci USA 94:14138–14143.

Halliwell B, Zhao KC, Whiteman M. 2000. The gastrointestinal tract: A major site of antioxidant action? Free Radic Res 33:819–830.

Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, Tujague M, Ström A, Treuter E, Warner M, Gustafsson J-Å. 2007. Estrogen receptors: How do they signal and what are their targets. Physiol Rev 87:905–931.

Hogan AM, Collins D, Sheehan K, Zierau O, Baird AW, Winter DC. 2010. Rapid effects of phytoestrogens on human colonic smooth muscle are mediated by oestrogen receptor β. Mol Cell Endocrinol 320:106–110.

Huang H, Cheville JC, Pan Y, Roche PC, Schmidt LJ, Tindall DJ. 2001. PTEN induces chemosensitivity in PTEN-mutated prostate cancer cells by suppression of Bcl-2 expression. J Biol Chem 276:38830–38836.

Janakiram NB, Steele VE, Rao CV. 2009. Estrogen receptor-β as a potential target for colon cancer prevention: Chemoprevention of azoxymethane-induced colon carcinogenesis by raloxifene in F344 rats. Cancer Prev Res 2:52–59.

Kim EK, Choi EJ. 2010. Pathological roles of MAPK signaling pathways in human diseases. Biochim Biophys Acta 1802:396–405.

Kim HP, Son KH, Chang HW, Kang SS. 2004. Anti-inflammatory plant flavonoids and cellular action mechanisms. J Pharmacol Sci 96:229–245.

Kim JY, Kim EH, Park SS, Lim JH, Kwon TK, Choi KS. 2008. Quercetin sensitizes human hepatoma cells to TRAIL-induced apoptosis via Sp1-mediated DR5 up-regulation and proteasome-mediated c-FLIPS down-regulation. J Cell Biochem 105:1386–1398.

Konstantinopoulos PA, Kominea A, Vandoros G, Sykiotis GP, Ricopoulos P, Varakis I, Sotiropoulou-Bonikou G, Papavassiliou AG. 2003. Oestrogen receptor β (ERβ) is abundantly expressed in normal colonic mucosa, but declines in colon adenocarcinoma paralleling the tumour's dedifferentiation. Eur J Cancer 39:1251–1258.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, Gustafsson J-Å. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 138:863–870.

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson J-Å. 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology 139:4252–4263.

Kumar R, Verma V, Jain A, Rajeev K, Jagdamba J, Maikhuri P, Gupta G. 2010. Synergistic chemoprotective mechanisms of dietary phytoestrogens in a select combination against prostate cancer. J Nutr Biochem (in press).

Kuo SM. 1996. Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells. Cancer Lett 110:41–48.

Kuo P-C, Liu H-F, Chao J-I. 2004. Survivin and p53 modulate quercetin-induced cell growth inhibition and apoptosis in human lung carcinoma cells. J Biol Chem 279:55875–55985.

Kyle JA, Sharp L, Little J, Duthie GG, McNeill G. 2010. Dietary flavonoid intake and colorectal cancer: A case-control study. Br J Nutr 103:429–436.

Lee JE, Safe S. 2000. 3',4'-dimethoxyflavone as an aryl hydrocarbon receptor antagonist in human breast cancer cells. Toxicol Sci 58:235–242.

Lee TJ, Kim OH, Kim S, Park JW, Kwon TK. 2006. Quercetin arrests G2/M phase and induces caspase-dependent cell death in cells. Cancer Lett 240:234–242.

Legler J, van den Brink CE, Brouwer A, Murk AJ, van der Saag PT, Vethaak AD, van der Burg B. 1999. Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. Toxicol Sci 48:55–66.

Leung YK, Mak P, Hassan S, Ho SM. 2006. Estrogen receptor (ER)-β isoforms: A key to understanding ER-β signaling. Proc Natl Acad Sci USA 103:13162–13167.

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

Marino M, Acconcia F, Bresciani F, Weisz A, Trentalance A. 2002. Distinct nongenomic signal transduction pathways controlled by 17β-estradiol regulate DNA synthesis and cyclin D(1) gene transcription in HepG2 cells. Mol Biol Cell 13:3720–3729.

Marino M, Acconcia F, Trentalance A. 2003. Biphasic estradiol-induced AKT phosphorylation is modulated by PTEN via MAP kinase in HepG2 cells. Mol Biol Cell 14:2583–2591.

Matter WF, Brown RF, Vlahos CJ. 1992. The inhibition of phosphatidylinositol 3-kinase by quercetin and analogs. Biochem Biophys Res Commun 186:624–631.

Mense SM, Hei TK, Ganju RK, Bhat HK. 2008. Phytoestrogens and breast cancer prevention: Possible mechanisms of action. Environ Health Perspect 116:426–433.

Moore JT, McKee DD, Slentz-Kesler K, Moore LB, Jones SA, Horne EL, Su JL, Kliewer SA, Lehmann JM, Willson TM. 1998. Cloning and characterization of human estrogen receptor β isoforms. Biochem Biophys Res Commun 247:75–78.

Murakami A, Ashida H, Terao J. 2008. Multitargeted cancer prevention by quercetin. Cancer Lett 269:315–325.

Murota K, Terao J. 2005. Quercetin appears in the lymph of unanesthetized rats as its phase II metabolites after administered into the stomach. FEBS Lett 579:5343–5346.

Nilsson S, Gustafsson J-Å. 2011. Estrogen receptors: Therapies targeted to receptor subtypes. Clin Pharmacol Ther 89:44–55.

Ogawa S, Inoue S, Watanabe T, Orimo A, Hosoi T, Ouchi Y, Muramatsu M. 1998. Molecular cloning and characterization of human estrogen receptor βcx: A potential inhibitor of estrogen action in human. Nucleic Acids Res 26:3505–3512.

O'Leary KA, Day AJ, Needs PW, Mellon FA, O'Brien NM, Williamson G. 2003. Metabolism of quercetin-7- and quercetin-3-glucuronides by an in vitro hepatic model: The role of human beta-glucuronidase, sulfotransferase, catechol-O-methyltransferase and multi-resistant protein 2 (MRP2) in flavonoid metabolism. Biochem Pharmacol 65:479–491.

Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J-Å, Kushner PJ, Scanlan TS. 1997. Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. Science 277:1508–1510.

Saville B, Wormke M, Wang F, Nguyen T, Enmark E, Kuiper G, Gustafsson J-Å, Safe S. 2000. Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements. J Biol Chem 275:5379–5387.

Shen YH, Zhang L, Gan Y, Wang X, Wang J, LeMaire SA, Coselli JS, Wang XL. 2006. Up-regulation of PTEN (phosphatase and tensin homolog deleted on chromosome ten) mediates p38 MAPK stress signal-induced inhibition of insulin signaling. A cross-talk between stress signaling and insulin signaling in resistin-treated human endothelial cells. J Biol Chem 281:7727–7736.

Shirai M, Kawai Y, Yamanishi R, Kinoshita T, Chuman H, Terao J. 2006. Effect of a conjugated quercetin metabolite, quercetin 3-glucuronide, on lipid hydroperoxide-dependent formation of reactive oxygen species in differentiated PC-12 cells. Free Radic Res 40:1047–1053.

Singh SU, Casper RF, Fritz PC, Sukhu B, Ganss B, Girard BJr, Savouret JF, Tenenbaum HC. 2000. Inhibition of dioxin effects on bone formation in vitro by a newly described aryl hydrocarbon receptor antagonist, resveratrol. J Endocrinol 167:183–195.
Stevenson DE, Hurst RD. 2007. Polyphenolic phytochemicals-just antioxidants or much more? Cell Mol Life Sci 64:2900–2916.
Surh YJ, Kundu JK, Na HK, Lee JS. 2005. Redox sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals. J Nutr 135:2993S–3001S.
Totta P, Acconcia F, Virgili F, Cassidy A, Weinberg PD, Rimbach G, Marino M. 2005. Daidzein-sulfate metabolites affect transcriptional and antiproliferative activities of estrogen receptor-β in cultured human cancer cells. J Nutr 135:2687–2693.
van der Woude H, Gliszczyńska-Swigło A, Struijs K, Smeets A, Alink GM, Rietjens IM. 2003. Biphasic modulation of cell proliferation by quercetin at concentrations physiologically relevant in humans. Cancer Lett 200:41–47.
van der Woude H, Ter Veld MG, Jacobs N, van der Saag PT, Murk AJ, Rietjens IM. 2005. The stimulation of cell proliferation by quercetin is mediated by the estrogen receptor. Mol Nutr Food Res 49:763–771.
van Erk MJ, Roepman P, van der Lende TR, Stierum RH, Aarts JM, van Bladeren PJ, van Ommen B. 2005. Integrated assessment by multiple gene expression analysis of quercetin bioactivity on anticancer-related mechanisms in colon cancer cells in vitro. Eur J Nutr 44:143–156.
Vijayababu MR, Arunkumar A, Kanagaraj P, Venkataraman P, Krishnamoorthy G, Arunakaran J. 2006. Quercetin downregulates matrix metalloproteinases 2 and 9 proteins expression in prostate cancer cells (PC-3). Mol Cell Biochem 287:109–116.
Wada-Hiraike O, Imamov O, Hiraike H, Hultenby K, Schwend T, Omoto Y, Warner M, Gustafsson J-Å. 2006. Role of estrogen receptor β in colonic epithelium. Proc Natl Acad Sci USA 103:2959–2964.
Wagner EF, Nebreda AR. 2009. Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer 9:537–549.
Warner M, Gustafsson J-Å. 2010. The role of estrogen receptor β (ERβ) in malignant diseases- a new potential target for antiproliferative drugs in prevention and treatment of cancer. Biochem Biophys Res Commun 396:63–66.
Williams RJ, Spencer JPE, Rice-Evans C. 2004. Flavonoids: Antioxidants or signalling molecules? Free Rad Biol Med 36:838–849.
Wong NA, Malcomson RD, Jodrell DI, Groome NP, Harrison DJ, Saunders PT. 2005. ERβ isoform expression in colorectal carcinoma: An in vivo and in vitro study of clinicopathological and molecular correlates. J Pathol 207:53–60.
Yue H, Yang B, Zhang H, Zhu SD, Du XJ, Feng XL, Yu Z, Xia YT, Yu JP. 2002. Clinical significance of TGF-β1 and β-glucuronidase synchronous detection in human pancreatic cancer. Hepatobiliary Pancreat Dis Int 1:309–311.