Peer-reviewed version available at *Nutrients* **2018**, *10*, 1950; doi:10.3390/nu10121950

Oleuropein, the main polyphenol of *Olea europaea* leaf extract, has an anti cancer effect on human BRAF melanoma cells and potentiates the cytotoxicity of current chemotherapies

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Abstract

Oleuropein (Ole), a secoiridoid glucoside present in *Olea europaea* leaves, gained the interest of many scientists thanks to its several biological properties, including the anticancer one. We verified whether Ole might potentiate cytotoxicity of conventional drugs used to treat melanoma, disclosing new potential therapeutic strategy.

We tested the cytotoxic action of Ole alone or in combination with chemotherapeutics on A375 human melanoma cells. We found that Ole was able, at a dose of 500 µM, to stimulate apoptosis in melanoma cells, while at a non-toxic dose of 250 µM, it affected cell proliferation and induced the downregulation of pAKT/pS6 pathway. 250 µM Ole did not potentiate the effect of Vemurafenib (PLX4032), but it succeeded in increase the cytotoxic effect of Dacarbazine (DTIC). The mayor effect was found in the association between Ole and Everolimus (RAD001), also on PLX4032-resistant BRAF melanoma cells, possibly cooperating in the inhibition of pAKT/pS6 pathway. Of interest, an olive leaf extract enriched in equimolar Ole was more effective and able to further improve DTIC and, particularly, RAD001 efficacy on BRAF melanoma cells than Ole alone.

Therefore, Ole represents a natural product able to potentiate a wide array of chemotherapeutics against BRAF melanoma cells affecting pAKT/pS6 pathway.

Keywords: BRAF melanoma, chemotherapeutics, extra virgin oil, Oleuropein, olive leaf extract.

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Introduction

Melanoma originates in skin and although represents one of the rarer forms of skin cancer, underlies the majority of skin cancer-related deaths [1] whose incidence is more than duplicated in the last 10 years. The most common oncogenic mutation in melanoma occurs in the serine/threonine kinase B-RAF gene at the 600 position, where a valine is replaced by either an arginine (V600K) or glutamic acid (V600E). Many studies have observed constitutive activation of extracellular signal-regulated kinases (ERK) signaling in nude mice harboring the B-RAFV600E mutation, leading to higher rates of proliferation and transformation as consequences [2–6]. Although this mutation commonly occurs in melanoma, it should be noted that the mutation itself is not sufficient to cause cancer since it is also found in benign melanocytic lesions [1,7,8]. Likewise, the phosphoinositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway is also involved in melanoma genesis, and its activation often leads to increased cell survival, proliferation, and motility [9]. Current protocol for melanoma treatment is dependent on the condition of the tumor at time of disease detection; if diagnosed early, melanoma may be removed by surgery, but if it has spread to lymph nodes, surgery will be more invasive and chemo- and immunotherapy will be associated to the treatment. Unfortunately, these therapies may undergo patient resistance and generate host tissue damages. In particular, intrinsic resistance to apoptosis of melanoma cells is one of the main causes of anticancer therapy failure. Until recently, the prognosis for advanced malignant melanoma was poor, but the discovery of the major pathways involved in melanoma progression and resistance prompted the use of new therapeutic agents.

Therefore, new strategies targeting melanoma cells, also reducing resistance and patient side effects, need to develop and a combination of conventional treatment with biological agents (the so-called complementary therapy) might be an important breakthrough.

Mediterranean diet is considered an important preventive instrument against chronic diseases, such as neurodegenerative and cardiovascular diseases, and cancer [10–12]. In particular, epidemiological studies indicate that dietary consumption of extra virgin olive oil (EVOO) has a protective effect in Mediterranean populations[13–16]. EVOO is a functional food with a high level of monounsaturated fatty acids and a minor of highly bioactive multiple components, including polyphenols, to which have been mainly attributed the beneficial effects [17–19]. Phenol composition of olive oil includes the phenolic alcohols HT (3,4dihydroxyphenylethanol, 3,4-DHPEA, DOPET) and tyrosol (p-hydroxyphenylethanol, p-HPEA) together with their secoiridoid precursors. The main secoiridoid in olive oil is

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3,4-dihydroxyphenylethanol-elenolic acid (3,4-DHPEA-EA), whose glycated form is also known as oleuropein, the main responsible of the bitter taste of olive leaves and drupes. Ole has been reported to have many pharmacological properties, among which antioxidant, anti-inflammatory, cardioprotective, neuroprotective and hepatoprotective [20,21]. Recently accumulating *in vitro* and *in vivo* experiments together with epidemiological and clinical data, have provided support to the anti-tumor properties of Ole toward different tumor histotypes, such as breast, colon and lung cancer [22,23]. What is of translational importance, Ole was found to be a powerful sensitizer of Doxorubicin-mediated killing of prostate and breast cancer cells [24,25]. In fact, it lowers the cytotoxic dose of Doxorubicin, while producing an anti-proliferative effect in cancer cells.

Aim of our work is to verify both Ole cytotoxic effect and its synergistic action with the current drugs used in BRAF melanoma. We have found that Ole promote cytotoxicity of Dacarbazine (DTIC), a guanine methylating agent, whose treatment, was approved by the Food and Drug Administration (FDA) and Everolimus (RAD001), mTOR inhibitor, and that its combination with RAD001 was also an effective strategy in treating Vemurafenib (PLX4032) (a BRAF inhibitor) - resistant BRAF melanoma cells. Overall, these findings disclose the wide possibility to use Ole in the complementary therapy of melanoma.

Materials and methods

Cell lines and culture conditions.

In this study, we used A375 human melanoma cell lines, obtained from American Type Culture Collection (ATCC, Rockville, MD). In some experiments we used also the human melanoma cell lines WM266-4 (from ATCC) and M21 (kindly provided by Dr. Antony Montgomery,The Scripps Research Institute, La Jolla, CA). Melanoma cells were cultivated in Dulbecco's Modified Eagle Medium high glucose (DMEM 4500, EuroClone, MI, Italy) supplemented with 10% fetal bovine serum (FBS, Boerhinger Mannheim, Germany), at 37°C in humidified atmosphere containing 90% air and 10% CO2. Viability of the cells was determined by trypan blue exclusion test. Cultures were periodically monitored for mycoplasma contamination using Chen's fluorochrome test.

A375 melanoma cells resistant to PLX4032 were kindly provided by Laura Poliseno from University of Pisa and they were obtained as explained in reference [26]. PLX4032 resistant A375 melanoma cells were maintained without PLX4032 overnight before the start of the experiment.

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According to the experiments, cells were treated with Oleuropein glucoside (purity ≥ 90%) (Extrasynthese S.A., Lyon, Nord-Genay, France), DTIC (Sigma Aldrich, Milano), RAD001 (MedChem Express, Stockholm, Sweden) or PLX4032 (MedChem Express, Stockholm, Sweden).

MTT assay

Cell viability was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay (Sigma Aldrich, Milano). Cells were plated into 96-multiwell plates in complete medium without red phenol. The treatment was added to the medium colture at different dose and times, according to the experiment. Then the MTT reagent was added to the medium and plates were incubated at 37°C. After 2 h, MTT was removed and the blue MTT–formazan product was solubilized with Dimethyl sulfoxide (DMSO) (Sigma Aldrich, Milano). The absorbance of the formazan solution was read at 595 nm using the microplate reader (Bio-Rad).

Sample preparation for Mass Spectrometry Analysis

Cells were washed with ice cold PBS containing 1 mM Na4VO3, scraped in PBS, centrifuged for 5 minutes at 1200 rpm and lysed with ice cold water. Cells were sonicated 3 times for 5 minutes and supernatants were collected for Mass Spectrometry Analysis.

The samples were measured using analytical HPLC coupled to API 4000 (AB SCIEX, Toronto, Canada) equipped with the TurboIonSpray source operated in negative ion mode. The capillary voltage of the mass spectrometer was set to -4500 V, the "turbo" gas flow was 10 L/min of air heated at 400°C. The following transitions were monitored in MRM mode (Multiple Reaction Monitoring): m/z 153.1>123.1 for HT; 377.4>307.3 for oleuropein aglycone; 539.5>275.3 for Ole. Optimal CE (Collision Energy) and CXP (Collision Cell Exit Potential) were found at -18 Volts and -8 Volts for HT; -16 V and -6 V for both Oleuropein aglycone and Ole, respectively. The resulting DP (Declustering Potential) was -70 Volts. The chromatographic experiments were undertaken by using a Series 1290 Infinity LC System (Agilent Technologies, Waldbronn, Germany) HPLC Capillary Pump coupled to an Agilent Micro ALS autosampler, both being fully controlled from the API 4000 data system. Liquid chromatography was performed using a Zorbax eclipse C18 3x150mm, 3.5 µm HPLC column (Agilent Techonologies, Waldbronn, Germany). Column flow was 0.4 mL/min using a water/acetonitrile (50:50) and 0.05 % formic acid in an isocratic elution system. The eluent from the column was directed to the TurboIonSpray probe without split ratio.

Plate colony forming assay

Approximately 100 cells/ml surviving the different treatments were selected by trypan blue exclusion test, seeded in fresh medium and incubated for 10 days at 37°C. Cells were washed with

PBS, fixed in cold methanol and stained using a Diff Quik kit (BD Biosciences). The stained colonies were photographed with a digital camera and the number of colonies in each well was counted.

Cell cycle analysis

Cell cycle distribution was analyzed by the DNA content using propidium iodide (PI) staining method. Cells were centrifugated and stained with a mixture of 50 µg/ml PI (Sigma-Aldrich, St. Louis, Missouri), 0.1% trisodium citrate and 0.1% NP40 (or triton x-100) in the dark at 4°C for 30 min. The stained cells were analyzed by flow cytometry (BD-FACS Canto) using red propidium-DNA fluorescence.

Western blotting analysis

Cells were washed with ice cold PBS containing 1 mM Na4VO3 and lysed RIPA lysis buffer (Merk Millipore, Vimodrone, MI, Italy) containing PMSF (Sigma-Aldrich), sodium orthovanadate (Sigma-Aldrich) and protease inhibitor cocktail (Calbiochem). Aliquots of supernatants containing equal amounts of protein in Laemmli buffer were separated on Bolt® Bis-Tris Plus gels 4-12% precast polyacrylamide gels (Life Technologies, Monza, Italy). Fractionated proteins were transferred from the gel to a PVDF nitrocellulose membrane using iBlot 2 system (Life Technologies, Monza, Italy). Blots were stained with Ponceau red to ensure equal loading and complete transfer of proteins, and then they were blocked for 1 hours, at room temperature, with Odyssey blocking buffer (Dasit Science, Cornaredo, MI, Italy). Subsequently, the membrane was probed at 4°C overnight with primary antibodies diluted in a solution of 1:1 Odyssey blocking buffer/T-PBS buffer. The primary antibodies were: rabbit anti poly (ADP-ribose) polymerase (PARP)1 and cleaved PARP1 (1:1000, Cell signaling Technology, Danvers, MA, US), rabbit anti-cleaved caspase 3 (1:1000, IDT, Tema Ricerca, Castenaso BO, Italy), rabbit anti pAKT (1:1000, Cell signaling Technology, Danvers, MA, US), rabbit anti AKT (1:1000, Cell signaling Technology, Danvers, MA, US), rabbit anti pERK (1:1000, Cell signaling Technology, Danvers, MA, US), rabbit anti ERK (1:1000, Cell signaling Technology, Danvers, MA, US). The membrane was washed in T-PBS buffer, incubated for 1 hour at room temperature with goat anti-rabbit IgG Alexa Flour 750 antibody or with goat anti-mouse IgG Alexa Fluor 680 antibody (Invitrogen, Monza, Italy), and then visualized by an Odyssey Infrared Imaging System (LI-COR® Bioscience). Mouse anti-β-Tubulin monoclonal antibody (1:1000, Cell signaling Technology, Danvers, MA, US) was used to assess equal amount of protein loaded in each lane.

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Olive leaf extract's preparation:

Plant material

Olea europaea L. (cultivar Moraiolo), organic green leaves, were collected in April 2018 in Tuscany (Vinci, Florence) and immediately processed.

Solvents and reagents

All the solvents (HPLC grade) and formic acid (ACS reagent) were purchased from Aldrich Chemical Company Inc. (Milwaukee, Wisconsin, USA). Tyrosol, luteolin 7-O-glucoside, chlorogenic and Ole were obtained from Extrasynthese S.A. (Lyon, Nord-Genay, France). The HPLC-grade water was obtained via double-distillation and purification with a Labconco Water Pro PS polishing station (Labconco Corporation, Kansas City, USA).

Extraction and lyophylisation

The extraction using 15% of *Olea* leaves (45g leaves/300 g double-distilled and purified water), was performed in water at a temperature of 50°C for 60 min and at room temperature over the night (12 h) [27]. The final powder is obtained by lyophilization with the LYOVAC GT 2, freeze-drying yield 1.85%.

Sample preparation for Mass Spectrometry Analysis

The identity of the phenolic compounds of *Olea* dry extract powder and the composition of the solution used for the test in vitro, enriched in Oleuropein, was ascertained using data from the HPLC/DAD and HPLC/MS analyses, in accordance with a previous paper [28].

Statistical analysis

Densitometric data are expressed as means ± standard errors of the mean (SEM) depicted by vertical bars from representative experiment of at least three independent experiments. Statistical analysis of the data was performed by ANOVA and Tukey's multiple comparisons test, and $p \leq 0.05$ was considered statistically significant.

Results

Ole efficacy on BRAF melanoma cells

The effect of Ole on the cell viability was assessed on BRAF mutant (V600E) A375, WM266-4 and M21 melanoma cells following incubation for 72 hours with an Ole concentration, ranging from 50 to 800 µM (corresponding to ~ 25-400 µg/ml), using the MTT assay protocol. At 500-800 µM Ole induced a very toxic effect able to reduce almost totally the viability of all melanoma cell lines (Fig. S1a); at 250 µM (~125 µg/ml) Ole caused a different but significantly decrease of viability (about 30% in A375, 50% in WM266-4 and 0% in M21, respectively). Starting from these results, we

decided to use, for the later experiments, the A375 melanoma cell line, in which 250 µM Ole showed an intermediate toxic effect. In consideration of the anticancer effects of Ole might be due to HT or its other metabolites [23,29,30], we performed some experiments to determine the presence of intracellular phenolic compounds after incubation from 15 min to 72 hours with 250 µM Ole in A375 cells. As shown in the figure S1b, only Ole glucoside was detected in the cytoplasm by mass spectrometry analysis already at 15 min of incubation and was present without being metabolized until the 72nd hour of exposure (data not shown).

Prompted by MTT assay results, we verified that using a dose of 500 µM Ole, but not 250 µM, a significant percentage of A375 melanoma cells undergoing apoptosis at 48 h, as demonstrated trough cytofluorimetric technique, (Fig. S2b). Based on these results, melanoma cells were exposed for 24-48 hours to a 250-500 µM Ole and level of apoptosis, assessed by western blotting of PARP1, confirmed that only 500 µM Ole was able to promote the expression of a significant level of cleaved PARP1 after 48 hours of treatment (Fig. S2c). With the aim to verify whether Ole might potentiate drug efficiency on melanoma cells, we decided to use a non-toxic 250 µM dose which affects cell viability, evaluated by MTT (Fig. S2a), without inducing cell apoptosis. On the other hand, 250 µM Ole reduces cell proliferation rate of treated melanoma cells (Fig. S2d) and inhibits pAKT/mTOR pathway, assessed by the decrease of AKT/S6 phosphorylation (Fig. S2e).

These results suggest that Ole alone might be effective in treating BRAF melanoma cells disclosing the possibility to analyze whether this polyphenol may also improve drug efficacy of advanced melanoma.

Combination of Ole with Vemurafenib (PLX4032)

First, we decided to investigate whether Ole might potentiate the activity of PLX4032, used for targeted therapy of $BRAF^{V600E}$ melanoma. Although this drug shows a very high clinical efficiency in metastatic melanoma patients harboring BRAF V600E mutation, a wide type of BRAF melanoma tumors do not respond to PLX4032 or, in some cases, important clinical side effects limit the use of this drug, such as skin lesions and even cutaneous squamous cell carcinoma. Furthermore, due to genetic heterogeneity of melanoma patients, the development of drug resistance represents a general phenomenon upon PLX4032 treatment. Several mechanisms of resistance to BRAF inhibitors have been proposed and most of them are based on pAKT/pS6 hyperactivation, the same pathway affected by Ole. Unfortunately, we did not find any potentiation of Ole on PLX4032 activity on A375 cells, as showed the results obtained testing cell viability and efficiency to form colonies (Fig. 1a and b)

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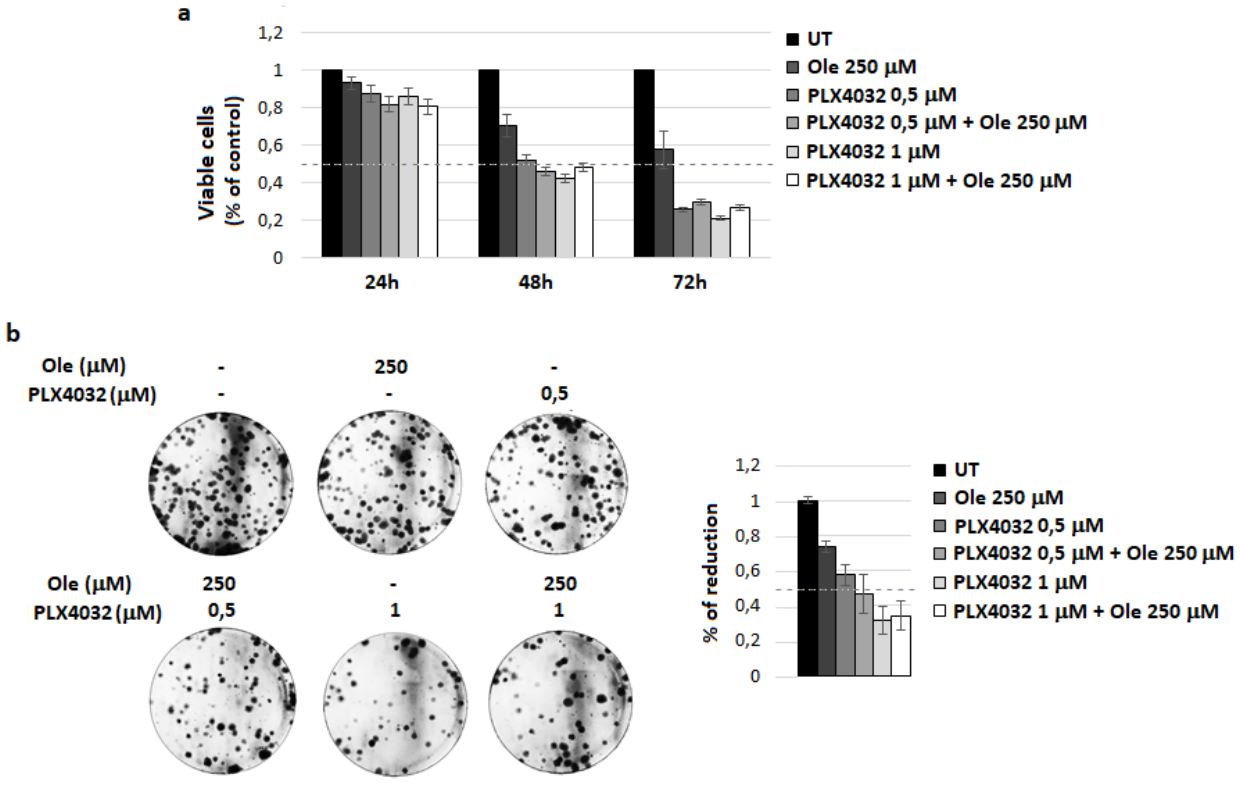

Figure 1. Ole-Vemurafenib (PLX4032) efficacy on A375 melanoma cells

a) Dose-time response evaluated by MTT assay; **b)** Colony Forming Units (CFU) assay of alive cells selected by trypan blue exclusion test after 250 µM (~125 µg/ml) Ole and/or d treatment for 72h; (right) Quantification data of the reduction of colony numbers respect to control.

Combination of Ole with Dacarbazine (DTIC)

DTIC, a DNA-methylation agent, is considered the main drug for the treatment of the advanced stage of melanoma. However, response rates are low, and 95% of the responses are partial with a 6-year median survival of 2%. Since the original approval of DTIC in 1975 for the treatment of metastatic melanoma, considerable effort has been expended in attempts to improve survival. However, treatment based on the combination of this agent with other cytotoxic drugs did not result in response rates of durable remission, enough to increase the median survival of the patients [31].

Combination of 250 µM Ole plus DTIC (270 and 550 µM corresponding to ~ 50 and 100 µg/ml) leads to a significant reduction in cell viability respect to the single treatments, particularly evident at 72 h of incubation (Fig. 2a). This finding was confirmed by the level of clonogenicity suggesting the effectiveness of Ole plus DTIC in reducing cloning efficiency of melanoma cells (Fig. 2b). Furthermore, combo treatment elicited a clear expression of cleaved PARP1 and caspase 3, signifying a promotion of apoptosis on treated cells. As above shown, Ole, by itself, reduced pAKT expression

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without any modification in pERK level, but when it was added to DTIC a significant and more pronounced reduction in pAKT was found (Fig. 2c), corroborating the hypothesis of a potential synergism of Ole with DTIC in pAKT pathway inhibition.

Overall, Ole cooperates with DTIC in melanoma treatment possibly participating in down regulation of pAKT signaling.

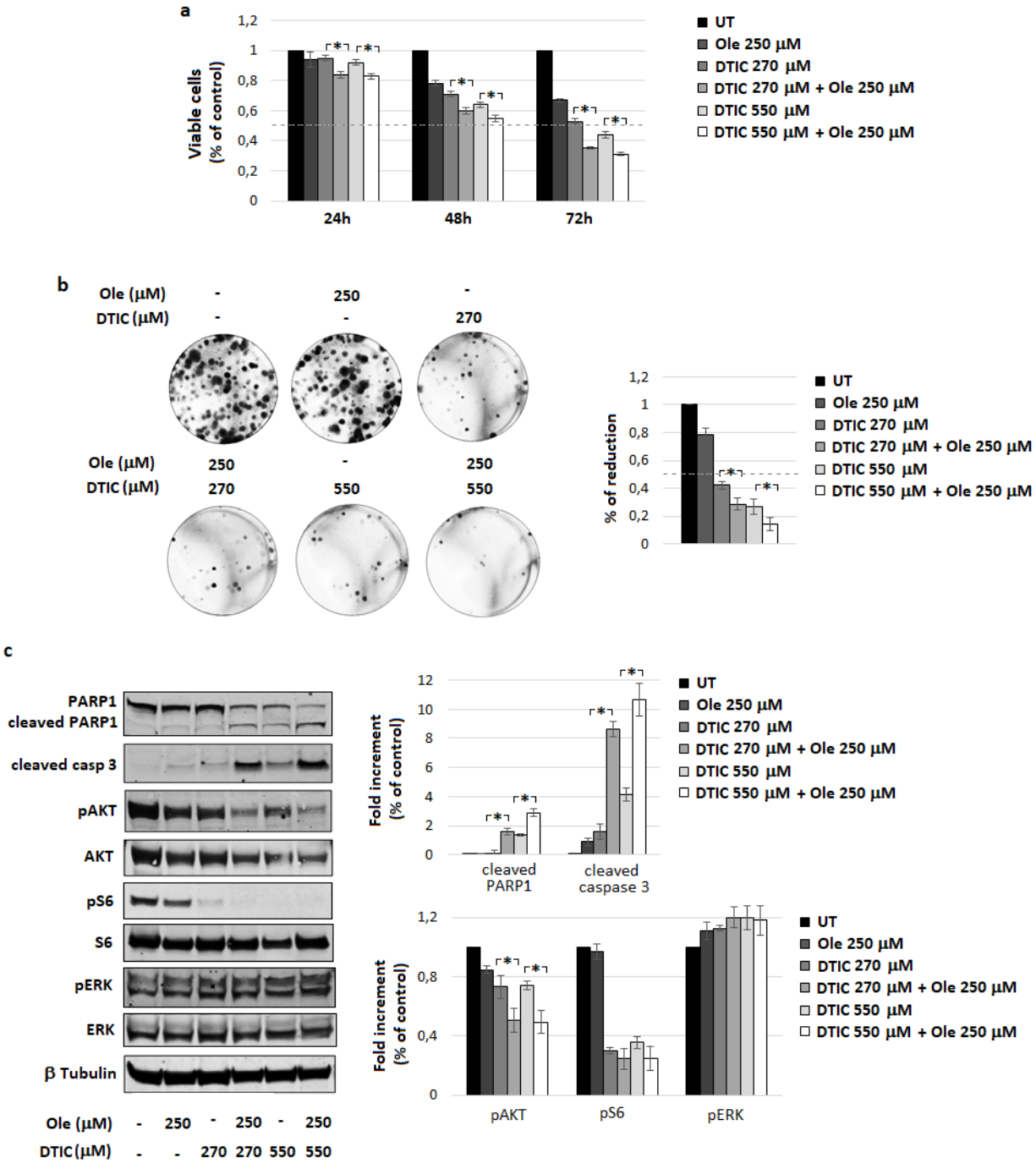

Figure 2. Ole-Dacarbazine (DTIC) efficacy on A375 melanoma cells

a) Dose-time response evaluated by MTT assay; **b)** Colony Forming Units (CFU) assay of alive cells selected by trypan blue exclusion test after the treatment with 250 µM (~125 µg/ml) Ole and/or DTIC 270 and 540 µM (~50 and 100 µg/ml) for 72h; (right) quantification data of the reduction of colony numbers respect to control; **c)** Western blot analysis of PARP1, cleaved PARP1, cleaved caspase 3, pAKT, AKT, pS6, S6, pERK and ERK after the treatment with 250 µM (~125 µg/ml) Ole and/or DTIC 270 and 540 µM (~ 50 and 100 µg/ml) for 48h. Levels of pERK and pAKT were quantified by densitometric analysis relative to AKT, S6 or ERK and β-Tubulin.

Significance is indicated with * and refers to the co-treatment Ole -DTIC compared to the treatment with DTIC alone.

Combination of Ole with Everolimus (RAD001)

In many cancers, including metastatic melanoma, PI3K/AKT signal transduction pathway regulates many basic cellular properties. mTOR inhibiting agents, including RAD001, are effective in inhibiting the PI3K/AKT/mTOR pathway, although several reports indicate that mTOR inhibitors have limited single-agent activity against melanoma [32]. Further, our recent research effort demonstrates that RAD001 might overcome PLX4032 resistance acquired by melanoma cells grown under a low extracellular pH [33]. These indications prompted us to study a possible cooperation between Ole and RAD001 and we found that Ole is a potent promoter of RAD001 cytotoxicity (Fig. 3a), also confirmed by a significant reduction of cloning efficiency of combo treated melanoma cells compared to single treatments (Fig. 3b). The enhanced cell death after combo treatment correlates with an enhanced level of cleaved PARP1 and caspase 3 (Fig. 3c). Further, Ole potentiates the inhibition of pAKT expression exerted by RAD001 at the 10 µM concentration (9,5 µg/ml) (Fig. 3c). In addition, Ole potentiation of RAD001 treatment might be useful to overcome PLX4032 resistance. In order to confirm this phenomenon, we used A375 PLX4032-resistant melanoma cells established by Dr. Poliseno [26], during a long period of exposure of A375 cells to PLX4032. These resistant cells grow and proliferate in the presence of PLX4032 in a similar way to control cells (Fig. 4a). Further, in contrast with the parental A375 cells, which stop proliferate and accumulate in G1 phase of cell cycle following exposure to PLX4032, resistant cells in the presence of the same amount of drug do not show any modification in cell cycle distribution (Fig. 4b). Considering signaling pathways, we found that, resistant cells express a higher level of AKT/S6 pathway and an unchanged level of pERK differently from PLX4032-treated cells, which instead undergo a reduction of pERK (at 2 h of treatment) and pS6 (at the 24 h of treatment) (Fig. 4c). When we determined the capacity of RAD001-Ole to affect resistant cells, we observed a higher percentage of death cells respect to that

found after the treatment with RAD001 alone (Fig. 4d), suggesting that the special combination of Ole plus RAD001 could represent a new strategy to treat also chronic addicted PLX4032-resistant melanoma cells.

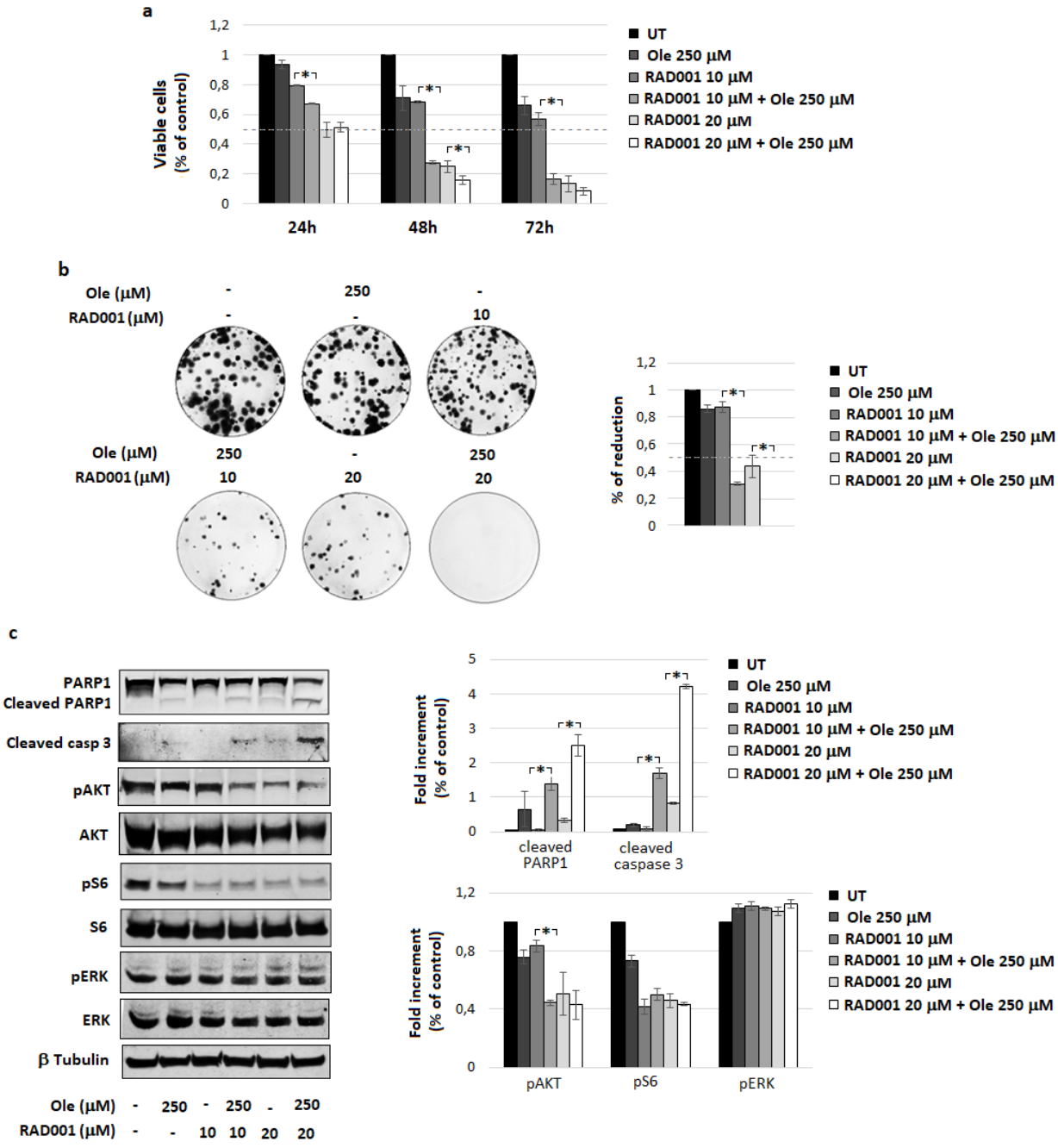

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Figure 3. Ole-Everolimus (RAD001) efficacy on A375 melanoma cells

a) Dose-time response evaluated by MTT assay; **b)** Colonies Forming Units (CFU) assay of alive cells selected by trypan blue exclusion test after 250 µM (~125 µg/ml) Ole treatment with and/or RAD001 10 and 20 µM (~9,5 and 19 µg/ml) for 48h; (right) quantification data of the reduction of colony numbers respect to control; **c)** Western blot analysis of PARP1, cleaved PARP1, cleaved caspase 3, pAKT, AKT, pS6, S6, pERK and ERK after 250 µM (~125 µg/ml) Ole treatment with and/or RAD001 10 and 20 µM (~9,5 and 19 µg/ml) for 24 h. Levels of pERK and pAKT were quantified by densitometric analysis relative to AKT, S6 or ERK and β-Tubulin.
Significance is indicated with * and refers to the co-treatment Ole -RAD001 compared to the treatment with RAD001 alone.

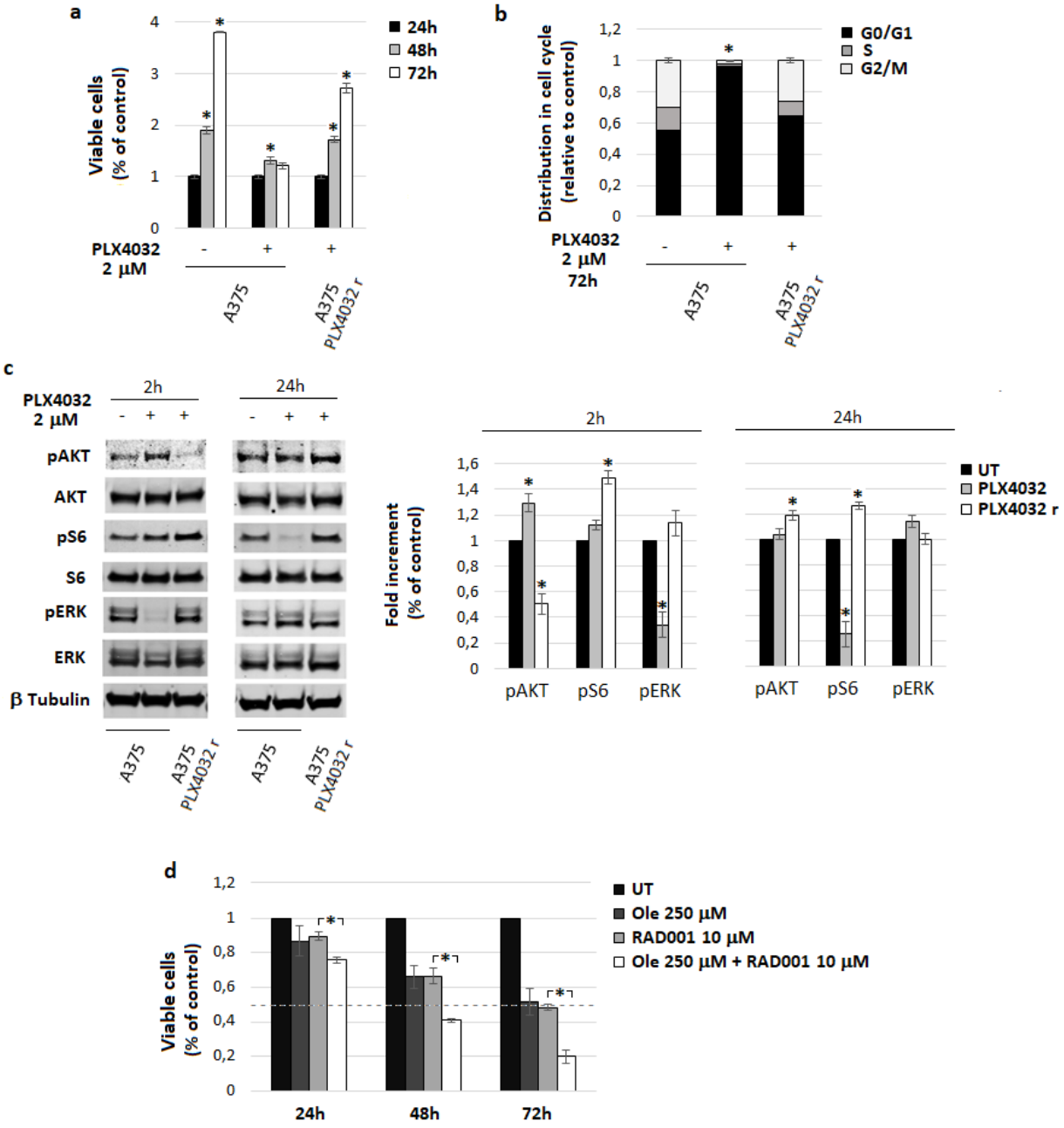

Figure 4. Characterization of A375 melanoma cells resistant to PLX4032 and effect of Ole-RAD001 treatment on these cells

a) Cell viability evaluated by MTT assay. Significance refers to the untreated control; b) Cell cycle distribution analyzed by FACS. Significance is indicated with * and refers to the untreated control; c) Western blot analysis of pAKT, AKT, pS6, S6, pERK and ERK in cells treated with PLX4032 2 μM (~980 ng/ml) for 2 or 24h. Levels of pERK, pS6 and pAKT were quantified by densitometric analysis relative to AKT, S6 or ERK and β-Tubulin. (Right) Representative Western blot panels. Significance is indicated with * and refers to the untreated control; d) Dose-time response evaluated on A375 PLX4032 resistant by MTT assay. Significance is indicated with * and refers to the co-treatment Ole -RAD001 compared to the treatment with RAD001 alone.

A freshly prepared olive leaf extract potentiates RAD001 treatment on BRAF melanoma cells.

This final investigation discloses the possibility that a freshly prepared olive leaf extract enriched in an equimolar concentration of Ole is endowed with the same or more ability to potentiate drug efficacy. Supplement Table S3 shows the dry extract composition of green leaves extract, enriched in Ole, with a final concentration of 51.99 % p/p (48.56% of secoiridoids (Oleuropein glucoside and Oleuropein aglycone); 0.92% hydroxytyrosol, tyrosol and derivatives; 1.93% of flavonoids; 0.57% of verbascoside and derivatives) while supplement Table S4 shows the quali-quantitative analyses of solution obtained by Olea powder extract after the solubilization of 56 mg of powder in 5 ml of physiological solution and used for the test in vitro.

The extract enriched in an equimolar concentration of Ole was more effective to potentiate DTIC and especially RAD001 cytotoxicity (Fig. 5a) compared to Ole alone (Fig 2a and Fig 3a). As reported in figure 5, the best combination in inducing cell death on BRAF A375 melanoma cells is represented by the Ole-enriched leaf extract, again, with RAD001, as shown by MTT assay and by the clonogenic assay (Fig. 5a and b). This finding confirms previous results adding a more potential translation impact to the clinic.

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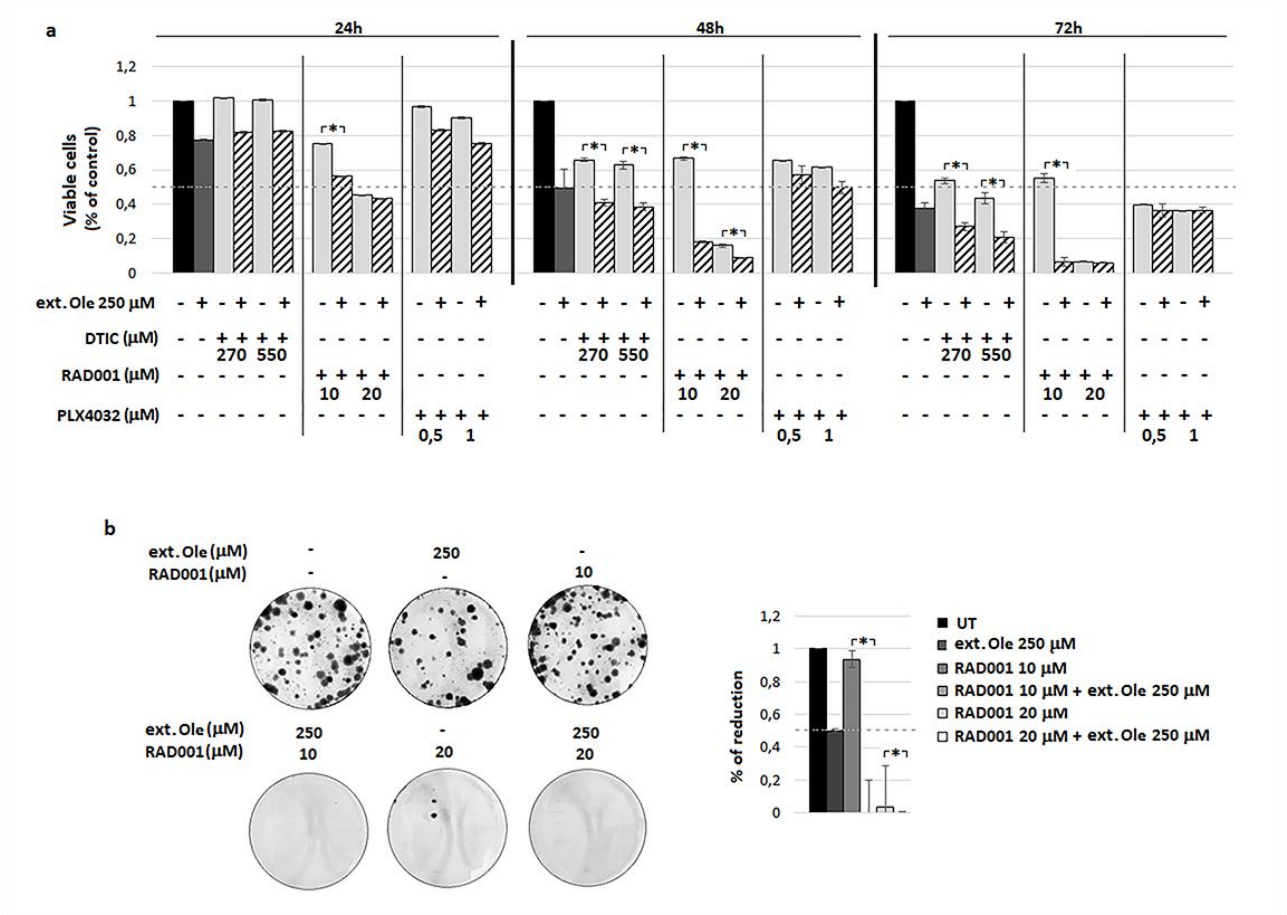

Fig. 4. Characterization of A375 melanoma cells resistant to PLX4032 and effect of Ole-RAD001 treatment on these cells

a) Cell viability evaluated by MTT assay. Significance refers to the untreated control; b) Cell cycle distribution analyzed by FACS. Significance is indicated with * and refers to the untreated control; c) Western blot analysis of pAKT, AKT, pS6, S6, pERK and ERK in cells treated with PLX4032 2 µM (~980 ng/ml) for 2 or 24h. Levels of pERK, pS6 and pAKT were quantified by densitometric analysis relative to AKT, S6 or ERK and β-Tubulin. (Right) Representative Western blot panels. Significance is indicated with * and refers to the untreated control; d) Dose-time response evaluated on A375 PLX4032 resistant by MTT assay. Significance is indicated with * and refers to the co-treatment Ole -RAD001

Discussion

The focus for cancer treatment has been shifted toward strategies of complementary therapy able to overcome the limitation of a single-agent treatment. Rational combination approaches are strongly preferred in order to improve the overall patient progression-free survival, overcome or delay the development of drug resistance and reduce the incidence of side effects. This is of special importance in the treatment of melanoma, particularly of the advanced metastatic form, often resistant to most of

the current drugs used in the clinic. Further, due to different transcription pathway activation in melanoma cells, several mechanisms of resistance also to BRAF inhibition have been identified. Melanoma tumors bearing wild type BRAF are intrinsically resistant to PLX4032 or Dabrafenib. Targeting MEK was consider a potential mechanism to overcome BRAF resistance [34], although the most favorable treatment schedule and sequence is still to be defined. Indeed, multiple levels of cross-talk among mitogen-activated protein kinase (MAPK) and PI3K/AKT pathways and the possibility that ERK can be phosphorylated by the pAKT pathway are demonstrated [35,36]. Thus, it is required to inhibit also AKT pathway in melanoma cells resistant to MAPK inhibitors [37]. Activation of PI3K/AKT/mTOR pathway represents one of the major mechanisms of acquired resistance to both targeted BRAF inhibitors and DTIC [38,39]. We have found that BRAF melanoma cells exposed to a low extracellular pH medium acquired a resistance to both BRAF and MEK inhibitors but were still sensitive to the inhibition of AKT/mTOR pathway induced by RAD001 [33]. It is possible, on the other hand, that during a prolonged mTOR inhibition, PI3K would be able to promote MAPK pathway through RAS activation. Overall, from these findings emerge the need to use a treatment involving the simultaneous inhibition of MAPK and AKT pathways in order to have a better drug efficiency and reducing drug resistance.

Although this anti cancer approach appeared very promising, it had not been as successful as believed. Indeed, most combined therapies are based on the combination of toxic compounds, leading to toxicity and unexpected side effects.

Given our previous evidence for the protective role of Ole in neurodegenerative and cardiovascular diseases [40–43], we have decided to investigate whether Ole might exert some role in melanoma treatment. The existing studies indicate that Ole express a well-demonstrated protective role against many types of cancer [22]. Most of the studies have investigated the anticancer effects of Ole on breast cancer, disclosing that the polyphenol may not only decrease cell viability and proliferation [44] and synergize with Doxorubicin in *in vitro* and *in vivo* model [25,45], but also may reverse resistance toward the chemotherapeutic agent, Trastuzumab [46]. In addition, Ole is effective in reducing cell proliferation increasing apoptosis in human colorectal cancer cells [47] and is able to sensitize the Doxorubicin-mediated killing of prostate cancer cells [24]. Ole also reduces cell viability of hepatocarcinoma, pancreatic, thyroid, neuroblastoma, mesothelioma and glioblastoma cancer cells [22]. Still to be clarified are Ole effects on BRAF human melanoma cells. Here, we demonstrate for the first time that Ole treatment represents a new non-toxic anti-cancer agent against BRAF melanoma cells as a suitable promoter of two major agents used in BRAF resistant melanoma cells, such as RAD001 and DTIC. In addition, the particular attitude of Ole to potentiate RAD001, was found appropriate to overcome PLX4032 resistance as demonstrated by the use of a

special PLX4032 resistant A375 melanoma cells developed in cultures. This finding discloses a complementary approach to therapy of BRAF inhibitor-resistant melanoma that harbor hyperactivation of AKT. Until now, only one study reported the reversing effect of Ole on chemotherapy-induced resistance [46]. Thus, these findings open up the chance to use Ole as a therapeutic molecule, due to its low toxicity in normal cells as previously reported [48], to improve the anticancer effects of current chemotherapeutics.

A375 melanoma cells, used in our study, and as most of human solid cancers (prostate, breast and colon cancer), express glucose transporter proteins GLUT1 and GLUT3 mRNA and protein, which likely may promote Ole uptake. Hamdi *et al*. found that the antiproliferative activity of Ole in normal fibroblasts was reduced by removing the glucose moiety by β-glycosidase. Furthermore D-glucose and Ole compete for the GLUTs, as it was demonstrated by the co-incubation of human melanoma cells with excess of D-glucose [49], so it is possible that GLUTs are involved in the transportation of Ole into cancer cells. In this study we found that Ole was present in the cytoplasm of A375 cells already at 15 min of incubation (Fig. S1b), suggesting a fast uptake of Ole inside the cells, probably due to their higher level of GLUTs than the normal ones. This is a very important aspect that we can exploit in thinking of a Ole topical application directly on tumor cells. However, we did not exclude that Ole could enter into the cells using other routes, in particular in areas of inflammatory reactions where several mediators are active.

Interestingly, Ole was able to induce in very short time (48h) a clear and significant induction of melanoma cell death, confirmed by an enhancement of markers of apoptosis. Ole affects viability of melanoma cells probably through the inhibition of phosphorylation of AKT and S6 pathway. This finding is in accordance with Liu's observation, which indicates that inhibition of AKT is the mechanism underlying the pro-apoptotic and anti-invasive process promoted by Ole in glioma cells [50] and with Yan's indication about the induction of apoptosis by Ole through PI3K/AKT pathway in HepG2 human hepatoma cell line [51].

Thus, we proceeded investigating whether Ole blocking AKT pathway may act synergistically with the current chemotherapy treatments and allow to decrease the doses that often lead to toxicity and severe side effects. We found that Ole potentiates the cytotoxic effect of DTIC reducing the effective dose by 50%; this might be related to an enhanced reduction in AKT phosphorylation. In parallel experiments, Ole was also able to potentiate an mTOR inhibitor such as RAD001, also in PLX4032-resistant melanoma cells. In the same way, Ole reduces the effective dose of RAD001 by 50% and this effect was linked to pAKT abrogation. Thus, Ole, by affecting the PI3K/AKT/mTOR pathway, might represent a new non-toxic agent of interest in the treatment of advanced melanoma. It is likely that Ole might boost up the inhibition of mTOR also through the AMPK/mTOR pathway, that in this

study was not investigated, but that was suggested by our previous finding and by other authors [42,52]. In addition, Ole may inhibit, tumor angiogenesis and *in vivo* tumor growth, respectively as recently found by Song's *et al.* [48] and Samara's *et al.* [53] studies.

Although the *in vitro* studies are very promising, they do not consider Ole metabolism and bioavailability, so that the *in vitro* used concentrations, despite in accordance with literature [25,54–57], could seem far greater than those that could be realistically achieved in *in vivo* models.

Ole inevitably undergoes a metabolic process *in vivo* models and after ingestion its metabolites were rapidly detected in plasma at different concentrations also depending on gender [58]; but in the clinic, most agents are typically given by repeated administration that may lead to accumulation [59], and this is quite closer to the high doses used in *in vitro* experiments.

Furthermore our results suggest that an easily available product as an olive leaf extract enriched in Ole could be even more effective than Ole alone, probably because of the co-presence of other polyphenols among which hydroxityrosol that many authors suggested to be the real active Ole metabolic product [23,29,30]. So olive leaf extracts seems to have a powerful anticancer property as also Samet I. *et al.* and Mijatovic SA *et al*. shown about human chronic myelogenous leukemia K562 and melanoma cells respectively [60,61], and their mixed phenolic composition with an enrichment in Ole could be useful to decrease the *in vitro* doses to obtain the same effect of Ole administration alone at higher concentration.

Netherless, polyphenols bioavailability is still a big drawback that many studies try to overcome through different approaches including the construction of granules containing probiotics and *Olea Europaea* extract in order to increase polyphenols bioavailability [62], and the synthesis of Ole analogs with various chemical properties and more active than Ole [53].

In conclusion, Ole, and even more olive leaf extracts, exhibited a promising potential as adjuvant in conventional anticancer therapies. Furthermore, it may reverse drug resistance of cancer cells to chemotherapeutics and reduce adverse effects of conventional therapies on nontarget cells.

The limited *in vivo* animal studies, as recently summarized by two reviews [22,63] and the paucity of in human studies, in particular randomized controlled clinical trials, still represents the major drawback. So preclinical evidence needs to be substantiated by an evidence-based approach to determine effective dose and best route of Ole administration, on the basis of its biodisponibility [64], and any side effects related to chronic administration.

Authors' contributions

L.C. and C.N. designed the experiments and wrote the paper, J.R. performed the experiments, S.P., E.A. and F.B. participated in experimental analyses, G.L.M. performed HPLC analysis of Ole's metabolites, A.R. and A.S. provided olive leaf extract and analyzed its composition. All authors performed critical review of the manuscript. All authors read and approved the final manuscript.

Funding

This study was financially supported by grants from Istituto Toscano Tumori and Ente Cassa di Risparmio di Firenze.

Conflict of interest

The authors declare that they have no competing interests.

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