RESEARCH ARTICLE

Oleuropein and hydroxytyrosol inhibit MCF-7 breast cancer cell proliferation interfering with ERK1/2 activation

Rosa Sirianni[1], Adele Chimento[1], Arianna De Luca[1], Ivan Casaburi[2], Pietro Rizza[2], Arianna Onofrio[1], Domenico Iacopetta[1], Francesco Puoci[3], Sebastiano Andò[2], Marcello Maggiolini[1] and Vincenzo Pezzi[1,2]

[1] Department of Pharmaco-Biology, University of Calabria, Arcavacata di Rende (CS), Italy
[2] Department of Cell Biology and Centro Sanitario, University of Calabria, Arcavacata di Rende (CS), Italy
[3] Department of Pharmaceutical Science, University of Calabria, Arcavacata di Rende (CS), Italy

The growth of many breast tumors is stimulated by estradiol (E2), which activates a classic mechanism of regulation of gene expression and signal transduction pathways inducing cell proliferation. Polyphenols of natural origin with chemical similarity to estrogen have been shown to interfere with tumor cell proliferation. The aim of this study was to investigate whether hydroxytyrosol (HT) and oleuropein (OL), two polyphenols contained in extra-virgin olive oil, can affect breast cancer cell proliferation interfering with E2-induced molecular mechanisms. Both HT and OL inhibited proliferation of MCF-7 breast cancer cells. Luciferase gene reporter experiments, using a construct containing estrogen responsive elements able to bind estrogen receptor alpha (ERα) and the study of the effects of HT or OL on ERα expression, demonstrated that HT and OL are not involved in ERα-mediated regulation of gene expression. However, further experiments pointed out that both OL and HT determined a clear inhibition of E2-dependent activation of extracellular regulated kinase1/2 belonging to the mitogen activating protein kinase family. Our study demonstrated that HT and OL can have a chemo-preventive role in breast cancer cell proliferation through the inhibition of estrogen-dependent rapid signals involved in uncontrolled tumor cell growth.

Received: March 12, 2009
Revised: June 18, 2009
Accepted: July 5, 2009

Keywords:
Cancer / Estrogen / Hydroxytyrosol / MCF-7 / Oleuropein

1 Introduction

Breast cancer is the most frequent tumor and the major cause of death among women in the United States [1]. The proliferation of many breast tumor cells is stimulated by the binding of 17β-estradiol (E2) to the estrogen receptor (ER) isoforms, ERα and ERβ. After binding their ligand, ERs modify their conformation, interacting with chromatin and regulating expression of genes involved in cell cycle regulation [2]. For these reasons, molecules named antiestrogens such as 4-hydroxytamoxifen (OHT), which have antagonistic effect on ERs and show chemical similarity with estrogens, are used in breast cancer therapy. However, recently it has been demonstrated that estrogens can activate rapid signals involving receptors other than ''classic'' ERs implicated in cell proliferation [3].

Several epidemiological studies pointed out that food rich in bioactive components, such as vitamins, flavonoids, and polyphenols, could contribute to a low incidence of tumors. This is due to the ability of these components to show

Correspondence: Professor Vincenzo Pezzi, Department of Pharmaco-Biology, Università della Calabria 87036 RENDE (CS), Italy
E-mail: v.pezzi@unical.it
Fax: +39-984-493271

Abbreviations: E2, estradiol; **EGF,** epidermal growth factor; **ERK1/2,** extracellular regulated kinase1/2; **HT,** hydroxytyrosol; **MAPK,** mitogen activated protein kinase; **OHT,** hydroxytamoxifen; **OL,** oleuropein; **PD,** PD98059; **RLU,** relative light units

protective actions or reverse pre-malignant lesions [4]. Among these substances, those with polyphenolic structure have arisen particular interest.

Olive oil, in particular extra-virgin type, is rich of three classes of polyphenoles (simple phenols such as hydroxytyrosol (HT), secoiridoid such as oleuropein (OL) and lignans) that exhibit a remarkable antioxidant action [5] and that are quickly absorbed by human intestine [6]. Lignans inhibit colon, lung, skin, and breast cell line growth [7, 8]. These data have been confirmed in animal models, in particular, breast cancer tumor growth was blocked by treatment with lignans [9]. It has been recently reported that HT may exert a pro-apoptotic effect modulating expression of genes involved in tumor cell proliferation of promyelocytes [10]. Moreover, recently it has been shown that OL and HT inhibit proliferation of human MCF-7 breast cancer cell [11]. However, to date there are no studies on the molecular mechanisms responsible for olive oil polyphenols chemopreventive effects on tumor cells. Olive oil polyphenols present an aromatic ring with a hydroxyl group, which is a feature of both E2 and its antagonist tamoxifen (Fig. 1), this feature can potentially be responsible for the observed anti-estrogenic effects [12]. Aim of this study was to investigate whether polyphenols HT and OL contained in extra-virgin olive oil, determine anti-proliferative effects on breast cancer cells, interfering with molecular mechanisms induced by estrogens.

2 Materials and methods

2.1 Cell cultures and treatments

Human MCF-7 (a gift from Dr. Ewa Surmacz, The Sbarro Institute for Cancer Research and Molecular Medicine, Philadelphia, USA) breast cancer cells were grown in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) supplemented with 10% fetal calf serum (Invitrogen, Milan, Italy) and 1 mg/mL penicillin–streptomycin (complete medium). Cell monolayers were subcultured onto 12-well culture plates for proliferation experiments (1×10^5 cells/well), onto 24-well culture plates for transfection experiments (5×10^4 cells/well) and 100 mm dishes for protein extraction (3×10^6 cells/plate) and used for experiments 48 h later. Cells were treated in serum-free medium for the indicated times with OHT (Tocris Bioscience, Ellisville, Missouri, USA), E2, glycosylated OL and HT (Sigma St Louis, MO, USA).

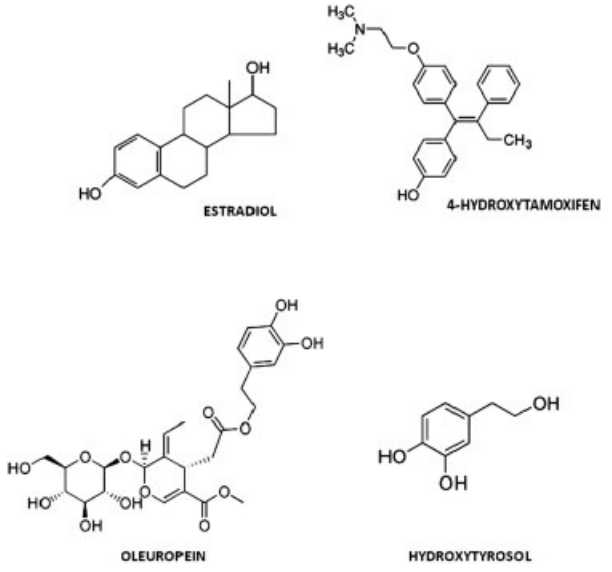

Figure 1. E2, OHT, olive oil polyphenols OL and HT chemical structures.

2.2 Cell proliferation assay

Before treatments cells were maintained in serum- and red phenol-free medium for 24 h. Treatments were allowed for 48 h, to control (basal) cells it was added the same amount of vehicle alone (DMSO) that never exceeded the concentration of 0.01% (v/v). [^{3}H]Thymidine incorporation was evaluated after a 6 h incubation with 1 μCi of [^{3}H]thymidine (Perkin-Elmer Life Sciences, Boston, MA, USA) *per* well. Cells were washed once with 10% trichloroacetic acid, twice with 5% trichloroacetic acid, and lysed in 500 μL of 0.1 mol/L NaOH at 37°C for 30 min. The total suspension was recovered and added to 10 mL optifluor fluid (Packard, USA) before being counted in a β-counter.

2.3 Transfections

Before transfection, complete medium was removed, and 0.5 mL of DMEM/F12 without phenol red, serum or antibiotics was added to the plates. Transfection was performed using Fugene6 reagent (Roche Diagnostics, Mannheim, Germany), following the manufacturer's instruction. Plasmids were used at the concentration of 0.5 μg/well for the XETL [13] promoter-luciferase reporter plasmid, of 10 ng/well for the β-galactosidase control vector (Promega, Promega Italia S.R.L.). Four hour after transfection, the medium was removed and replaced with DMEM/F12 without phenol red, serum or antibiotics and supplemented with the indicated concentrations of treatments for 24 h. Cells were lysed using the passive lysis buffer (Promega), and enzymatic activities were assayed using the Luciferase (Promega) and β-galactosidase (Ambion, Austin, TX, USA) assay systems following the manufacturer's instructions. The firefly luciferase values of each sample were normalized by β-galactosidase activity and data were reported as relative light units (RLU) values.

2.4 Western blot analysis

Cells were maintained in serum- and phenol red-free medium for 24 h before being treated in order to study ERα

expression, and for 72 h before being treated to evaluate ERK1/2 phosphorylation. After treatments, MCF-7 cells were lysed in ice-cold RIPA buffer containing protease inhibitors (20 mmol/L Tris, 150 mmol/L NaCl, 1% Igepal, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 0.1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 0.15 units/mL aprotinin, and 10 μmol/L leupeptin). Lysates were sonicated for 2 min in an ultrasounds waterbath in ice-cold water (Progen Scientific), and centrifuged at 13 000 rpm for 10 min at 4°C. Protein content was determined by the Bradford method. Proteins were separated on 11% SDS-polyacrylamide gel and then electro-blotted onto a nitrocellulose membrane. Blots were incubated overnight at 4°C with (a) anti-ERα (F-10) antibody (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), (b) anti-pERK1/2 antibody (1:1000) (Cell Signaling Technology, Celbio, Milan, Italy) (c) anti-ERK1/2 antibody (1:1000) (Cell Signaling Technology) and (d) anti-β-tubulin antibody (1:1000) (Santa Cruz Biotechnology). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Cologno Monzese, Italy) and immunoreactive bands were visualized with the enhanced chemiluminescence Western blotting detection system (Amersham Biosciences). Anti-ERK1/2 or anti-β-tubulin antibodies were used on stripped membranes to ensure equal loading of proteins.

2.5 Data analysis and statistical methods

Pooled results from triplicate experiments were analyzed using one-way ANOVA with Student-Newman–Keuls multiple comparison methods, using SigmaStat.

3 Results

3.1 HT and OL inhibit E2-induced MCF-7 cell growth

The first aim of this study was to investigate if both HT and OL are able to influence E2-induced breast cancer cells proliferation. As expected, 10 nM E2 was capable of significantly increasing MCF-7 cells growth, while 1 μM OHT was able to completely inhibit E2 effect (Fig. 2). The use of increasing doses of HT or OL determined statistically significant dose-dependent inhibition of E2-induced proliferation when used in the 10–75 μM range (Fig. 2). Concentration equal or above 100 μM were toxic. The presence of ERK1/2 inhibitor PD98059 (PD) reproduced the effect previously reported [14, 15].

3.2 HT and OL effects on ERα transactivation

The proliferation data led us to investigate if HT and OL could have an anti-estrogenic action. To demonstrate this hypothesis we used a reporter plasmid named XETL [13] containing the firefly luciferase gene driven by a tyrosine kinase promoter containing an ER responsive element. Transfection of MCF-7 cells with XETL followed by treatment with 10 nM E2 determined a 15-fold induction compared with transfected but untreated (basal) cells, while the anti-estrogen OHT (1 μM) did not determine any change in luciferase activity (Fig. 3).

No statistically significant differences in luciferase activity were determined after treatment with OL (10 μM) (Fig. 3), while 10 μM HT determined a significant induction comparable to that obtained with E2 used at 1000-fold lower concentration (10 nM) (Fig. 3).

When OHT was used in combination with E2, it was able of inhibiting luciferase reporter gene induction by 80% (Fig. 4). Co-treatments of E2 plus HT or OL, at concentrations that showed anti proliferative effects (10–75 μM), did not change E2-induced luciferase expression (Fig. 4).

Western analysis after 24 h treatment with 10 nM E2 determined as expected a decrease in ERα expression compared with untreated cells (Fig. 5). OHT 1 μM used in combination with E2 was able to block E2 effect allowing for ERα normal expression (Fig. 5). The presence of HT and OL (10–75 μM) did not interfere with E2 action and ERα expression was maintained at the same levels observed with E2 alone (Fig. 5). These results suggest that OL and HT are not able to interfere with estrogen action through competition with ERs, which are responsible for activation of gene expression.

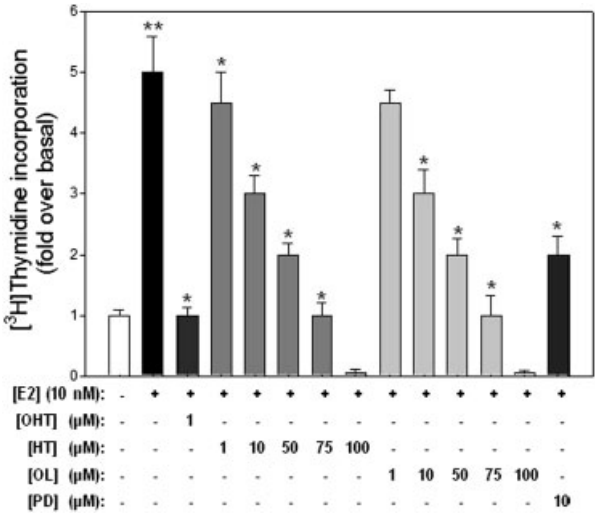

Figure 2. HT and OL effects on E2-inducted MCF-7 cell proliferation. MCF-7 cells were treated with 10 nM E2 in the absence or in the presence of the indicated concentrations of HT, OL, OHT and PD. Cellular proliferation was evaluated by [3H] Thymidine incorporation analysis. Columns, mean of three independent experiments each performed with triplicate samples expressed as percent of basal; bars, SE. (**$p<0.01$ compared with basal and *$p<0.01$ compared with E2 treated sample).

3.3 OL and HT inhibit E2-dependent ERK1/2 activation

Recently it has been demonstrated that estrogens can activate alternative pathways, in addition to mechanisms of regulation of gene expression, capable of inducing, within a few minutes, MAP kinases involved in cell proliferation [3]. For this reason we decided to investigate the possible involvement of OL and HT in E2-dependent rapid activated pathways. In particular, we studied ERK1/2 activation by looking to their phosphorylation status. Figure 6 clearly shows that E2-induced ERK1/2 activation was blocked by both OL and HT, at all tested doses. However, only 10 μM OL was able to reproduce inhibition levels similar to those obtained with PD98089, a specific ERK1/2 inhibitor. As previously shown OHT was able to only partially inhibit E2-dependent kinases activation [15].

4 Discussion

In this study we have demonstrated that both OL and HT are able to inhibit E2-dependent proliferation in human breast cancer cells through a non-classic mechanism.

In the last decade the molecular mechanisms related to the hormone dependency of breast tumors have been extensively investigated [16]. There is a large body of epidemiological and experimental evidences that support the hypothesis that estrogens are strongly implicated in the etiology of breast cancer with some carcinomas requiring estrogen for continued growth and progression [17]. Upon entering cells, E2 binds nuclear receptors, available as different isoforms (estrogens receptors α and β; ERα and ERβ), inducing their dimerization and interaction with DNA sequences that regulate gene transcription [18, 19]. Alternatively, rather than interacting directly with DNA, ERs bind DNA-associated transcription factors stimulating or repressing transcription [20].

The ER is quite promiscuous in ligand binding [21, 22], and many non-steroidal compounds of dietary and environmental origin have been found to possess estrogenic activity [23, 24]. Molecules contained in foods, such as quercetin and genestein, active ingredients in soy products,

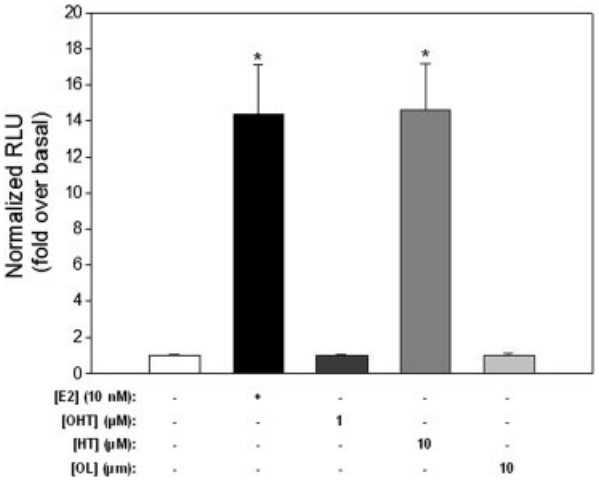

Figure 3. HT and OL effects on ERα basal activation. MCF-7 cells were transiently transfected with XETL reporter plasmid and treated with E2, HT, OL and OHT, at the indicated concentrations. Data were normalized to the co-expressed β-galactosidase expression vector and expressed as RLU (RLU). Results represent the mean ± SD of data from three independent experiments, each performed in triplicate. $^{*}p<0.01$ compared with basal.

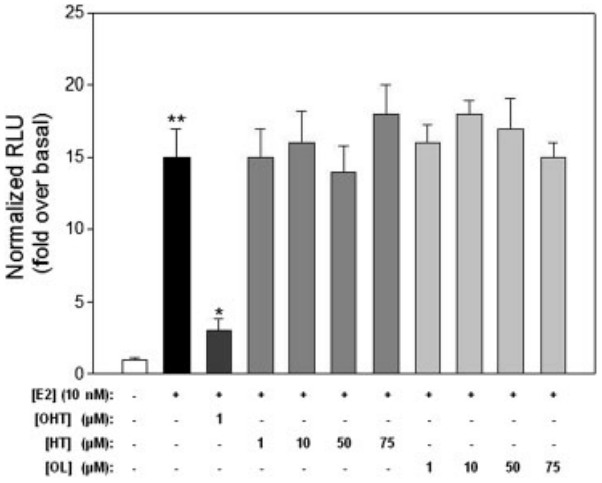

Figure 4. HT and OL effects on E2 induced ERα activation. MCF-7 cells were transiently transfected using XETL reporter plasmid and treated with E2 alone or combined with HT, OL and OHT, at the indicated concentrations. Data were normalized to the co-expressed β-galactosidase expression vector and expressed as RLU. Results represent the mean ± SD of data from three independent experiments, each performed in triplicate. ($^{**}p<0.01$ compared with basal and $^{*}p<0.01$ compared with E2 treated samples).

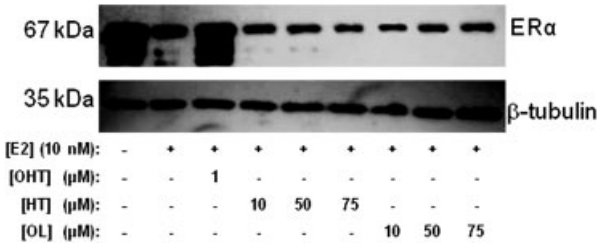

Figure 5. HT and OL effects on ERα protein expression. MCF-7 cells were left untreated (basal) or treated for 48 h with E2 alone or in combination with HT, OL and OHT at the indicated concentration. Total cell extracts were submitted to western blot analysis, using anti-ERα (dil. 1:4000) antibody. β-tubulin was used as loading control. These results are representative of three independent experiments with similar results.

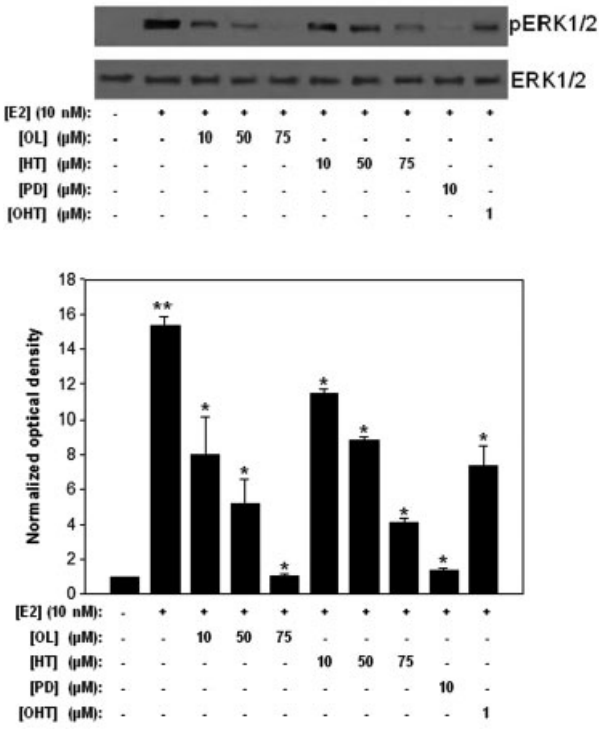

Figure 6. OL and HT effects on ERK1/2 activation. MCF-7 cells were maintained in basal (untreated) conditions or treated with E2 in the presence or absence of HT, OL, PD and OHT at the indicated concentrations for 15 min. Total cellular extracts were subjected to western blot analysis, using anti-pERK1/2 antibody. Total ERK1/2 was used as loading control. These results are representative of at least three independent experiments with similar results. Normalized optical densities were subjected to statistical analysis, statistically significant differences are indicated ($^{**}p<0.01$ compared with basal and $^{*}p<0.01$ compared with E2).

for their structural analogy with estrogens have been named ''phytoestrogens''. These compounds exert a wide range of hormonal and non-hormonal activities in animals, suggesting that diets rich in these compounds may have physiological effects in humans.

OL and HT are phenolic compounds present in olive oil. The aromatic ring present in both molecules is a feature common to E2 (Fig. 1). This common structure can lead to suppose a possible mechanism of action of these compounds correlated to their capacity to compete with estrogens for ER binding sites. This hypothesis arises from the observation that phytoestrogens with an analogue phenolic structure, such as quercetin and genestein, can modulate E2 effect on MCF-7 breast cancer cell proliferation, as a consequence of competition with ERs [25].

It has previously been reported that genistein and quercetin, exert their estrogenic effects through direct binding and activation of the ERs [26, 27].

Quercitin and genistein behave differently at different concentrations in human MCF-7 breast cancer cells. A previous study provided mechanistic insights into the mode of action of the two abundant dietary phytoestrogens [25]. At relatively low concentrations, genistein and quercetin are full agonists for ERα and ERβ, and induce proliferation of ER-dependent breast cancer cells. In the same cells, they are cytotoxic in an ER-independent fashion at concentrations that are reached only in humans with a soy-rich diet.

To classify distinct molecules as weak agonists, selective ER modulators or antiestrogens, it is essential to study their action also in the presence of endogenous estrogens. Therefore, we have examined the dose-dependent effects of OL and HT in comparison to E2-induced cell proliferation in human MCF-7 breast cancer cells.

Similarly to what previously published [25], proliferation studies showed that MCF-7 cells increase their rate of proliferation in response to E2, the presence of OL and HT are able to interfere with E2-dependent MCF-7 proliferation in a dose-dependent manner, however when used concentrations higher than 100 μM the two compounds show cytotoxic effects. Recent studies pointed out that similar concentrations of HT (100 μM) determine apoptotic phenomena in promyelocytes in leukemia models but not in normal cells [10].

The observed anti-proliferative effects suggest an anti-estrogenic action of the two molecules. To show ability of different molecules to activate endogenous ERα a reporter gene for nuclear ERs named XETL has been utilized in transfection experiments for trans-activation studies [25]. In our trans-activation assay E2 was able to induce XETL transactivation at levels similar to those previously seen in breast cancer cells [25]. The same experiment demonstrated that only HT, but not OL, is a strong activator of ERα, determining an induction of reporter gene similar to that seen with E2. However when used in combination with E2 neither HT nor OL were able to interfere with E2-induced gene transactivation. These data suggest that HT could be able to bind ERα but with a low affinity. For this reason HT is not able to compete with E2, a competition that becomes effective in the presence of OHT.

E2 is known to down-regulate the levels of ERα in breast cancer cell lines through an increased turnover of the E2-activated ERα protein and a reduced transcription rate of its own gene [28]. This down-regulation represents an additional hallmark of ERα activation by an agonist. While OHT behaves as an ER antagonist preventing E2-induced receptor down-regulation, HT and OL do not show antagonistic effects since they do not interfere with ERα protein expression. These data further support the hypothesis that OL and HT inhibit E2-mediated MCF-7 cell proliferation through a mechanism which do not involve the classic mechanism of regulation of gene expression.

Instead of directly activating gene expression, after activation with E2 ERα or β can interact with c-Src and activate the c-Src/Shc/Ras/Erk1/2 signal transduction pathway [3, 29–33]. Interference with this activation pathway abolishes

the hormone-dependent cell growth [31]. Furthermore, E2 can also activate the PI3-kinase/AKT pathway, a critical component of the cell growth regulation [31, 34].

Recently, it has been revealed in a variety of cell lines that the transmembrane receptor GPR30 can mediate estrogen action [35, 36]. GPR30 couples to a trimeric G protein, stimulating the cAMP pathway most likely through a Gαs [36] and Src through Gβγ [37]. Subsequently, Src promotes the shedding of heparin-binding EGF-like growth factor and activation of the EGF receptor [38]. This in turn activates a whole series of intracellular signalling events, most notably the activation of mitogen-activated protein kinases (MAPK) ERK1/2, PI3 kinase and phospholipase C (reviewed in [39]). Further cellular responses lie downstream of these signals, including the activation of the gene fos [40].

E2 was shown to activate ERK1/2 phosphorylation in MCF-7 cells [40]. Here we confirm these data, showing also the ability of OL and HT to interfere with this activation. The inhibitory effect of HT on ERK1/2 is in agreement with a previous report indicating the inability of this compound to induce expression of ERK1/2 dependent genes such as COX-2 [41]. Moreover, the observation that PD blocks completely ERK1/2 activation, but only partially MCF-7 E2-dependent proliferation, suggests that other kinases can play a role in rapid signals activated by E2 and controlling cell growth. Our data address the hypothesis that HT and OL could inhibit the estrogen-activated signal transduction pathway involving GPR30 and epidermal growth factor (EGF) receptor.

This hypothesis is supported by the observation that other natural substances with a phenolic structure, such as genestein interfere with proliferation at different levels, inhibiting tyrosine kinase activity of EGF receptor [42]. Moreover we have recently shown that the xenoestrogen atrazine stimulated proliferation of ovarian cancer cells through the GPR30-EGF receptor transduction pathway and through a cross talk with ERα [43]. However further experiments will be necessary to determine the mechanism of action of HT and OL in inhibiting MAPK phosphorylation. Experiments aimed to study this mechanism are currently ongoing in our lab.

In conclusion, this study pointed out that both OL and HT are able to inhibit E2 induced proliferation in human breast cancer cells. This event requires the achievement of high concentrations of polyphenols (in the μM range), concentrations hardly reached in humans [6]. In fact, a regular consumption of olive oil, like it happens in a Mediterranean diet, allows to rich blood concentrations of HT equivalent to 50–160 nM [44–46].

Moreover we cannot exclude that, *in vivo*, their action may synergize with other molecules present in the diet with a similar structure. Our data exclude OL and HT involvement in classic estrogen-activated regulation of gene expression, but point out a clear inhibition of E2-dependent rapid signaling pathways which are able to induce, within a few minutes, molecules involved in proliferation events, such as MAPK.

Our study demonstrates that the intake of molecules with phenolic structure (HT and OL) contained in extra virgin olive oil may contribute to a chemopreventive action through rapid modification of mitogen signals.

This work was supported by C.R.A-ISOL, grant on "Ricerca ed Innovazione dell'Olivocultura Meridionale" (RIOM). This work was also supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC).

The authors have declared no conflict of interest.

5 References

[1] Landis, S. H., Murray, T., Bolden, S., Wingo, P. A., Cancer statistics. *CA Cancer J. Clin.* 1999, *49*, 8–31.

[2] Jensen, E. V., Ann, N. Y., Steroid hormones, receptors, and antagonists. *Acad. Sci.* 1996, *784*, 1–17.

[3] Levin, E. R., Integration of the extranuclear and nuclear actions of estrogen. *Mol. Endocrinol.* 2005, *19*, 1951–1959.

[4] Hong, W. K., General keynote: the impact of cancer chemoprevention. *Gynecol. Oncol.* 2003, *88*, S56–S58.

[5] Owen, R. W., Giacosa, A., Hull, W. E., Haubner, R. *et al.*, Bartsch, H. Olive-oil consumption and health: the possible role of antioxidants. *Lancet Oncol.* 2000, *1*, 107–112.

[6] Vissers, M. N., Zock, P. L., Roodenburg, A. J. C., Leenen, R., Katan, M. B., Olive oil phenols are absorbed in humans. *J. Nutr.* 2002, *132*, 409–417.

[7] Kardono, L. B., Tsauri, S., Padmawinata, K., Pezzuto, J. M., Kinghorn, A. D., Cytotoxic constituents of the bark of plumeria rubra collected in Indonesia. *J. Nat. Prod.* 1990, *53*, 1447–1455.

[8] Hirano, T., Fukuoka, K., Oka, K., Naito, T. *et al.*, Antiproliferative activity of mammalian lignan derivatives against the human breast carcinoma cell line, ZR-75-1. *Cancer Invest.* 1990, *8*, 595–602.

[9] Thompson, L. U., Seidl, M. M., Rickard, S. E., Orcheson, L. J., Fong, H. H., Antitumorigenic effect of a mammalian lignan precursor from flaxseed. *Nutr. Cancer* 1996, *26*, 159–165.

[10] Fabiani, R., De Bartolomeo, A., Rosignoli, P., Servili, M. *et al.*, Virgin olive oil phenols inhibit proliferation of human promyelocytic leukemia cells (HL60) by inducing apoptosis and differentiation. *J. Nutr.* 2006, *136*, 614–619.

[11] Goulas, V., Exarchou, V., Troganis, A. N., Psomiadou, E. *et al.*, Phytochemicals in olive-leaf extracts and their antiproliferative activity against cancer and endothelial cells. *Mol. Nutr. Food Res.* 2009, *53*, 600–608.

[12] Adlercreutz, H., Bannwart, C., Wähälä, K., Mäkelä, T. *et al.*, Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens. *J. Steroid Biochem. Mol. Biol.* 1993, *44*, 147–156.

[13] Bunone, G., Briand, P. A., Miksicek, R. J., Picard, D., Activation of the unliganded estrogen receptor by EGF involves

the MAP kinase pathway and direct phosphorylation. *EMBO J.* 1996, *15*, 2174–2183.

[14] Razandi, M., Pedram, A., Rosen, E. M., Levin, E. R., BRCA1 inhibits membrane estrogen and growth factor receptor signaling to cell proliferation in breast cancer. *Mol. Cell. Biol.* 2004, *24*, 5900–5913.

[15] Reddy, K. B., Glaros, S., Inhibition of the MAP kinase activity suppresses estrogen-induced breast tumor growth both in vitro and in vivo. *Int. J. Oncol.* 2004, *30*, 971–975

[16] Subramanian, A., Salhab, M., Mokbel, K., Oestrogen producing enzymes and mammary carcinogenesis: a review. *Breast Cancer Res. Treatment* 2008, *111*, 191–202.

[17] Henderson, B. E., Ross, R., Bernstein, L., Estrogens as a cause of human cancer: the Richard and Hinda Rosenthal foundation award lecture. *Cancer Res.* 1988, *48*, 246–253.

[18] Beato, M., Herrlich, P., Schutz, G., Steroid hormone receptors: many actors in search of a plot. *Cell* 1995, *83*, 851–857.

[19] Parker, M. G., White, R., Nuclear receptors spring into action. *Nat. Struct. Biol.* 1996, *3*, 113–115.

[20] McKenna, N. J., Lanz, R. B., O'Malley, B. W., Nuclear receptor coregulators: cellular and molecular biology. *Endocr. Rev.* 1999, *20*, 321–344.

[21] Anstead, G. M., Carlson, K. E., Katzenellenbogen, J. A., The estradiol pharmacophore: ligand structure-estrogen receptor binding affinity relationships and a model for the receptor binding site. *Steroids* 1997, *62*, 268–303.

[22] Blair, R. M., Fang, H., Branham, W. S., Hass, B. S. *et al.*, The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. *Toxicol. Sci.* 2000, *54*, 138–153.

[23] Sohoni, P., Sumpter, J. P., Several environmental oestrogens are also anti-androgens. *J. Endocrinol.* 1998, *158*, 327–339.

[24] Akingbemi, B. T., Hardy, M. P., Oestrogenic and anti-androgenic chemicals in the environment: effects on male reproductive health. *Ann. Med.* 2001, *33*, 391–403.

[25] Maggiolini, M., Bonofiglio, D., Marsico, S., Panno, M. L. *et al.*, Estrogen receptor alpha mediates the proliferative but not the cytotoxic dose-dependent effects of two major phytoestrogens on human breast cancer cells. *Mol. Pharmacol.* 2001, *60*, 595–602.

[26] Kuiper, G. G. J. M., Carlsson, B., Grandien, K., Enmark, E. *et al.*, Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology* 1997, *138*, 863–870.

[27] Barkhem, T., Carlsson, B., Nilsson, Y., Enmark, E. *et al.*, Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. *Mol. Pharmacol.* 1998, *54*, 105–112.

[28] Santagati, S., Gianazza, E., Agrati, P., Vegeto, E. *et al.*, Oligonucleotide squelching reveals the mechanism of estrogen receptor autologous down-regulation. *Mol. Endocrinol.* 1997, *11*, 938–949.

[29] Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A. *et al.*, Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO J.* 1996, *15*, 1292–1300.

[30] Migliaccio, A., Piccolo, D., Castoria, G., Di Domenico, M. *et al.*, Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. *EMBO J.* 1998, *17*, 2008–2018.

[31] Castoria, G., Migliaccio, A., Bilancio, A., Di Domenico, M. *et al.*, PI3-kinase in concert with Src promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. *EMBO J.* 2001, *20*, 6050–6059.

[32] Castoria, G., Migliaccio, A., Di Domenico, M., Lombardi, M. *et al.*, Role of atypical protein kinase C in estradiol-triggered G1/S progression of MCF-7 Cells. *Mol. Cell. Biol.* 2004, *24*, 7643–7653.

[33] Migliaccio, A., Di Domenico, M., Castoria, G., Nanayakkara, M. *et al.*, Steroid receptor regulation of epidermal growth factor signaling through Src in breast and prostate cancer cells: steroid antagonist action. *Cancer Res.* 2005, *65*, 10585–10593.

[34] Carpenter, C. L., Cantley, L. C., Phosphoinositide 3-kinase and the regulation of cell growth. *Biochim. Biophys. Acta* 1996, *1288*, M11–M16.

[35] Revankar, C. M., Cimino, D. F., Sklar, L. A., Arterburn, J. B., Prossnitz, E. R., A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* 2005, *307*, 1625–1630.

[36] Thomas, P., Pang, Y., Filardo, E. J., Dong, J., Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* 2005, *146*, 624–632.

[37] Filardo, E. J., Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. *J.Steroid Biochem. Mol. Biol.* 2002, *80*, 231–238.

[38] Filardo, E. J., Quinn, J. A., Bland, K. I., Frackelton, A. R. Jr., Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol. Endocrinol.* 2000, *14*, 1649–1660.

[39] Prossnitz, E. R., Arterburn, J. B., Smith, H. O., Oprea, T. I. *et al.*, Estrogen signaling through the transmembrane G protein coupled receptor GPR30. *Annu. Rev. Physiol.* 2008, *70*, 165.

[40] Maggiolini, M., Vivacqua, A., Fasanella, G., Recchia, A. G. *et al.*, The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17beta-estradiol and phytoestrogens in breast cancer cells. *J. Biol. Chem.* 2004, *279*, 27008–27016.

[41] Martinez-Gonzalez, J., Rodriguez-Rodriguez, R., Gonzalez-Diez, M., Rodriguez, C. *et al.*, Oleanolic acid induces prostacyclin release in human vascular smooth muscle cells through a cyclooxygenase-2-dependent mechanism. *J. Nutr.* 2008, *138*, 443–448.

[42] Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H. *et al.*, Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* 1987, *262*, 5592–5595.

[43] Albanito, L., Lappano, R., Madeo, A., Chimento, A. *et al.*, G-protein-coupled receptor 30 and estrogen receptor-alpha are involved in the proliferative effects induced by atrazine in ovarian cancer cells. *Environ. Health Perspect.* 2008, *116*, 1648–1655.

[44] Weinbrenner, T., Fito, M., de la Torre, R., Saez, G. T. *et al.*, Olive oils high in phenolic compounds modulate oxidative/antioxidative status in men. *J. Nutr.* 2004, *134*, 2314–2321.

[45] Miro-Casas, E., Covas, M.I., Farre, M., Fito, M. *et al.*, Hydroxytyrosol disposition in humans. *Clin. Chem.* 2003, *49*, 945–952.

[46] Rietjens, S. J., Bast, A., de Vente, J., Haenen, G. R. M. M., The olive oil antioxidant hydroxytyrosol efficiently protects against the oxidative stress-induced impairment of the NO response of isolated rat aorta. *Am. J. Physiol. Heart Circ. Physiol.* 2007, *292*, 1931–1936.

