Antioxidant and antigrowth action of peracetylated oleuropein in thyroid cancer cells

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Abstract

The olive tree phenolic component oleuropein (OLE) and its derivatives have shown many biological properties, thus representing promising novel therapeutics for the treatment of several diseases, including neoplasia. In this study, we evaluated the activities of OLE and its peracetylated derivative (peracetylated oleuropein, Ac-OLE) against two thyroid tumor cell lines that host genotypic alterations detected in human papillary thyroid cancer. TPC-1 and BCPAP cells were treated with OLE and Ac-OLE, and the effects on viability were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, cell counting, and trypan blue exclusion assays. Antioxidant effects were analyzed by measuring the reactive oxygen species (ROS) in basal conditions and after treatment with hydrogen peroxide (H_2O_2). Activity of MAP kinase and PI3K–Akt signaling pathways was evaluated by examining the levels of phosphorylated ERK and Akt by western blot. We found that OLE significantly inhibited the proliferation of both cell lines. This effect was paralleled by a reduction of basal phospho-Akt and phospho-ERK levels and H_2O_2-induced ROS levels. A stronger effect was elicited by Ac-OLE either in inhibiting cell growth or as an antioxidant, in particular on BCPAP cells. Our results demonstrate that OLE and especially Ac-OLE inhibit *in vitro* thyroid cancer cell proliferation acting on growth-promoting signal pathways, as well as exerting antioxidant effects. Further studies will reveal the potential application as novel targeted therapeutics in thyroid cancer.

Key Words
- oleuropein
- thyroid cancer
- MAP kinase
- Akt
- ROS

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Introduction

The last decades have registered an increased incidence of differentiated thyroid cancer (DTC), including those unable to concentrate iodide and, for this reason, unresponsive to the current radioiodine-based treatment (Schlumberger *et al.* 2007, Hall *et al.* 2009). For these tumors, the search for novel molecular targets and therapeutics still represents a big challenge. Promising results have been obtained by targeting known oncogene-driven alterations of signal transduction pathways mainly involved in thyroid tumor development and progression (Knauf & Fagin 2009). Thus, novel 'targeted' drugs are currently undergoing clinical testing (Schlumberger & Sherman 2012). Additional potential therapeutic targets are represented by other molecular alterations detected in

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thyroid cancer cells (Fagin & Mitsiades 2008). Recently, attention has been focused on the role of oxidative stress and reactive oxygen species (ROS) production in neoplastic transformation and progression (Duracková 2010). Strong evidence suggests a close relationship even between oxidative stress and thyroid cancer (Wang *et al.* 2011, Xing 2012), encouraging testing of antioxidant molecules as potential novel targeted therapeutics.

Many reports have stated the ability of oleuropein (OLE) and its derivatives, seco-phenolic compounds present in considerable amounts in olive leaves, drupes, and virgin olive oils, to scavenge ROS (Visioli *et al.* 2002, El & Karakaya 2009, Cicerale *et al.* 2010) and inhibit the proliferation of several tumor cell lines (Hamdi & Castellon 2005, El & Karakaya 2009, Goulas *et al.* 2009, Cicerale *et al.* 2010, Bulotta *et al.* 2011). Using an innovative extractive method starting from renewable sources of primary matter and adopting sustainable synthetic strategies, we obtained in our laboratory OLE and some acetylated derivatives in good yields and very mild conditions (Procopio *et al.* 2008, 2009). The latter compounds showed an improved capacity to permeate the molecular membrane, as well as their 'drug likeness' (Procopio *et al.* 2009). Moreover, when tested against two breast cancer cell lines, the peracetylated compounds revealed a stronger antioxidant and growth-inhibitory activity than OLE (Bulotta *et al.* 2011).

In this work, we investigated the antiproliferative and antioxidant effects of OLE and its peracetylated derivative (peracetylated oleuropein, Ac-OLE) on two thyroid cancer cell lines, TPC-1 and BCPAP. These cells are validated models of human papillary thyroid cancer (PTC), in that they host the RET/PTC1 rearrangement and *BRAF* V600E mutation respectively (Ribeiro *et al.* 2008, Schweppe *et al.* 2008), the most common genetic alterations detected in human PTC (Fagin & Mitsiades 2008). Their tumorigenic action is related to activation of intracellular signaling pathways controlling cell growth and survival (Fagin & Mitsiades 2008, Schlumberger & Sherman 2009). For this reason, the effects of OLE and Ac-OLE on the activation of ras-raf-MAPK- and PI3K–Akt-dependent pathways were also investigated.

Subjects and methods

Chemicals

OLE and Ac-OLE (Fig. 1) were obtained using a novel sustainable synthetic strategy, as described by Procopio *et al.* (2009). The following reagents were purchased as indicated: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue solution (0.4%), propidium iodide, Triton X-100, RNase A, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), anti-β-actin MAB, and anti-poly ADP-ribose polymerase (PARP) MAB were from Sigma–Aldrich; Hybond-ECL-PVDF membranes and ECL-PRIME from GE Healthcare (Milan, Italy); anti-ERK polyclonal antibody and anti-phospho-ERK (Tyr204) MAB from Santa Cruz Biotechnology (DBA, Segrate, Milan, Italy); anti-Akt and anti-phospho-Akt (Ser473) polyclonal antibodies from Cell Signaling (Euroclone, Milan, Italy); goat anti-mouse and goat anti-rabbit HRP-conjugated antibodies from Transduction Laboratories (Lexington, KY, USA); protease inhibitor mixture from Roche Diagnostics; and BCA protein assay kit from Pierce (Celbio, Milan, Italy).

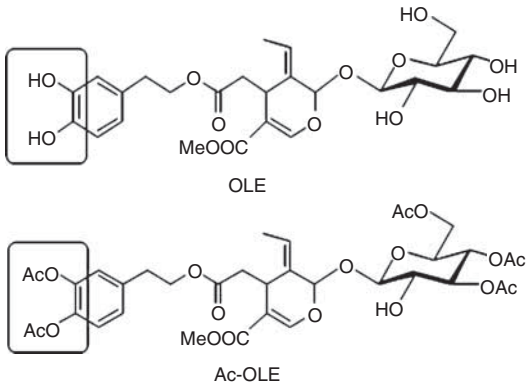

Figure 1
Chemical structures of OLE and Ac-OLE. The free catechol moiety (evidenced in squares), responsible for the antioxidant activity of OLE, is protected in Ac-OLE with the acetyl group.

Cell cultures

TPC-1 and BCPAP cell lines from PTC were provided by Prof. A Fusco (University of Naples) and Dr E Puxeddu (University of Perugia) respectively and confirmed to have maintained their original genotype. TAD-2 cell line (immortalized, but not tumorigenic thyroid cells, obtained by Simian virus 40 infection of human fetal thyroid cells) was a gift of Prof. M Vitale (University of Salerno). TPC-1 and TAD-2 cells were grown in DMEM and BCPAP in RPMI culture medium (Gibco), each enriched with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 μg/ml), and amphotericin B (2.5 μg/ml) (Sigma–Aldrich). Cells were kept in incubation at 37 °C in an atmosphere of 5% CO_2.

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Cell growth assays

To monitor cell viability, an MTT assay was performed as described previously (Celano *et al*. 2008) with small modifications. Briefly, cells were implanted in 96-well plates at a density of 6×10^3 for TPC-1 and 8×10^3 for BCPAP and TAD-2. After 24 h, cells were treated with different concentrations (10, 50, and 100 μM) of OLE and Ac-OLE or equivalent dilution of ethanol (control) for 48 h. Eight replicates were performed for each sample. Then medium was replaced with phenol red-free medium containing MTT (0.5 mg/ml). After 4-h incubation, 100 μl 10% SDS was added to each well and optical density was measured with a microplate spectrophotometer reader (X MARK Spectrophotometer Microplate Bio-Rad) at $\lambda=540$.

Cellular growth was also evaluated by counting the viable cells using the trypan blue exclusion method. TPC-1, BCPAP, and TAD-2 cell lines were seeded in 12-well plates at a density of 10^4 for TPC-1 and 4×10^4 for BCPAP and TAD-2. For non-proliferating cells, the experiments were performed in medium supplemented with only 0.1% FBS: in these conditions, the increase in cell number after 72 h was always lower than 5%. After 24 h, cells were treated as described earlier, resuspended in 0.4% trypan blue solution, and counted in a Burker chamber. Cellular proliferation was expressed as percent of live (non-stained) cells over control. Cell death rates were calculated as the percentage of stained cells over total cells and expressed as the ratio between treated and untreated (control) cells.

Cell cycle analysis

Cell cycle distribution was analyzed as described previously (Bulotta *et al*. 2011). Briefly, TPC-1 and BCPAP cells were either treated with ethanol and used as control, or with OLE and Ac-OLE (50 and 100 μM) for 48 h. Then cells were harvested, washed twice with PBS, and fixed in 70% cold ethanol for at least 2 h at −20 °C. Fixed cells were washed with PBS, incubated with 1 ml PBS containing 0.5 μg/ml RNase A and 0.5% Triton X-100 for 30 min at 37 °C, and stained with 50 μg/ml propidium iodide. Cells were analyzed for DNA content by flow cytometry (FACScan, Becton Dickinson, San Jose, CA, USA) and the percentage of cells in various cell cycle phases was determined using Cell Quest software (Becton Dickinson).

ROS assay

Determination of ROS was founded on the oxidation of the cell-permeable non-fluorescent probe H_2DCF-DA. Upon cleavage of the acetate groups by intracellular esterases and oxidation, it is converted to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). For both TPC-1 and BCPAP cells, the experiments were performed in RPMI culture medium for its lower content of sodium bicarbonate (Odiatou *et al*. 2013). Cells were incubated for 48 h with 50 and 100 μM OLE and Ac-OLE. At the end of the treatment, the cells were washed with PBS, trypsinized, resuspended in phenol red-free medium containing H_2DCF-DA (25 μM) and incubated for 30 min at 37 °C. Subsequently, the cells were centrifuged, resuspended in PBS and, in the presence or not of H_2O_2 (100 μM, 30 min of incubation), the fluorescence was evaluated by flow cytometric analysis using a FACScan laser flow cytometer equipped with Cell Quest software (Becton Dickinson).

Protein extraction and western blot

TPC-1 and BCPAP cells were seeded in six-well plates; after 24 h, the medium was replaced with fresh medium containing OLE or Ac-OLE (100 μM) or vehicle (ethanol) alone. After 30 min, 1-h, and 24-h incubation, cell monolayers were washed and solubilized in preheated denaturing buffer containing 50 mM Tris-phosphate, pH 6.8, 2% SDS, 10 mM NaF, and a protease inhibitor mixture and immediately boiled as described previously (Bulotta *et al*. 2009). The cell lysates were quantified spectrophotometrically using the bicinchoninic acid procedure and samples were loaded and run onto 12% SDS–polyacrylamide gels. After electrophoresis, proteins were transferred to Hybond-ECL-PVDF membranes and incubated with antibodies specific for β-actin or the phosphorylated and non-phosphorylated forms of ERK and Akt proteins (D'Agostino *et al*. 2012). After incubation with the appropriate secondary antibodies, blots were developed with ECL-PRIME reagent.

Statistical analysis

The results are expressed as mean ± S.D. of at least three independent experiments. The differences among treatments were evaluated with one-way ANOVA followed by the Tukey–Kramer multiple comparisons test.

Results

Effects of OLE and Ac-OLE on thyroid cell proliferation

MTT assays were performed to evaluate the effects of OLE and Ac-OLE on cellular proliferation by treating TPC-1,

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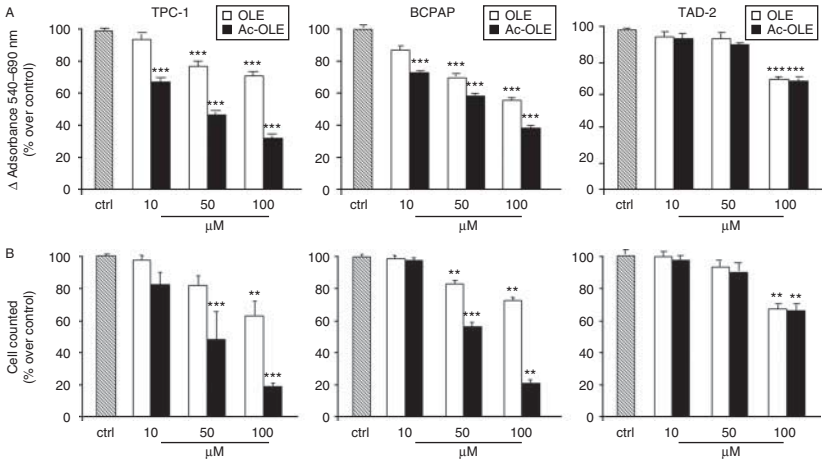

Figure 2

OLE and Ac-OLE effects on TPC-1, BCPAP, and TAD-2 cell viability. Cells were treated in the absence (ctrl) or presence of the indicated concentrations of the compounds for 48 h. Viability was evaluated by MTT assay (A) and cell count (B), as described in the Subjects and methods section. Values represent the mean ± S.D. of three independent experiments. **,***$P<0.01$ and <0.001 vs control respectively.

BCPAP, and TAD-2 cells at various concentrations of the compounds for 48 h. The treatment with OLE resulted in a significant dose-dependent growth inhibition in TPC-1 and BCPAP cell lines (~30 and 40% with 50 and 100 μM vs control respectively) (Fig. 2A). Even stronger effects were detected with Ac-OLE, which determined a significant reduction in cell viability already at 10 μM (~30% reduction vs control) and elicited a ~70% reduction at 100 μM in both cell lines (Fig. 2A). Instead in non-tumor TAD-2 cells, we observed a significant effect (~25% reduction vs control) only with 100 μM of both OLE and Ac-OLE (Fig. 2A). These results were confirmed using cell count (Fig. 2B). In addition, to test the effects of two compounds in non-proliferating cells, TPC-1 and BCPAP cells were grown in low-serum conditions (0.1% FBS). In such circumstances, Ac-OLE was still effective on BCPAP cells at 100 μM with Ac-OLE (Fig. 3). By contrast, TPC-1 presented a higher rate of mortality so that the results could not be informative (data not shown).

To determine whether cellular growth inhibition was due to a block on cell cycle progression, TPC-1 and BCPAP cells were treated for 48 h with OLE and Ac-OLE and subjected to flow cytometric analysis (Fig. 4). In TPC-1 cells, OLE induced a mild block in S phase (Fig. 4A and C), while Ac-OLE induced a stronger dose-dependent S phase arrest (Fig. 4A and C). Instead, BCPAP cells showed a minimal arrest in G2/M phase after treatment with both compounds (Fig. 4B and C); in addition, 100 μM concentration of Ac-OLE determined a cytotoxic effect (Fig. 4B and C). Increase in BCPAP cell mortality after 100 μM Ac-OLE was confirmed by trypan blue exclusion assay (Fig. 5A). Cellular death was not associated with apoptosis as shown by immunoblot analysis that revealed absence of PARP cleavage (Fig. 5B).

Antioxidant effects of OLE and Ac-OLE

Antioxidant effects were analyzed by determining ROS levels in TPC-1 and BCPAP cells treated with OLE and Ac-OLE using a cytofluorimetric analysis. In cells exposed to extracellular ROS attack (H_2O_2, 100 μM) (Fig. 6B), increase in ROS levels was revealed by rightward shift of

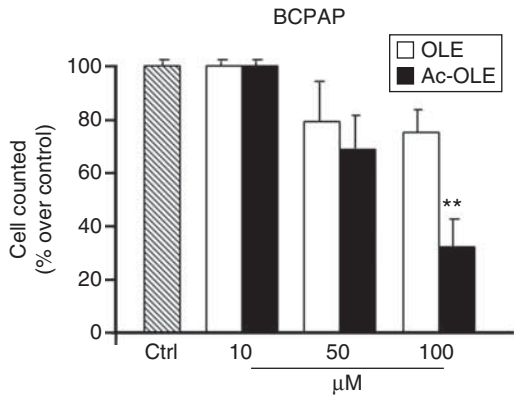

Figure 3

Effects of OLE and Ac-OLE on BCPAP cell viability in low-serum conditions. BCPAP cells were cultured in medium supplemented with only 0.1% FBS and treated or not (ctrl) with the compounds for 48 h. Viability was determined by cell count, as described in the Subjects and methods section. **$P<0.01$ vs control.

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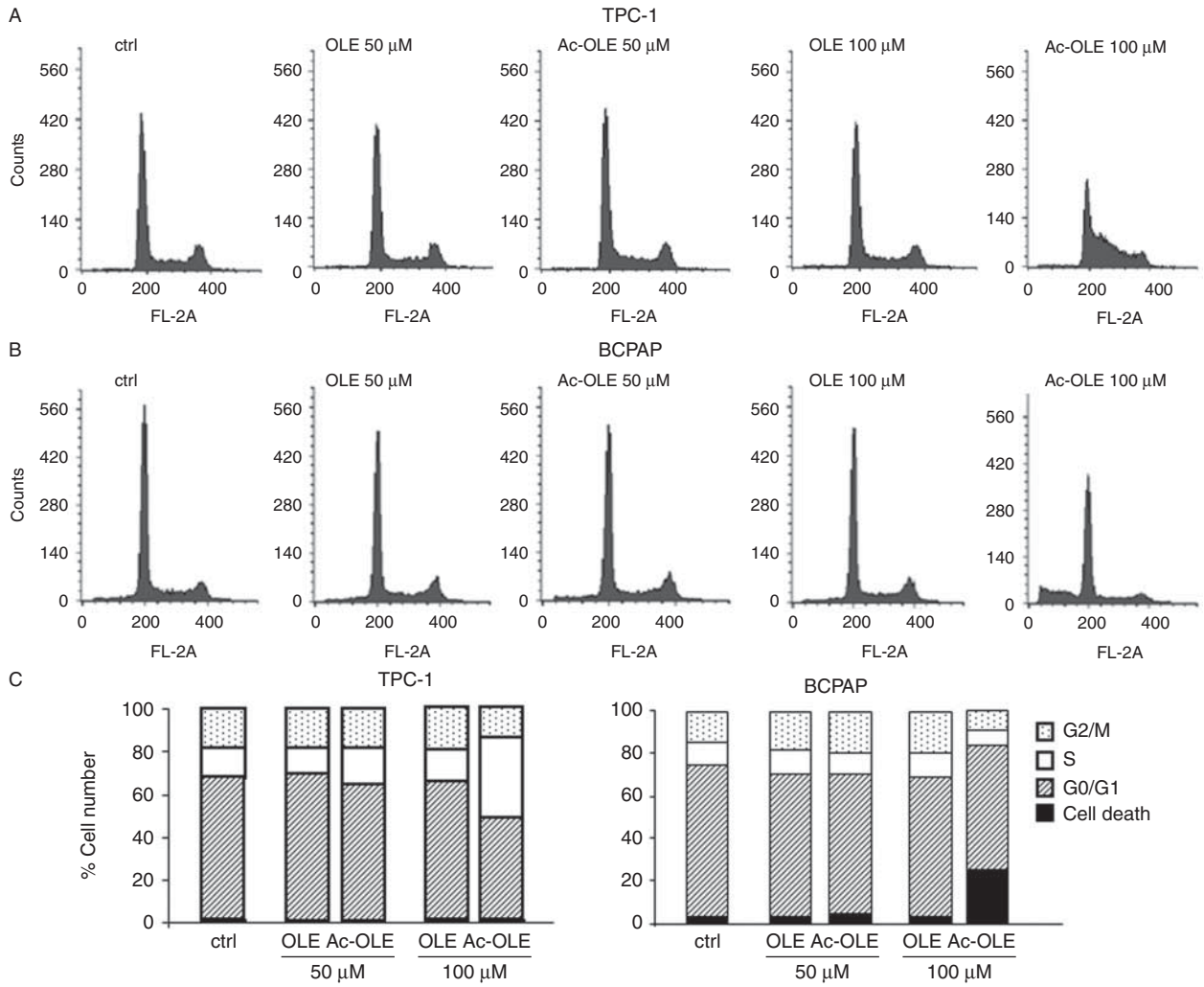

Figure 4

Effects of OLE and Ac-OLE on cell cycle progression. Cell lines were treated with the indicated concentrations of the compounds for 48 h and then analyzed by FACS (A and B) as indicated in the Subjects and methods section. ctrl, cells without treatment. The bar graphs in C are representative of three independent experiments.

the fluorescence. In both cell lines, OLE treatment caused a decrease in ROS levels and a stronger effect appeared after incubation with Ac-OLE. In addition, Ac-OLE and not OLE treatment also caused a decrease in endogenous ROS levels in a dose-dependent way in both cell lines, as revealed by a leftward shift of fluorescence (Fig. 6A).

OLE and Ac-OLE effects on ERK and Akt activation

TPC-1 and BCPAP cells are characterized by RET/PTC1 rearrangement and *BRAF* V600E mutation respectively (Ribeiro *et al.* 2008, Schweppe *et al.* 2008) and present high-basal activation of the MEK/ERK and Akt signaling pathways. Thus, we investigated whether OLE and Ac-OLE would affect these intracellular pathways by looking at the phosphorylation status of ERK and Akt. In TPC-1 cells, immunoblot experiments indicated that OLE at 100 μM inhibited both ERK and Akt phosphorylation at 30 and 60 min followed by almost complete recovery after 24 h (Fig. 7, left panel). A stronger effect was observed treating the cells with Ac-OLE 100 μM (Fig. 7, left panel). A similar inhibition occurred in BCPAP cells (Fig. 7, right panel): again both compounds induced an early reduction of phospho-ERK and phospho-Akt levels, but the effect of Ac-OLE on ERK phosphorylation was maintained even after 24 h. Parallel immunoblots with antibodies against Akt and ERK proteins showed no changes in the total levels of these proteins (Fig. 7).

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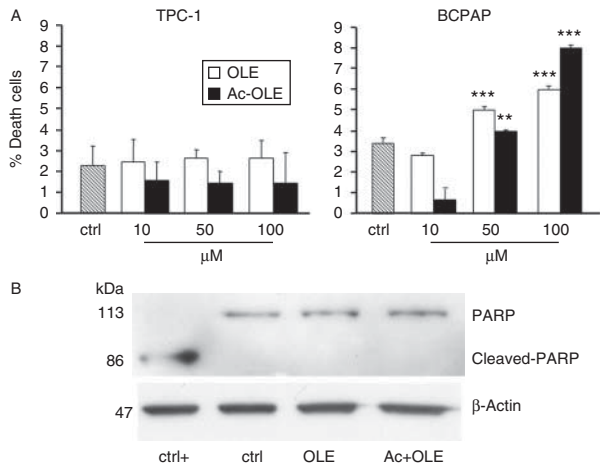

Figure 5
OLE and Ac-OLE effects on cell death and apoptosis. (A) Mortality rates in TPC-1 and BCPAP cell lines treated with different concentrations of the compounds for 48 h were evaluated by trypan blue exclusion assay. ctrl, cells without treatment. **,***$P<0.01$ and <0.001 vs control respectively. (B) Analysis of PARP cleavage after 48 h of treatment with 100 μM OLE and Ac-OLE was performed in protein extract by immunoblot. BCPAP cells treated with U0126 (10 μM) were used as positive control (ctrl+). Protein load was checked by β-actin immunostaining.

Discussion

Elucidation of the molecular abnormalities determining the development and progression of the most aggressive DTCs has helped to define novel targeted approaches against this subgroup of thyroid tumors. In particular, alterations of the RET-ras-raf-MAP kinase pathway, especially when associated with simultaneous activation of the PI3K–Akt pathway, characterize most of the less-differentiated thyroid tumors, unable to concentrate the iodine and therefore refractory to radioiodine treatment (Schlumberger & Sherman 2012). Indeed, a direct link has been demonstrated between the presence of ras, BRAF, and RET/PTC activating alterations and the loss of the sodium/iodide symporter expression and/or function in the thyrocyte (Trapasso *et al*. 1999, Puxeddu *et al*. 2008). In any case, among the novel therapeutic approaches emerging from the results of the clinical trials including the radioiodine-refractory metastatic DTC (www.clinicaltrials.gov), the kinase inhibitors of the above-mentioned pathways do represent the most promising new targeted agents (Schlumberger & Sherman 2012). However, the clinical trials have also revealed that the kinase inhibitors, even when initially effective, do not fully cure all DTC patients; moreover, there is yet a percentage of patients unresponsive to such a treatment, which is not completely free of side effects (Gild *et al*. 2011, Harris & Bible 2011, Bible 2012). Thus, the search for novel approaches, other molecular targets, and potential new drugs for a better management of these unresponsive tumors is necessarily ongoing. Selected cytotoxic drugs are still demonstrating effectiveness in individual patients (Crouzeix *et al*. 2012, Spano *et al*. 2012), and the possibility to reduce the effective dosage to minimize their side effects by improving their delivery into cancer cells is also currently under investigation (Celano *et al*. 2004, Paolino *et al*. 2010). In addition, there are other molecular alterations that have been and are being taken into consideration as targets for novel treatments: among them, emphasis has been given to epigenetic changes involving DNA methylation or histone acetylation (Xing 2007, Russo *et al*. 2011) and, more recently, to damages induced by ROS production resulting by oxidative stress (Xing 2012). Interestingly, the ROS protumorigenic action has been proposed to be associated with the activation of MAPK- and Akt-dependent pathways (Xing 2012). In this view, antioxidant molecules appear as good candidates as therapeutic agents for refractory DTC hosting such alterations. Among the 'natural' antioxidant compounds under investigation as anticancer agents for various neoplasia, we focused our attention on OLE, a phenolic derivative of virgin olive oil, whose antioxidant activity and antiproliferative effects have been demonstrated in many preclinical models of cancer disease (Visioli *et al*. 2002, El & Karakaya 2009, Cicerale *et al*. 2010).

Herein, we demonstrate that OLE is able to reduce the proliferation of TPC-1 and BCPAP thyroid cancer cells at micromolar concentrations, doses similar to those used in other tumor cellular models (Goulas *et al*. 2009, Notarnicola *et al*. 2010), while only mild effects have been detected in the non-tumor TAD-2 cell line. This effect is associated with a reduction of H_2O_2-induced ROS levels and phosphorylation of ERK and Akt. Regarding the latter finding, it should be remembered that TPC-1 and BCPAP cells host the RET/PTC rearrangement and *BRAF* V600E mutation respectively (Ribeiro *et al*. 2008, Schweppe *et al*. 2008), two genetic alterations detected in PTCs resulting in the oncogene-driven activation of the growth-promoting and anti-apoptotic pathways occurring in thyroid tumorigenesis (Knauf & Fagin 2009). Interestingly, the capacity of OLE to block ERK activation has also been reported in breast cancer MCF-7 cells stimulated by estradiol (Sirianni *et al*. 2010).

Moreover, our data show that OLE effects are further improved by its peracetylation. Such a chemical modification has been previously demonstrated to improve its

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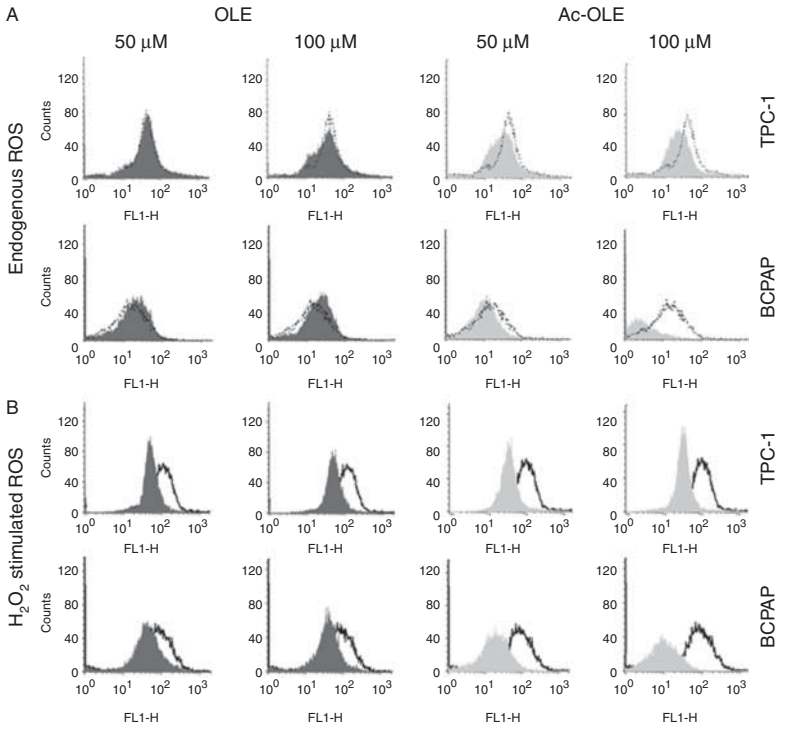

Figure 6

ROS determination in TPC-1 and BCPAP cell lines. Cells were pretreated for 48 h without or with the indicated concentrations of OLE and Ac-OLE, incubated with H_2DCF-DA and the fluorescence of DCF was evaluated by flow cytometry. (A) Endogenous ROS levels. (B) ROS levels were evaluated after oxidative stimulus (H_2O_2, 100 μM) for 30 min (dotted areas, no treatment; open space areas, H_2O_2; dark grey areas, OLE; light grey areas, Ac-OLE).

permeability (Procopio *et al*. 2009), still maintaining the biological antioxidant activity of the parent compound, probably because of extensive deacetylation of hydroxytyrosol acetate by carboxylesterases. Such deacetylation can take place either within the cell, upon absorption of the acetylated molecule, or in the extracellular space by secreted esterases (Mateos *et al*. 2005). Furthermore, the abundance of OLE in olive leaves, a discard product of the olive oil production industry, together with the Green Chemistry-based technique of extraction and semi-synthetic processes, accomplish many Green Chemistry tasks and demonstrate the possibility to develop sustainable complex protocols using some non-conventional techniques. It is noteworthy to remind the absence of toxicity of OLE, as documented by *in vivo* studies (Hamdi & Castellon 2005, El & Karakaya 2009, Cicerale *et al*. 2010). Enrichment of olive oil or other food components with such compounds has been already efficaciously tested,

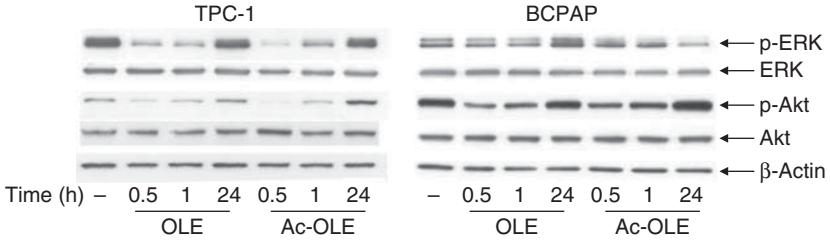

Figure 7

Effects of OLE and its peracetylated form, Ac-OLE, on ERK and Akt phosphorylation. TPC-1 and BCPAP cells were treated in the absence or presence of the compounds at 100 μM. Immunoblots were performed as described in the Subjects and methods section. At the indicated time, cell lysates were prepared and analysis of active, phosphorylated ERK (p-ERK) and phosphorylated Akt (p-Akt), and total form of the enzymes (ERK, Akt), was performed by western blotting. Protein load was checked by β-actin immunostaining.

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providing preliminary useful information in view of their administration to patients (Markopoulos *et al*. 2009, Achat *et al*. 2012). Further studies, using *in vivo* experimental models of thyroid tumors, will shed more light on the possibility of including these molecules in the class of targeted therapeutics to test alone and/or in combination for treatment of radioiodine-refractory DTCs.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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