Resveratrol downregulates Akt/GSK and ERK signalling pathways in OVCAR-3 ovarian cancer cells†‡

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Phytochemicals constitute a heterogeneous group of substances with an evident role in human health. Their properties on cancer initiation, promotion and progression are well documented. Particular attention is now devoted to better understand the molecular basis of their anticancer action. In the present work, we studied the effect of resveratrol on the ovarian cancer cell line OVCAR-3 by a proteomic approach. Our findings demonstrate that resveratrol down-regulates the protein cyclin D1 and, in a concentration dependent manner, the phosphorylation levels of protein kinase B (Akt) and glycogen synthase kinase-3β (GSK-3β). The dephosphorylation of these kinases could be responsible for the decreased cyclin D1 levels observed after treatment. We also showed that resveratrol reduces phosphorylation levels of the extracellular signal-regulated kinase (ERK) 1/2. Chemical inhibitors of phosphatidylinositol 3-kinase (PI3K) and ERK both increased the in vitro therapeutic efficacy of resveratrol. Moreover, resveratrol had an inhibitory effect on the AKT phosphorylation in cultured cells derived from the ascites of ovarian cancer patients and in a panel of human cancer cell lines. Thus, resveratrol shows antitumor activity in human ovarian cancer cell lines targeting signalling pathway involved in cell proliferation and drug-resistance.

Introduction

Ovarian cancer is one of the leading causes of cancer death worldwide. The mortality that results from this type of cancer is still high considering the enormous advances in the understanding of the underlying biology. In comparison with other cancer types, such as breast and colorectal cancers, the ovarian cancer death rate has remained unchanged between 1930 and 2005.1,2 The poor efficacy of the currently available therapies and the difficulty detecting cancer in the early phase can in part explain this high death rate. The diagnosis in the first stage is usually elusive; tumors are in fact diagnosed at an advanced stage when cancer cells have metastasized to the pelvic organs making difficult the complete eradication of secondary tumors. This scenario is further complicated by the failure of the current drugs, which show only a limited efficacy, even in chemo-sensitive tumors.3

Moreover, the presence of several histological types and subtypes with distinct clinical presentations and multiple genetic abnormalities contribute to make the picture so complex. In fact, more than seven signalling pathways are activated in ovarian cancer and several genetic abnormalities have been clearly detected.4

With this in mind, it seems clear that improvement in the current approaches with the identification of new therapeutic molecules is pivotal in the management of the disease. Ideally, to address the major clinical needs, these drugs should be able to (i) show therapeutic efficacy when used alone or in combination...
with the available drugs, in order to overcome chemoresistance, and (ii) target specific or multiple molecular pathways involved in the ovarian cancer physiopathology.

Today, a number of epidemiological and genetic evidence indicates that several types of cancer can be prevented by the lifestyle and an appropriate diet. More than 25 000 different bioactive components are thought to occur in foods and several of these components may arise from plants (phytochemicals).

Phenolic compounds comprise one of the largest groups of metabolites produced from plants to protect themselves against several stresses including reactive species and herbivores. Although of great importance for plant physiologists, phenolic compounds attracted the attention of scientists working on the topics related to cancer, due to their health benefits including the ability to interfere with different steps of the tumorigenesis.

Resveratrol (trans-3,4',5-trihydroxystilbene), a stilbene abundant in red grapes, responds to the description of a molecule with a potential clinical use, not only for the prevention of cancer but also for its treatment. In fact, extensive research during recent years has well described the ability of resveratrol to cause an increase in the expression of the enzyme inducible factor (VEGF) expression was also reported. All these properties are explained considering the uniqueness of resveratrol to modulate several signalling pathways that mediate invasion, metastasis and angiogenesis.

However, data regarding the properties of resveratrol in ovarian cancer are still limited and the mechanisms of action are not fully known. Previous works reported that resveratrol treatment induced autophagocytosis in ovarian cancer cells, activation of both ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia-Rad3-related) proteins, and down-regulation of the insulin- or serum-induced expression of eukaryotic elongation factor 1A2 (eEFIA2). Additionally, resveratrol causes an increase in the expression of the enzyme cyclooxygenase (COX)-2, associated with COX-2 nuclear localization, p53 phosphorylation and apoptosis. A role in limiting ovarian cancer progression by inhibiting hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) expression was also reported.

In this study, we use an approach that combines two-dimensional electrophoresis (2-DE) and mass spectrometry (MS/MS) to identify new potential targets of resveratrol action in ovarian cancer cell lines. We identified some proteins whose expression was significantly modulated by resveratrol treatment. In particular, the resveratrol-induced down-regulation of the protein cyclin D1 was further confirmed by western blotting. The phosphorylation level of the protein kinase B (Akt) and the combination of phosphatidylinositol 3-kinase (PI3K) and ERK inhibitors with resveratrol further enhanced resveratrol-effects on cell proliferation.

**Experimental methods**

**Chemicals**

Resveratrol was purchased from Sigma-Aldrich. A 100 mM stock solution in 70% ethanol of resveratrol was prepared and kept frozen (−20 °C). Phallolidin-fluorescein isothiocyanate (FITC) and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyles-trazolium bromide) were from Sigma-Aldrich. LY294002 and UO126 were from Santa Cruz. Both inhibitors were dissolved in DMSO and stored at −20 °C. The following reagents were obtained from GE Healthcare: IPG strips pH 3–10 NL, IPG (Imobilized PH Gradient) buffer 3–10, dry strip cover fluid and Hybond ECL membrane and ECL western blotting detection reagents. Anti-phospho-Akt threonine 308 (pAkt Thr308) and anti-phospho-GSK-3 tyrosine 279 and tyrosine 216 (pGSK3 Tyr279/Tyr216) were from Millipore. Anti-phospho-mTOR serine 2448 (pmTOR Ser2448), anti-ERK1/2 and anti-phospho-ERK 1/2 threonine 202 and tyrosine 204 (pERK Thr202/Tyr204) were from Cell Signaling. All other primary and secondary antibodies (HRP-conjugated) were from Santa Cruz. All other reagents were from standard commercial sources and were of the highest grade available.

**Cell cultures and ascites-derived tumor cells**

The human ovarian cancer cell lines OVCAR-3 and SKOV-3, the human breast cancer cell line MCF-7, the human cervical cancer cell line HeLa, the human osteosarcoma cell lines MG-63 and SAOS2, and the human neuroblastoma cell lines SHSY-5Y and IMR32 were all cultured in DMEM medium supplemented with 10% foetal bovine serum (FBS), 100 U mL⁻¹ penicillin and 100 μg mL⁻¹ streptomycin at 37 °C in an atmosphere of 5% CO₂.

Ascites were collected from five chemotherapy-naïve patients, who ranged in age from 51 to 72 years, diagnosed with serous adenocarcinoma and presented as stage III (FIGO classification). Following appropriate consent and according to institutional guidelines, ascites were collected at the time of clinical intervention. Approval for the study was obtained from the local Institutional Review Board. Ascites-derived tumor cells were isolated according to the protocol of Shepherd and collaborators. Cells were allowed to grow for several weeks and used for our research purposes after three passages.

Tumor cells derived from ascites grew in culture with the typical cobblestone-like morphology and maintained the expression of epithelial markers including cytokeratins, as detected by reverse transcription-PCR (RT-PCR) (data not shown). Only ascites-derived cells with these characteristics were further processed.

**Experiment design**

OVCAR-3 cells were treated with resveratrol at the concentrations of 25, 50, 100 and 200 μM for 30 min. Controls were treated with the same volume of 70% ethanol (vehicle). To evaluate the combined use of resveratrol with PI3K and ERK inhibitors, OVCAR-3 cells were treated with vehicle or treated...
with resveratrol (100 μM) and/or LY294002 (10 μM) and/or UO126 (10 μM) for 24 h. Ascites-derived tumor cells were treated with resveratrol at the concentrations of 25, 50, 100 and 200 μM for 48 h. HeLa, IMR32, MCF-7, SAOS-2, SHSY-5Y and SKOV-3 were treated with resveratrol at the concentrations of 50, 100 and 200 μM for 24 h, 48 h and 72 h.

**Cell viability assay**

Cells were seeded at a density of $5 \times 10^3$ per well in a 96-well plate containing 100 μL of full medium and allowed to adhere to the plate overnight. For determining cell viability, the MTT assay was used. After treatment with resveratrol, the culture medium was aspirated and 100 μL of fresh medium containing 10 μL of MTT stock solution, 5 mg mL$^{-1}$ in phosphate-buffered saline (PBS) solution, was added to each well. Cells were then incubated for further 2–3 h. After removal of MTT solution, 100 μL of DMSO was added to the wells, maintaining in agitation for 15 min to dissolve MTT–formazan crystals. Absorbance of the converted dye was measured at a wavelength of 570 nm with background subtraction at 690 nm. The relative cell viability was expressed as a percentage of the untreated control wells.

**Confocal microscopy**

Exponentially growing ovarian cancer cells were seeded on 25 mm square glass cover slips placed in 35 mm diameter culture dishes. After treatment, cells were fixed for 5 min with 3.7% formaldehyde in PBS solution, permeabilized with a 0.1% solution of Triton X-100 in PBS, and incubated for 30 min in 25 mm square glass cover slips placed in 35 mm diameter culture dishes. After treatment, cells were fixed for 5 min with 3.7% formaldehyde in PBS solution, permeabilized with a 0.1% solution of Triton X-100 in PBS, and incubated for 30 min in agitation for 15 min to dissolve MTT–formazan crystals. Absorbance of the converted dye was measured at a wavelength of 570 nm with background subtraction at 690 nm. The relative cell viability was expressed as a percentage of the untreated control wells.

**2-DE and image analysis**

Cell pellets were resuspended in lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, protease inhibitor cocktail, 1 mM sodium orthovanadate, 10 mM sodium fluoride and sonicated on ice for three rounds of 10 s. The lysates were centrifuged for 15 min at 12 000 rpm to separate the undissolved cell debris. Protein concentration was determined using the Bradford protein assay (BioRad).

Protein 2-DE was performed as described before. In detail, protein samples (80 μg) were diluted up to 250 μL with rehydration buffer (7 M urea, 2 thiourea, 4% CHAPS (3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonate), 65 mM DTT and 0.5% v/v IPG buffer) and applied to IPG strips (13 cm, pH 3–10 NL). IEF (isoelectric focusing) and second dimension (12% SDS-PAGE) were performed on a Protean III xi Cell System (BioRad). After electrophoresis, gels were stained with Coomassie Blue and destained using water with 0.2% formic acid. The gels were then cut into small pieces and sent for in-gel trypsin digestion and LC-MS/MS analysis.

**Mass spectrometry analysis**

Protein bands were excised from gels, and cysteines were reduced with dithiothreitol and alkylated with iodoacetamide. After derivatization, the samples were incubated overnight at 37 °C with 20 μL of a buffer solution, 50 mM ammonium bicarbonate, that contained 10 mg mL$^{-1}$ sequence-grade trypsin (Sigma, St. Louis, MO). The reaction was stopped by adding a final concentration of 0.1% trifluoroacetic acid and by freezing gel plugs at −20 °C. After digestion, samples were used for protein identification by LC-MS/MS, using a CapLC system (Micromass, Waters) coupled on-line with a nano-ESI-Q-TOF instrument (Micromass, Waters). The sample (6 μL) was first concentrated into a Waters Symmetry 300 C18 5 μm OPTI-PAK trap column, 0.35 × 5 mm, by a carrier solvent (water with 0.2% formic acid) at 20 μL min$^{-1}$ for 3 min and subsequently, it was eluted at 0.2 μL min$^{-1}$ (using a pre-column splitter) on a C18 column LC-Packings DIONEX PepMap 5 μm, 100 Å 75 μm inner diameter × 250 mm, with a water/acetonitrile gradient in the presence of 0.2% formic acid. Argon was used as the collision gas. The collision energy was as a function of the precursor ion mass. MS/MS spectra were acquired by automatic switching between MS and MS/MS mode. Acquired MS/MS data were converted in a compatible format (PKL files) using default parameters of ProteinLinx 2.0 software (Micromass) and analyzed using Mascot MS/MS ion search engine (http://www.matrixscience.com) with SwissProt database. The query was restricted to the Homo sapiens (human) database. The maximal tolerance for peptide masses was 100 ppm and the maximal tolerance for MS/MS data was 0.3 Da, searching peptide charge of 2+ and 3+. At most one mis-cleavage for tryptic peptides was allowed, and the variable modification accepted was oxidation of methionines. Identifications were accepted based on significant Mascot Mowse scores over the threshold of 32.
Statistical analysis

Data were analysed using the statistical software GraphPad (version 4.0). The two-tailed Mann–Whitney t-test was used to test statistical significance. Differences were considered statistically significant for p-values < 0.05.

Pathway analysis

Network analysis was performed using the Ingenuity pathway analysis (IPA) software (www.ingenuity.com). This program builds protein networks based on known direct and indirect interactions described in the literature and defines common functional and canonical pathways. Significance was evaluated by exact Fisher’s test. A p-value of 0.01 corresponding to a score of 2 was considered the cut-off for the analysis.

RT-PCR

Total cellular RNA was isolated by Illustra TriplePrep extraction kit following the manufacturer’s instruction and immediately used. Purified DNA and protein pellet were stored at –80 °C for further analysis.

Total RNA (1 μg) was reverse transcribed into cDNA using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystem). PCR was conducted on a MyCycler thermal cycler (Bio-Rad). The final volume of 25 μL included 1 μL of cDNA template, 12.5 μL of PCR Master Mix (Promega), and 1 μL of a mix containing primers.

PCR was carried out using the following conditions: denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s. PCR samples were loaded onto a 1.2% agarose gel containing ethidium bromide and analysed. The primers used for PCR amplification were designed using the Primer blast program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were as follows: cyclin D1 sense 5′-CGGTTCTGTCGCTGGAGCC 3′, antisense 5′-CCTCTCGGCCTCAGGGGA 3′ (111 bp, Tm 60 °C); β-actin sense 5′-CCACGGCTTCCAGCTCC 3′, antisense 5′-GGAGGCGCCCGACTCGTCAT 3′ (422 bp, Tm 60 °C).

Western blot analysis

Whole proteins were extracted in RIPA buffer (50 mM Tris-base, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM sodium orthovananadate, 10 mM sodium fluoride, 1% protease inhibitor cocktail) and quantified by the Bradford protein assay. Samples were separated by 12% SDS-PAGE and transferred to the Hybond ECL nitrocellulose membrane. The membranes were blocked overnight in Blotto A (Santa Cruz) at 4 °C, and subsequently probed by the appropriately diluted primary antibodies for 2 h at room temperature. After three washes with a solution of 0.1% (v/v) Tween 20 in PBS, the blots were incubated with secondary antibody HRPC-conjugated for 2 h at room temperature (1 : 2000 dilution). Immunoblots were developed using the ECL system.

Results and discussion

To test the antiproliferative effects of resveratrol we selected the ovarian cancer cell line OVCAR-3, a chemoresistant cell line established from an ovarian adenocarcinoma of a treated patient; OVCAR-3 cells express high levels of PI3K.22

The treatment with resveratrol inhibited OVCAR-3 cell proliferation in a dose- and time-dependent manner (Fig. 1). This effect was more pronounced for drug-concentrations higher than 50 μM, while for lower concentrations, significant effects were observed only after prolonged treatments.

Phalloidin-FITC staining of OVCAR-3 cells was carried out to visualize actin morphological modifications after resveratrol treatment (Fig. 1b). Cells were treated with resveratrol at the concentration of 100 μM and observed after 24 h, 48 h and 72 h. Cells maintained under normal growth conditions showed a classic cobblestone epithelial morphology with close cell–cell contacts and a predominant actin staining in the cell borders. Actin cytoplasmic protrusions were also evident. After 24 h and 48 h of treatment, cells displayed a different morphology; cell–cell contacts are less evident as well as cytoplasmic protrusions. On the contrary, long echinoid protrusions became evident.

Fig. 1 Effects of resveratrol on cell proliferation and actin organization. (a) Resveratrol inhibits cell proliferation of human ovarian cancer cells. OVCAR-3 cells were treated with resveratrol for 24 h, 48 h and 72 h. Control cells (CTR) were treated with vehicle (70% EtOH). MTT test was used to measure cell proliferation. *p < 0.05; **p < 0.01; ***p < 0.001 compared with untreated group. (b) Ovarian cancer cells plated on glass cover slips were treated with resveratrol at the concentration of 100 μM for 24 h, 48 h, 72 h (B, C, D) and then fixed. Control cells were treated with vehicle (A). Cells were stained with phalloidin-FITC.
After 72 h of treatment, cells appeared rounded lacking the actin structures normally seen in control cells and having fully lost their cobblestone morphology.

To better characterise the molecular mechanisms of resveratrol action, total proteins were extracted from control and treated cells (100 μM, 24 h), subjected to proteome analysis by 2-DE and visualized by silver staining (Fig. 2). Spot analysis was performed using the software Image Master 5.0. Gels were divided in two groups that were subjected to spot detection, matching, and statistical analysis. Nineteen protein spots were found to be differentially expressed. Spots were manually excised, trypsin-digested and subjected to MS/MS analysis. Of these 19 spots, 9 were successfully identified, whereas the others remain unidentified probably due to their low-abundance levels.

Proteins identified by MS/MS were listed in Table 1 and ESI Table 1.†

Among these, the possible mechanism of cyclin D1 regulation is highlighted in this work, considering the clinical impact of this protein. In fact, the overexpression of cyclin D1 is frequently observed in epithelial ovarian cancer samples, and associated with a more aggressive tumor phenotype and poor prognosis.

Western blot analysis with a cyclin D1 specific antibody was performed to validate changes in cyclin D1 expression in OVCAR-3 cells in response to resveratrol. This experiment confirmed that resveratrol had an effect on cyclin D1 protein expression level; in contrast, resveratrol had no effect on cyclin D1 expression at the mRNA level, as determined by RT-PCR (Fig. 3a).
This discrepancy between the mRNA level and the corresponding protein level suggests that post-transcriptional mechanisms are involved in the regulation of cyclin D1 by resveratrol.

The proto-oncogene cyclin D1 is a positive regulator of the cell cycle and promotes G1 to S phase transition in cooperation with its binding partners cyclin-dependent kinases (CDKs) 4 and 6.25

Expression of cyclin D1 is regulated by growth factor stimulation through the activation of a cascade of kinases involving the mitogen-activated protein kinase (MAPK)/ERK kinase and PI3K signalling pathway.26 A further level of regulation of cyclin D1 occurs post-transcriptionally. Cyclin D1 degradation is mediated by phosphorylation-triggered, ubiquitin-dependent proteolysis.27,28 GSK-3β has been reported to enhance cyclin D1 degradation by phosphorylating the cyclin D1 threonine residue 286 thereby promoting the nuclear-to-cytoplasm redistribution of cyclin D1 and triggering its turnover.29,30

The activity of GSK-3β is regulated by the phosphorylation of serine 9 and tyrosine 216 and several lines of evidence indicate that Akt is an important determinant of GSK Ser9 phosphorylation, which becomes inactivated by Akt-dependent phosphorylation.31 Taken together, these observations suggest that alterations in the levels of cyclin D1 may be Akt/GSK dependent. Having shown that resveratrol influences cyclin D1 levels, we tested if these kinases were modulated after resveratrol treatment.

OVCAR-3 cells were treated for 30 min with resveratrol at a concentration ranging from 25 to 200 μM and levels of phospho-Akt and phospho-GSK were examined by western blotting. Treatment of OVCAR-3 cells with resveratrol decreased the levels of phosphorylated Akt at serine 473 (pAkt Ser473), but not at Thr308, and phosphorylated GSK-3β at Ser9 in a concentration-dependent manner, particularly at concentrations above 50 μM (Fig. 3b). In contrast, levels of total Akt and GSK-3β and phospho-GSK Tyr279/Tyr216 did not change during treatment. We next asked whether other members of the Akt pathways were modulated after resveratrol treatment. We selected mTOR, a direct substrate for Akt. As observed in Fig. 3b, the serine 2448 phosphorylation of mTOR decreased in ovarian cancer cells in response to resveratrol treatment.

In summary, these results clearly demonstrate that the inhibitory effect of resveratrol on cyclin D1 is dependent on the inhibition of Akt-dependent signaling by this polyphenolic compound.

Together with the Akt/GSK pathway, the literature documents the important role of other signalling pathways in the regulation of cell proliferation. The MAPK cascade is one of the most characterized. Several studies have demonstrated that suppression of MAPK activity by inhibitors or silencing reduces the proliferation rate of several cell lines.32–34

We therefore sought to determine if resveratrol could affect the phosphorylation status of this pathway. In doing this, we used specific antibodies against two kinases belonging to this pathway: ERK 1/2 and p38. ERK1 (p44 MAPK) and ERK2 (p42 MAPK) are usually activated by a variety of mitogenic receptors (e.g., receptors for epidermal growth factor and insulin-like growth factor), whereas p38 is activated primarily by environmental stress such as osmotic stress, ionizing radiation or by inflammatory cytokines.35

Substantial evidence points to the central role of both these kinases in promoting cancer cell growth and drug-resistance in ovarian cancer36–38 making them attractive targets for a therapeutic intervention. As shown in Fig. 3, resveratrol reduces phosphorylation of ERK but has no effects on the phosphorylation status and total level of the kinase p38.

As shown in Fig. 1b, resveratrol induces the re-arrangement of the actin architecture in OVCAR-3 when assessed by confocal microscopy, using phalloidin-FITC (Fig. 1b).

The mechanism by which resveratrol induces actin-remodelling could involve rapid modifications in the phosphorylation status of proteins controlling actin dynamics. To test this, we decided to investigate possible modifications of cofilin, an actin binding protein, because this protein is strictly involved in actin remodelling39 and its levels were previously reported to be modified by resveratrol treatment in other cell models.40

Furthermore, cofilin seems to be regulated through the Akt pathway41 and, as previously shown, Akt is modulated after resveratrol treatment in these cells. Based on these observations, we investigated total cofilin (COF) and phospho-cofilin (pCOF) levels since phosphorylation at serine 3 (pCOF Ser3) regulates

**Table 1** Protein spots differentially expressed between control and resveratrol-treated cells

<table>
<thead>
<tr>
<th>Spot number</th>
<th>Swiss Prot Accession No</th>
<th>Protein name</th>
<th>Gene name</th>
<th>Matched peptides</th>
<th>Coverage (%)</th>
<th>Protein score</th>
<th>Fold variation control vs. treated</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>Spot 1</td>
<td>Q14203</td>
<td>Dynactin subunit 1</td>
<td>DCTN1</td>
<td>4</td>
<td>3</td>
<td>54</td>
<td>-2.3</td>
<td>*</td>
</tr>
<tr>
<td>Spot 3</td>
<td>P18206</td>
<td>Vinculin</td>
<td>VCL</td>
<td>5</td>
<td>5</td>
<td>36</td>
<td>-2.1</td>
<td>*</td>
</tr>
<tr>
<td>Spot 7</td>
<td>P10809</td>
<td>60 kDa heat shock protein, mitochondrial</td>
<td>HSPD1</td>
<td>20</td>
<td>44</td>
<td>438</td>
<td>-2.1</td>
<td>**</td>
</tr>
<tr>
<td>Spot 8</td>
<td>P23526</td>
<td>Adenosylhomocysteinase</td>
<td>AHCY</td>
<td>16</td>
<td>32</td>
<td>262</td>
<td>2.2</td>
<td>**</td>
</tr>
<tr>
<td>Spot 12</td>
<td>P37837</td>
<td>Transaldolase</td>
<td>TALDO1</td>
<td>9</td>
<td>25</td>
<td>186</td>
<td>2.5</td>
<td>**</td>
</tr>
<tr>
<td>Spot 13</td>
<td>P24385</td>
<td>G1/S-specific cyclin-D1</td>
<td>CCND1</td>
<td>2</td>
<td>12</td>
<td>35</td>
<td>2.7</td>
<td>**</td>
</tr>
<tr>
<td>Spot 14</td>
<td>P04406/P54920</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase/alpha-soluble NSF attachment protein</td>
<td>GAPDH/NAFA</td>
<td>14/9</td>
<td>36/48</td>
<td>405/292</td>
<td>2.4</td>
<td>**</td>
</tr>
<tr>
<td>Spot 15</td>
<td>P04406/M1555</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase/microtubule-associated protein</td>
<td>GAPDH/ MAPRE2</td>
<td>2</td>
<td>5/7</td>
<td>225/124</td>
<td>2.4</td>
<td>**</td>
</tr>
<tr>
<td>Spot 18</td>
<td>P60981</td>
<td>Destrin</td>
<td>DSTN</td>
<td>3</td>
<td>22</td>
<td>74</td>
<td>2.0</td>
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</tr>
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</table>

*<p < 0.05; **<p < 0.01.
the severing activity of cofilin. As shown in Fig. 3b, no modifications were observed either in the phosphorylation and total levels of cofilin after 30 min of treatment, thus suggesting that cofilin is not a primary target of resveratrol. Since a short-term exposure to resveratrol does not inhibit cofilin, it might act on this pathway only after a longer treatment. Furthermore, the lack of correlation between phospho-Akt and phospho-cofilin levels (pAkt decreases while the pCOF remain unchanged after treatment) indicates that changes in the phosphorylation of Akt are not correlated with the phosphorylation state of cofilin in these cells.

Next, given the ability of resveratrol to modulate both Akt/GSK and ERK pathways, we tested whether the combination treatment with cytotoxic agents that target these two pathways could heighten its antiproliferative potential. OVCAR-3 cells were treated with resveratrol alone, or with resveratrol in combination with LY294002 (an inhibitor that blocks the PI3K-dependent Akt phosphorylation) and UO126 (a MEK 1/2 inhibitor) (Fig. 4). MTT test shows that the simultaneous administration of resveratrol with drugs that modulate the Akt/GSK and ERK pathways enhances anti-tumor activity above that seen by treating with either agent alone.

Given the remarkable heterogeneity of ovarian cancer at molecular and cellular level, this broad mechanism of action marks a goal toward the potential translation of these results into the clinic.

To further test the potential antiproliferative effects of resveratrol we extended our experiments to cancer cells isolated from the ascites of five patients with metastatic ovarian cancer (Fig. 5). The treatment of cells with resveratrol for 48 h resulted in a significant reduction of their viability at concentrations higher than 25 μM. On the contrary, the effect observed at 25 μM was modest, only two cellular isolates (AOvC1 and AOVc-3) responded significantly to the treatment. To explain this result we should take into account that at low concentrations (<50 μM) resveratrol usually exhibits a cytostatic effect (unpublished data) and that ascites-derived cells have a slow proliferation rate compared to OVCAR-3. Using low concentrations of resveratrol, longer times of stimulation are probably required to have a significant impact on cell viability. In addition, to ensure that the effect of resveratrol on Akt and ERK pathways is not unique to cell lines, we repeated the experiments described above using ascites-derived tumor cells. In these cells, resveratrol treatment provoked a clear decrease in the phosphorylation of Akt and ERK, confirming our previous observations.

Next, we wondered if resveratrol was able to reduce cell viability and target Akt phosphorylation of other cancer cell lines showing different origin and genotypes respect to OVCAR-3. We selected the cancer cell line SKOV-3 (PI3K overexpression, no p53 expression), the breast cancer cell line MCF-7
In the present study, we described the antiproliferative action of resveratrol in the cancer cell line OVCAR-3. Resveratrol acts in a dose- and time-dependent manner to reduce cell viability, and phalloidin-FITC staining also revealed that these effects are associated with profound modifications in the actin cytoskeleton organization. Using a proteomic approach we identified some differentially expressed proteins including cyclin D1 for which we described a possible mechanism of regulation. On the basis of the results described above, and considering previously published data, the resveratrol-induced down-regulation of the Akt pathway and activation of GSK-3β appear to be responsible for cyclin D1 phosphorylation and degradation. For these reasons, the antiproliferative effect of resveratrol can be mainly identified with the ability of this molecule to modulate Akt pathway. This observation is consistent with the reduction in the phosphorylation of Akt observed after resveratrol treatment in osteosarcoma, neuroblastoma and cervical cancer cell lines.

Furthermore, another important observation of this study is that resveratrol showed pleiotropic effects in OVCAR-3 cells. In fact, together with the ability to modulate Akt/GSK/mTOR, resveratrol inhibits another kinase, ERK 1/2. Although not directly involved in the degradation of cyclin D1, ERK 1/2 kinase is a downstream component of a pathway involved in the regulation of cell proliferation and survival. Resveratrol markedly and rapidly reduced both Akt/GSK and ERK phosphorylation in a dose-dependent manner. The effect of resveratrol on these proteins was evident already after 30 s of treatment (data not shown).

The mechanism by which resveratrol decreases the phosphorylation of these two kinases remains an open issue. In fact, it is still not clear if the regulation is through a common upstream activator or occurs independently. This aspect regards principally the understanding of the incomplete and complex relationship between the Akt/GSK and ERK pathways and it is beyond the aim of this work.

The inhibition of multiple, rather a single, pathways represents an approach largely investigated in cancer therapy and a more
effective means to induce cancer death. For this reason, resveratrol is likely to be used in association with drugs that target Akt, ERK or other signalling cascades. Here, we showed that the enhanced anti-proliferative effects of resveratrol in association with LY29004 and UO126 proved the possibility of these combinations. Future works are warranted to explore the combination of resveratrol with traditional chemotherapeutics such as paclitaxel.

Disclosing the molecular targets of phytochemicals action is probably the first step towards their potential clinic application. However, assessing the impact of such chemicals is difficult, considering that in many cases their mechanism of action is cell-type specific, with transformed cells being particularly sensitive. Furthermore, epidemiological data are often confounding, supporting or not the chemopreventive effects of chemicals found in food. For example, a large meta-analysis recently reported the lack of association between wine consumption and risk of ovarian cancer. In addition, in vivo indications still lack about the therapeutic use of such chemicals, in particular resveratrol, in cancer patients. Data coming from the literature that report a possible combination of resveratrol with drugs currently used from the treatment of ovarian cancer should encourage possible clinical trials.

Another observation regards the proteomic approach used in this work. It becomes clear that only a few proteins were identified by 2-DE as differentially expressed after resveratrol treatment. The explanation can be found in the cellular response to resveratrol that involves the modulation of proteins at the post-transcriptional level rather than at the expression level.

Fig. 6 Resveratrol suppresses the proliferation of several human cancer cell lines targeting the Akt pathway. Resveratrol affects cell viability in several human cancer cell lines as assessed by MTT test (left). The antiproliferative effect of resveratrol on the cell line SAOS-2 was comparable to that of MG-63 and is not shown here. Whole protein extracts of HeLa, IMR32, MCF-7 and SKOV-3 cells were obtained as described above after treatment with resveratrol at a concentration of 100 μM for 30 min. Western blotting analysis showed that resveratrol reduces the phosphorylation status of Akt serine 473 compared with untreated control cells (right).
To resolve this problem we are planning on focusing our attention only to the fraction of phosphorylated proteins adopting for their identification mass spectrometry based techniques.

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References