

## *trans*-Resveratrol induces apoptosis in human breast cancer cells MCF-7 by the activation of MAP kinases pathways

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**Abstract** Polyphenols represent a large class of plant-derived molecules with a general chemical structure that act as potent free radical scavengers. They have long been recognized to possess several therapeutic activities ranging from anti-thrombotic to antioxidant. Moreover, the capability of polyphenols to act as reducing or oxidizing molecules depends on the presence of environmental metals and on the concentrations used. In this work we demonstrated that the stilbene *trans*-resveratrol was able to commit human breast cancer MCF-7 cells to apoptosis. Mainly, we evidenced a pivotal role of the mitochondria in this phenomenon as cytochrome *c* release into the cytosol was found after the treatment. We further showed that *trans*-resveratrol was able to affect cellular redox state. In particular, it induced an early production of ROS and lipid oxidation, and only later compromised the GSH/GSSG ratio. This mode of action was mirrored by a temporally different activation of JNK and p38<sup>MAPK</sup>, with the former rapidly induced and the latter weakly activated at long intervals. The results obtained demonstrate a pro-apoptotic activity for *trans*-resveratrol, and suggest a preferential activation of different classes of MAP kinases in response to different oxidative stimuli (ROS *versus* GSH/GSSG alteration).

**Keywords** Resveratrol · Apoptosis · Glutathione · ROS · MAP kinases · Oxidative stress

### Introduction

Apoptosis is a programmed mode of cell death induced physiologically either during organ development, or for the maintenance of cellular homeostasis by means of the elimination of damaged or unnecessary cells [54, 61]. Recently, it has been demonstrated that phosphorylation/de-phosphorylation state of some regulatory proteins are crucial events along the pathways controlling cell growth and apoptosis. A well established apoptotic signaling cascade is that regulated by mitogen activated protein (MAP) kinases, such as the p42/44 extracellular signal-related kinases (ERK1/2), c-Jun N-terminal protein kinase (JNK) and p38<sup>MAPK</sup>, which directly modulate the phospho-active levels of pro-apoptotic factors [38]. MAP kinases are serine/threonine kinases that are regulated by a variety of extracellular stimuli including growth factors, mitogens, cytokines and environmental stresses [39]. Oxidative stress-mediated apoptosis is induced by phosphorylative cascades; indeed, in the last few years a hypothesis has emerged that reactive oxygen species (ROS) are not only the downstream damaging species produced by radical chain reactions, but also the second messengers of signaling networks [15].

Considerable scientific interest in the anticancer therapy has been focused, for long time, on the identification of compounds able to efficiently commit cells to apoptosis; in this context several chemotherapeutic agents can generate ROS at high extent, by catalyzing one-electron redox cycles with oxygen [55]. The specificity of action of these compounds towards tumor histotypes is supposed

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to be due to: (i) low concentration of antioxidant enzymes in transformed cells; (ii) high rate of proliferation with respect to differentiated cells, a feature that does not allow tumor cells repairing oxidative injuries, especially those involving DNA [23, 32, 49]. More recently, there has been a focus of attention on natural anti-oxidant molecules that, besides their role in scavenging free radicals, could function as pro-oxidants, when highly concentrated or in the presence of transition metals [51]. Previous studies have indicated that some nutraceuticals exhibit potent anti-tumor properties and can modulate apoptosis, differentiation and cell cycle, probably by virtue of their anti-oxidant functions. However, in vitro experiments reported that most of them behave as potent pro-oxidant molecules [6, 9, 40]. For example, organosulfur molecules from garlic, such as the oil-soluble diallyl disulfide [12, 13], diallyl trisulfide [22], or the water-soluble *S*-allylmercaptocysteine [63] have been demonstrated to induce growth arrest and apoptosis dose-dependently by increasing ROS production.

Among nutraceuticals, polyphenols represent the most intriguing and studied class of compounds that can be therapeutics for a large spectrum of the most common diseases including cancer [20]. Nevertheless, despite several studies regarding their function, the anti-oxidant/pro-oxidant properties of these compounds remain somewhat debatable and the detailed molecular mechanisms of their effects continue to be largely unknown. A pro-apoptotic function has been often proposed for many polyphenols [21, 35, 42]; however, although a huge amount of data support these assumptions, the molecular mechanism(s) by which apoptosis is triggered are long to be elucidated.

One of the well-characterized polyphenols is *trans*-resveratrol (3,5,4'-trihydroxy-*trans*-stilbene), a phytoalexin found in edible material, such as grape skin, peanuts and red wine. Jang and coworkers demonstrated that *trans*-resveratrol is an anticancer compound able to inhibit each phase of cell transformation both in in vitro and in in vivo models. Particularly, by acting as antioxidant, anti-mutagen as well as by inducing phase II drug-metabolizing enzymes, *trans*-resveratrol inhibits the initiation phase of tumorigenesis; concomitantly, it mediates the anti-inflammatory response and induces cell differentiation, thereby inhibiting tumor promotion and progression, respectively [28]. Subsequently, other groups reported the anti-proliferative and pro-apoptotic activity of *trans*-resveratrol in several tumor cell lines [2, 27, 41, 56], suggesting that it can function by modulating and interacting with a broad range of cellular targets, ranging from cell surface receptors [5, 7] to caspases [43]; from mitochondria [8] and mitochondria-associated proteins [43, 50,

60], to intracellular protein kinases, such as protein kinase B (PKB)/Akt, PKC, MAP kinases [34, 57, 58, 62, 65] and transcription factors (e.g. p53, pRb, c-Jun and NF- $\kappa$ B) [30, 37, 45, 59].

Although resveratrol, and polyphenols in general have been reported to be antioxidants, the idea that they can function as pro-oxidant compounds is emerging. Galati and colleagues demonstrated that several flavonoids, including *trans*-resveratrol, show anti-cancer properties when present at high concentration, by virtue of their capability to react with cellular peroxidases or with cellular thiols, thereby transforming into highly reactive phenoxyl radicals [17, 18]. The capability of these phenoxyl radicals to selectively deplete the cells of glutathione (GSH), to form GSH-conjugates or to oxidize GSH into the disulfide form of the tripeptide (GSSG) mainly depends on their chemical structure, in particular it seems to correlate with the number and the position of hydroxyl groups [19].

Besides this hypothesis of a direct production of oxy-radicals (especially superoxide,  $O_2^-$ ), *trans*-resveratrol and other polyphenols could induce the production of ROS by means of several indirect pathways, such as those downstream of surface receptors, which have been often suggested to be associated with an NAD(P)H-dependent ROS production. It has been also demonstrated that, under the phenoxyl radical form, polyphenols can cause mitochondrial toxicity by collapsing the mitochondrial transmembrane potential, thereby allowing ROS being generated at high extent [19]. Moreover it has been reported that several natural-occurring flavonoids, and *trans*-resveratrol itself, are able to affect mitochondrial respiration [24] by inhibiting NADH oxidase, succinoxidase and ATP synthesis [3], thus inducing the generation of partially reduced oxygen species [26, 48]. Particularly, a direct relationship between flavonoids redox potential and the above mentioned mitochondrial phenomena, has been proposed [25].

An intimate relationship between ROS and activation of phosphorylative cascades in the apoptotic response downstream polyphenols treatment has been argued [52] and, especially for what *trans*-resveratrol concerns, several pathways involving MAP kinases have been proposed to be activated upon pharmaceutical (micromolar) doses, as modulators of apoptosis [24]. Therefore, in this study, we have focused on the role of oxidative stress and oxidative stress-induced MAPK as downstream effectors of high (pharmaceutical) doses of *trans*-resveratrol. In particular, we demonstrate that both JNK and p38<sup>MAPK</sup> were involved in the cytotoxic effects of *trans*-resveratrol, although with a temporally different

activation, and sensitivity to ROS production or GSH/GSSG alteration.

## Materials and methods

### Cell culture

Human breast cancer cells MCF-7 were purchased from the American Type Culture Collection and grown in minimum essential medium (SIGMA, St.Louis, MO, USA) supplemented with 1% sodium pyruvate, 1% non-essential amino acids, 1 mg/l bovine insulin 10% fetal calf serum, at 37°C in an atmosphere of 7.5% CO<sub>2</sub> in air. Cells were routinely trypsinized, plated at 4 × 10<sup>4</sup>/cm<sup>2</sup> flasks. Cell viability was assessed by Trypan blue exclusion.

### Treatments

About 5 mg/ml solution of *trans*-resveratrol (SIGMA) was prepared just before the experiments by dissolving the powder in dimethyl sulfoxide. Treatments were performed with different amounts of *trans*-resveratrol ranging from 6.25 to 50 µg/ml at 37°C in medium supplemented with serum. Unless specified, the concentration of *trans*-resveratrol selected for all the experiments was 50 µg/ml since it gives a valuable degree of apoptosis on MCF-7. As control, equal amount of dimethyl sulfoxide (0.1%) was added to untreated cells.

Treatment with the cell permeable JNK and p38<sup>MAPK</sup> inhibitors, SP600125 and SB203580 (Calbiochem-Novabiochem, La Jolla, CA, USA), respectively, were performed at concentration of 10 µM because lower concentrations did not show significant inhibition of MAP kinases phosphorylation and higher concentrations were toxic. SP600125 and SB203580 were added 1 h before the addition of *trans*-resveratrol and maintained throughout the experiment.

### Analysis of cell viability and apoptosis

Adherent (after trypsinization) and detached cells were combined, washed in PBS and counted after Trypan blue staining by optic microscope on hemocytometer. Nuclei were detected by fluorescent microscopy using vital staining assay with DNA-specific cell-permeable dye Hoechst 33342. Images of cells were rapidly digitized with a Cool Snap video camera connected to Nikon Eclipse TE200 epifluorescence microscopy. All images were captured under constant exposure time, gain, and offset. Cells were also stained with 50 µg/ml propidium iodide (dissolved in 0.1% Triton X-100) prior to analysis by a FACScalibur instrument (Becton Dickinson, San José, CA,

USA). The percentages of cells in each phase of cell cycle were evaluated according to Nicoletti et al. [47] by calculating peak areas of nuclei with different amounts of DNA. Alternatively, cells were washed with PBS, stained with an annexin V-FITC/propidium iodide kit (Bender MedSystem, Vienna, Austria) and analyzed by FACScalibur instrument.

### Measurements of ROS levels and oxidative damages

Detection of intracellular ROS was performed as previously described [9]. Briefly, cells were incubated with 50 µM 2',7'-dichlorodihydrofluorescein diacetate (DHFDA) (Molecular Probes, Eugene, OR, USA) (dissolved in dimethyl sulfoxide) for 30 min at 37°C followed by treatment with *trans*-resveratrol. Treatment with 100 µM *tert*-butyl hydroperoxide was used as a positive control.

Carbonylated proteins were detected using the Oxyblot Kit (Intergen, Purchase, NY, USA) after reaction with 2,4-dinitrophenylhydrazine (DNP) for 15 min at 25°C. Samples were resolved on 12% SDS-polyacrylamide gels and DNP-derivatized proteins were identified by immunoblot using an anti-DNP antibody.

Levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were measured by a colorimetric method using the Lipid Peroxidation Assay Kit (Calbiochem-Novabiochem) according to manufacturer instructions. Lipid peroxidation was evaluated with reference to standard curves obtained with known amounts of MDA and 4-HNE and expressed as µmol MDA+4-HNE/mg protein.

### Glutathione determination

Intracellular glutathione was assayed upon formation of S-carboxymethyl derivatives of free thiols with iodoacetic acid, followed by the conversion of free amino groups to 2,4-dinitrophenyl derivatives by the reaction with 1-fluoro-2,4-dinitrobenzene. Cells were lysed by repeated cycles of freezing and thawing under liquid nitrogen. Lysates were then utilized for GSH and GSSG assay as previously described [16]. Data are expressed as nmoles of GSH or GSSG/mg protein.

### Preparation of cell lysates and Western blot analyses

#### *Cytosolic caspase-9, cytochrome c and β-actin determination*

Cells were washed with PBS and collected by centrifugation at 700×g for 7 min at 4°C. The cell pellet was

resuspended in extraction buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (DTT), and protease inhibitors [11]. After 30 min incubation on ice, cells were homogenized with a glass Dounce. Cell homogenates were spun at 14,000×g for 15 min at 4°C; supernatants and pellets were collected and stored at –80°C until analysis by gel electrophoresis; 20 µg of cytosolic protein or 10 µg of pellets extracts were loaded onto each lane of a 12% SDS-polyacrylamide gel, separated, and then blotted to nitrocellulose membrane (Bio-Rad). Purified mouse anti-cytochrome *c* (PharMingen, San Diego, CA, USA), anti-caspase-9 (Upstate Biotechnology, Lake Placid, NY, USA) and anti-β-actin (SIGMA) monoclonal antibodies were used as primary antibodies (1:3,000).

#### *Phospho-JNK, phospho-p38<sup>MAPK</sup>, phospho-c-Jun*

Cell pellet was resuspended in lysis buffer containing 62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol blue, sonicated for 15 s to shear DNA and reduce sample viscosity. About 20 µl of sample was loaded on 12% polyacrylamide gel and transferred onto a nitrocellulose membrane (BioRad). Polyclonal anti-phospho-p38<sup>MAPK</sup> (Cell Signaling, Beverly, MA, USA), and monoclonal anti-phospho-JNK and c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as primary antibodies (1:1,000). The protein complex formed upon specific secondary antibody treatments (1:5,000), was identified using a Fluorchem Imaging system (Alpha Innotech–Analytica De Mori, Italy) after incubation with ChemiGlow chemiluminescence substrate (Alpha Innotech). Densitometric analyses were calculated using Quantity One Software (Bio-Rad) and data were normalized with respect to the actin band. Data are reported as arbitrary units.

Proteins were determined by the method of Lowry et al. [36].

#### Data presentation

All experiments were done at least 5 different times ( $n = 5$ ) unless otherwise indicated. The results are presented as means ± SD. Statistical evaluation was carried out by ANOVA, followed by correction with Bonferroni's. Comparisons were considered to be significant at  $P < 0.05$ .

## Results

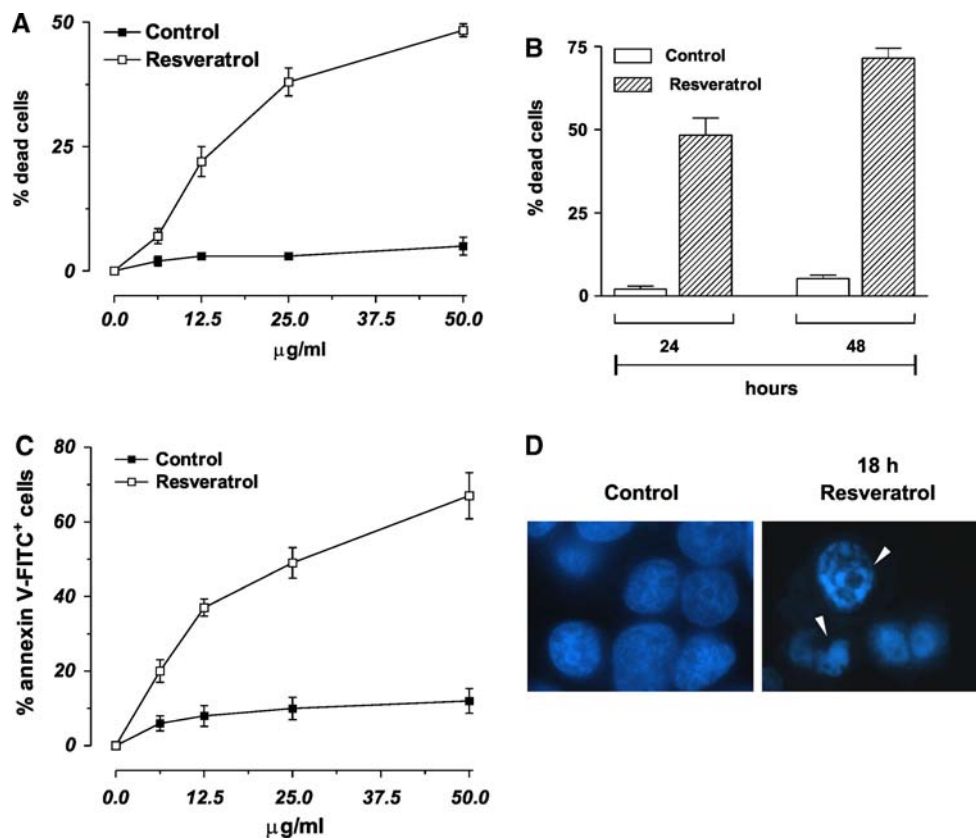
### *trans*-Resveratrol induces apoptosis in MCF-7 cells via the mitochondrial pathway

We studied the effects of *trans*-resveratrol treatment on cell viability of human breast cancer cells MCF-7. Figure 1a shows the percentages of Trypan blue-positive cells after 24 h of treatment with different concentrations of *trans*-resveratrol. The data reported evidenced that *trans*-resveratrol decreased cell viability in a dose- and time-dependent manner (Fig. 1a, b). In particular, at concentration of 50 µg/ml, the percentage of dead cells at 24 h was about 48%, phenomenon that became more evident after 48 h of treatment, reaching values of about 72% (Fig. 1b).

In order to distinguish which kind of cell death was induced under our experimental conditions (apoptosis versus necrosis), MCF-7 cells were treated for 24 h with the different concentrations of *trans*-resveratrol and then utilized for cytofluorimetric analyses upon staining with annexin V-FITC. Figure 1c shows the percentages of annexin V-FITC positive cells the amount of which paralleled the data previously obtained by direct counts upon Trypan blue exclusion (Fig. 1a). Moreover, fluorescence microscope analysis of nuclei upon staining with DNA-specific dye Hoechst 33342, showed that MCF-7 cells, treated for 18 h with 50 µg/ml *trans*-resveratrol, exhibited areas of condensed chromatin confirming that this compound is able to commit MCF-7 cell to death with the features of apoptosis (Fig. 1d).

MCF-7 cells represent a well-established histotype lacking of caspase-3, for this reason, they undergo apoptosis without showing inter-nucleosomal cleavages [29]. This was confirmed by cytofluorimetric analyses of cell cycle carried out upon staining with propidium iodide. As shown in Fig. 2a the representative histograms of MCF-7, cells treated for 24 h with 50 µg/ml *trans*-resveratrol, evidenced a low but significant arrest of the cell cycle in G<sub>2</sub>/M phase (see table below). As a consequence of the deficiency of caspase-3 activity, the rate of sub-G1 (apoptotic) cells was lower with respect to the data obtained by the previous analyses (see Figs. 1, 2).

To characterize the mechanism through which apoptosis occurs, MCF-7 cells were treated with 50 µg/ml *trans*-resveratrol and used for sub-cellular fractionation in order to isolate mitochondria and the cytosolic fractions. Figure 2b shows the Western blot analyses of cytosolic cytochrome *c* and caspase-9 after 18 h of treatment; in particular, cytochrome *c* was efficiently released from mitochondria, and caspase-9 activated, indicating an



**Fig. 1** *trans*-Resveratrol triggers cell death with the feature of apoptosis in MCF-7 cells. **a** MCF-7 cells were treated for 24 h with different concentrations of *trans*-resveratrol, ranging from 6.25 to 50 µg/ml. At indicated time points, cells were harvested and counted upon Trypan blue staining. Data show the percentage of Trypan blue-positive cells and are expressed as means  $\pm$  SD. All the values reported are significantly different with respect to control ( $P < 0.001$ ). **b** MCF-7 cells were treated for 24 and 48 h with 50 µg/ml *trans*-resveratrol. At indicated time points, cells were harvested and counted upon Trypan blue staining. Data show the percentage of Trypan blue-positive cells and are expressed as means  $\pm$  SD. The values reported are significantly different with respect to control ( $P < 0.001$ ). **c** MCF-7 cells were treated for 24 h with different concentrations of

efficient induction of the intrinsic mitochondrial pathway. These results suggest that, also in the absence of an active caspase-3, the execution of the apoptotic program could occur as already demonstrated [29].

#### *trans*-Resveratrol induce alteration of intracellular redox state

As previously reported, *trans*-resveratrol can produce ROS, directly by reacting as phenoxy radical with  $O_2$  [17, 18], or indirectly by impairing mitochondrial integrity [19, 26, 48]. In order to investigate whether ROS could be produced under our experimental conditions, we performed cytofluorimetric analyses of MCF-7 cells pre-loaded with the ROS-sensitive fluorophore DHCF-DA. Figure 3a shows

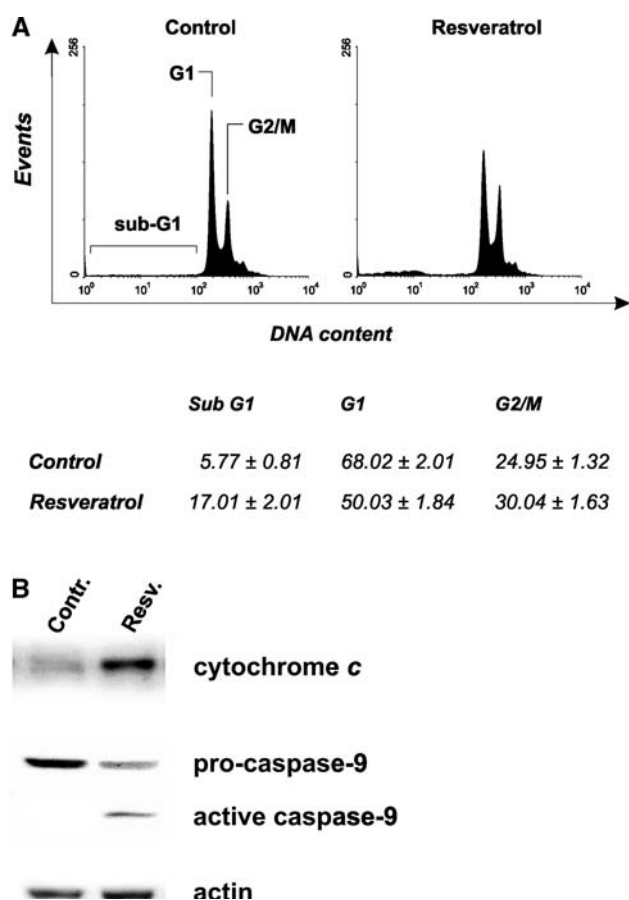
*trans*-resveratrol, ranging from 6.25 to 50 µg/ml, and stained with annexin V-FITC and propidium iodide. Fluorescence was analysed by a FACScalibur instrument and percentages of staining-positive cells were calculated using WinMDI version 2.8 software. Data show the percentage of total annexin V-FITC-positive cells and are expressed as means  $\pm$  SD. All the values reported are significantly different with respect to control ( $P < 0.001$ ). **d** MCF-7 cells were grown on chamber-slices and treated for 18 h with 50 µg/ml *trans*-resveratrol. After washing with PBS and fixing with 0.4% paraformaldehyde, cells were stained with Hoechst 33342 and visualized by fluorescence microscopy. *White arrows* indicate condensed chromatin characteristic of apoptotic nuclei

that *trans*-resveratrol was able to induce a high production of ROS (+37.2%) that was maintained up to 3 h with a slow decrease only after 6 h of treatment.

This result prompted us to investigate on the molecular targets of ROS by determining the content of protein oxidation and the extent of lipid peroxidation. No increase in carbonyl content was evidenced by Western blot analyses (data not shown); conversely, the levels of lipid peroxides significantly changed. Figure 3b shows that 24 h of treatment with 50 µg/ml *trans*-resveratrol induced a fivefold increase of lipid peroxides, indicating that lipids were one of the main targets of this compound under our experimental conditions.

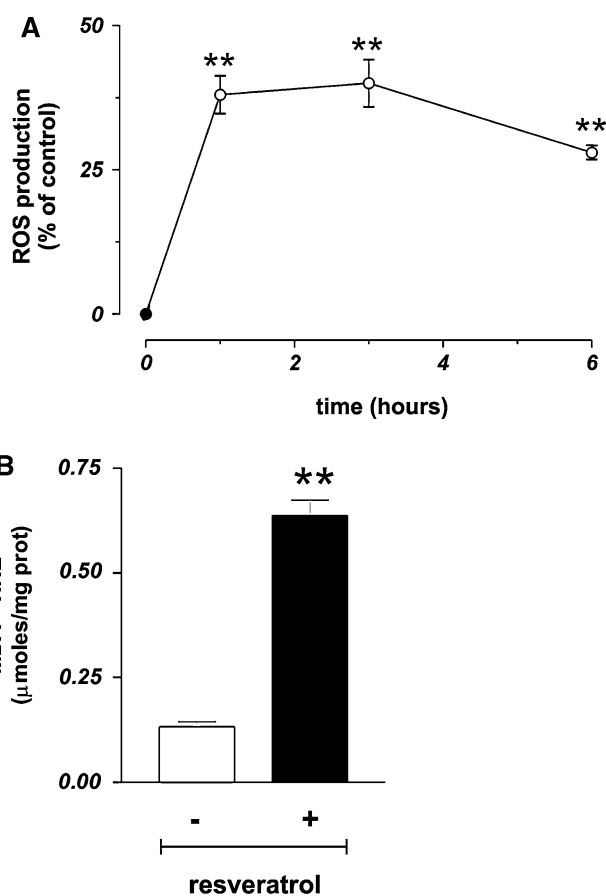
A sustained flux of ROS usually results in an imbalance of the intracellular redox state, which is promptly buffered by glutathione, the most abundant low molecular weight





**Fig. 2** *trans*-Resveratrol induces apoptosis via the mitochondrial pathway. **a** MCF-7 cells were treated for 24 h with 50  $\mu\text{g/ml}$  *trans*-resveratrol, washed and stained with propidium iodide. Analysis of cell cycle and apoptosis was performed by a FACScalibur instrument and percentages of staining-positive cells were calculated using WinMDI version 2.8 software. The cell cycle plots reported are from a typical experiment done in triplicate out of five that gave similar results (*sub-G1*, apoptotic cells). A table indicating the percentages of nuclei in the different phases of cell cycle is shown at the bottom. **b** MCF-7 cells were treated for 18 h with 50  $\mu\text{g/ml}$  *trans*-resveratrol; 20  $\mu\text{g}$  of cytosolic extracts were loaded onto each lane for the detection of cytochrome *c* or pro-caspase-9 (inactive) and caspase-9 (active) protein levels.  $\beta$ -actin was used as loading control. Immunoblots are from one experiment representative of three that gave similar results

thiol inside the cell [14]; therefore we analysed the concentration of both GSH and GSSG. MCF-7 cells were treated with 50  $\mu\text{g/ml}$  *trans*-resveratrol and used for the determination of the tripeptide by HPLC technique. Figure 4a shows that *trans*-resveratrol was able to profoundly influence the ratio [GSSG]/[2GSH], since GSH levels decreased, while GSSG concentration increased in a time-dependent manner (Fig. 4b). Such a phenomenon was particularly significant starting from 3–6 h of treatment, suggesting a sequence of oxidative events in which the upstream production of ROS represents the primary

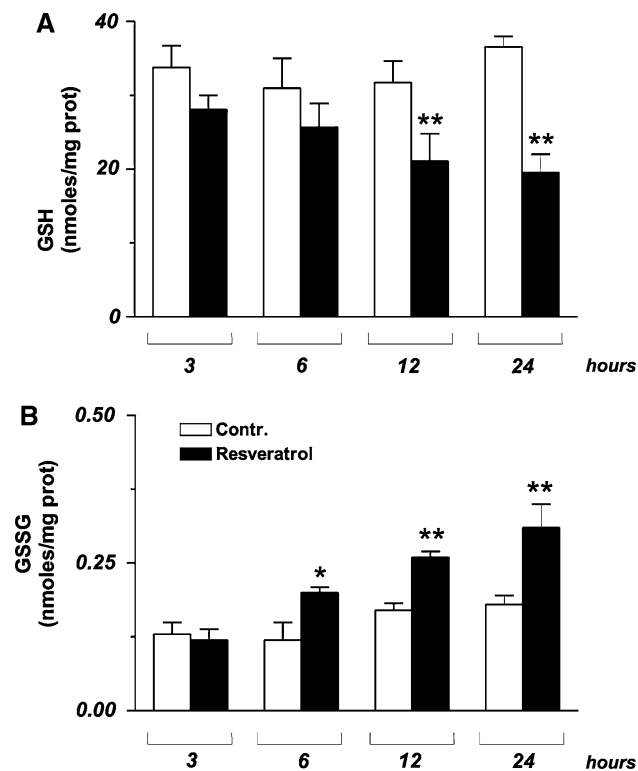


**Fig. 3** *trans*-Resveratrol induces ROS production and lipid peroxidation. **a** MCF-7 cells were incubated with 50  $\mu\text{M}$  DHCF-DA at 37°C for 30 min and then treated with 50  $\mu\text{g/ml}$  *trans*-resveratrol up to 6 h. At the indicated time points, cells were washed with PBS and ROS production was analysed by FACScalibur instrument. Data are expressed as mean  $\pm$  SD ( $n = 3$ )  $**P < 0.001$ . **b** MCF-7 cells were treated for 24 h with 50  $\mu\text{g/ml}$  *trans*-resveratrol. Lipid peroxidation was evaluated by measuring the levels of lipid peroxidation by-products malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) using a colorimetric method. Data are expressed as means  $\pm$  SD. ( $n = 5$ ).  $**P < 0.001$  versus untreated cells

inducer, and changes in GSH redox state the downstream outcome.

*trans*-Resveratrol induce a selective activation of JNK and p38<sup>MAPK</sup>

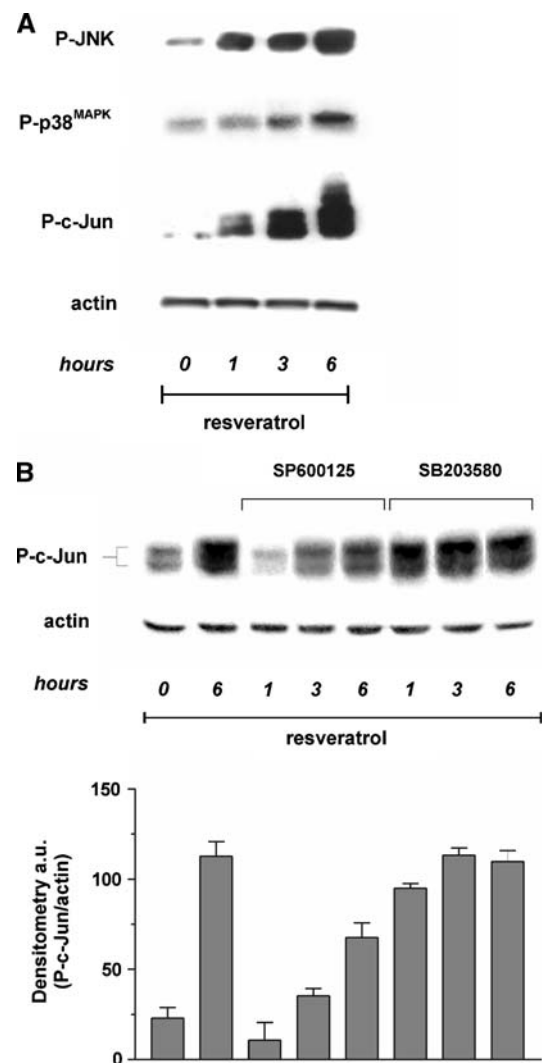
We have previously demonstrated that different oxidizing agents can trigger apoptosis by inducing JNK or p38<sup>MAPK</sup>-governed phosphorylative cascades. In order to investigate whether apoptosis was related to the activation of the MAP kinases pathway, we treated MCF-7 cells with 50  $\mu\text{g/ml}$  *trans*-resveratrol for 6 h and then performed Western blot analyses of phospho-activated isoforms of the pro-apoptotic members of the MAP kinases family, JNK and



**Fig. 4** *trans*-Resveratrol affect glutathione redox state MCF-7 cells were treated up to 24 h with 50  $\mu\text{g/ml}$  *trans*-resveratrol. At the indicated time points, cells were collected, exhaustively washed with PBS and used for GSH (a) and GSSG (b) assay by HPLC. Data are expressed as nmol of GSH or GSSG/mg total protein. Values represent means  $\pm$  SD ( $n = 5$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ ; versus untreated cells

$\text{p38}^{\text{MAPK}}$ . Figure 5a shows that *trans*-resveratrol was able to rapidly activate JNK and  $\text{p38}^{\text{MAPK}}$ , although with different kinetics and to different extent. Particularly, the phosphorylation of JNK was time-dependent and started to be operative after 1 h of treatment, conversely the lower induction of  $\text{p38}^{\text{MAPK}}$  could be observed only at 3–6 h of treatment. The results obtained allow to speculate that, while the activation of JNK is very rapid and associated with the increase of ROS concentration, the activation of  $\text{p38}^{\text{MAPK}}$  could reasonably represent an event principally related to late alterations of the intracellular redox state, such as those in [GSSG]/[2GSH] ratio, thus suggesting a specific response to different types of oxidative stress, i.e. production of ROS *versus* alteration of [GSSG]/[2GSH] ratio.

To investigate the specific contribution of JNK and  $\text{p38}^{\text{MAPK}}$  on c-Jun phosphorylation, we incubated MCF-7 cells with the specific inhibitors of JNK and  $\text{p38}^{\text{MAPK}}$ , SP600125 and SB203580, respectively, and further analysed the phospho active levels of c-Jun. Western blot reported in Fig. 5b shows that, the incubation with SP600125 determined a time-shifted phosphorylation of



**Fig. 5** *trans*-Resveratrol induces preferential activation of MAP kinases. **a** MCF-7 cells were treated for 6 h with 50  $\mu\text{g/ml}$  *trans*-resveratrol; 20  $\mu\text{g}$  of cytosolic extracts were loaded onto each lane for detection of phospho-isoforms of JNK,  $\text{p38}^{\text{MAPK}}$  and c-Jun.  $\beta$ -actin was used as loading control. Immunoblots are from one experiment representative of three that gave similar results. **b** MCF-7 cells were incubated with 10  $\mu\text{M}$  SP600125 or SB203580, the specific inhibitors of JNK and  $\text{p38}^{\text{MAPK}}$ , respectively, for 1 h before and throughout the treatment with 50  $\mu\text{g/ml}$  *trans*-resveratrol; 20  $\mu\text{g}$  of cytosolic extracts were loaded onto each lane for detection of phospho-c-Jun.  $\beta$ -actin was used as loading control. Immunoblots are from one experiment representative of three that gave similar results. Densitometric analyses of each lane was calculated using Quantity One Software and data are expressed as arbitrary units

c-Jun, suggesting that JNK was the earlier, but not the sole mediator of *trans*-resveratrol-induced apoptosis. Conversely, the inhibition of  $\text{p38}^{\text{MAPK}}$  did not prevent the phosphorylation of c-Jun (Fig. 5b), suggesting that, under our experimental conditions,  $\text{p38}^{\text{MAPK}}$  had an additive, rather than a principal role in phosphorylating c-Jun.

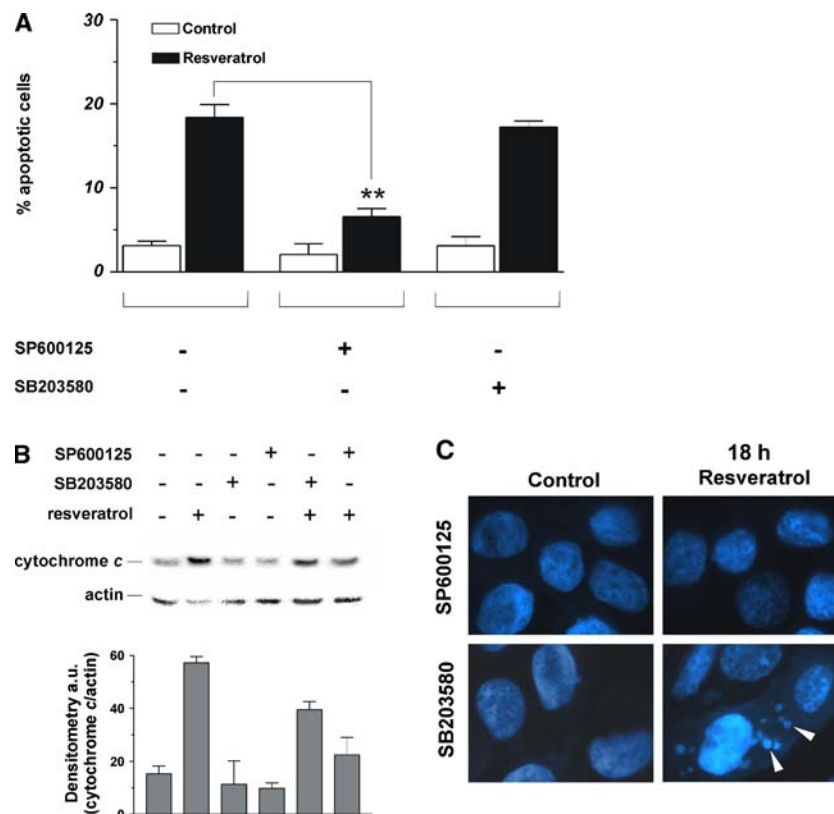
The inhibition of JNK and p38<sup>MAPK</sup> inhibits apoptosis induced by *trans*-resveratrol

The involvement of JNK and p38<sup>MAPK</sup> in the induction of apoptosis upon treatment with *trans*-resveratrol was investigated by incubating MCF-7 cells with specific inhibitors of JNK and p38<sup>MAPK</sup>. Figure 6a shows the cytofluorimetric analyses upon propidium iodide staining; in particular, the inhibition of JNK induced a significant decrease of apoptotic cells, indicating that JNK-dependent phosphorylative cascade plays a pivotal role in the cell death induced by *trans*-resveratrol. On the other hand, the inhibition of p38<sup>MAPK</sup> did not affect the content of apoptotic cells after treatment with *trans*-resveratrol, indicating that p38<sup>MAPK</sup> pathway was not effective in the induction of cell death. Moreover, Western blot analyses showed that the inhibition of p38<sup>MAPK</sup> only partially decreased the level of cytosolic cytochrome *c* (–30%), whereas the inhibition of JNK induced a significant decrease of the protein in the cytosol (–65%), confirming the pivotal role played by the

latter kinase in the apoptotic process. This trend was confirmed by fluorescence microscopy analyses of MCF-7 nuclei stained with Hoechst 33342. Figure 6c shows that, after incubation with SP600125, chromatin distribution within the nuclei was not affected by *trans*-resveratrol, whereas the inhibition of p38<sup>MAPK</sup>-mediated pathway was not able to efficiently prevent the canonical apoptotic changes of chromatin. In particular, apoptotic bodies were still visible after 18 h of treatment (white arrows).

## Discussion

*trans*-Resveratrol has been recently reported to possess several beneficial effects for human health. Besides its antioxidant activity, it has been suggested that *trans*-resveratrol inhibits each phase of tumor growth, by interacting with several cellular targets, thereby activating cell cycle arrest and death. At molecular level, it has been suggested that high concentration of polyphenols can induce the



**Fig. 6** JNK and p38<sup>MAPK</sup> are differently responsible for *trans*-resveratrol-induced apoptosis. MCF-7 cells were incubated with 10  $\mu$ M SP600125 or SB203580, the specific inhibitors of JNK and p38<sup>MAPK</sup>, respectively, for 1 h before and throughout the treatment with 50  $\mu$ g/ml *trans*-resveratrol. **a** After 24 h treatment, cells were stained with propidium iodide for cytofluorimetric analyses. Percentages of apoptotic cells are expressed as means  $\pm$  S.D. ( $n = 5$ ).  $**P < 0.001$ . **b** After 18 h 20  $\mu$ g of cytosolic extracts were loaded onto each lane for detection of cytochrome *c*.  $\beta$ -actin was used as loading

control. Immunoblots are from one experiment representative of three that gave similar results. Densitometric analyses of each lane were calculated using Quantity One Software and data are expressed as arbitrary units. **c** Alternatively, cells were grown on chamber-slices and treated for 18 h. After washing with PBS and fixing with 0.4% paraformaldehyde, cells were stained with Hoechst 33342 and visualized by fluorescence microscopy. *White arrows* indicate condensed chromatin or apoptotic bodies after *trans*-resveratrol treatment



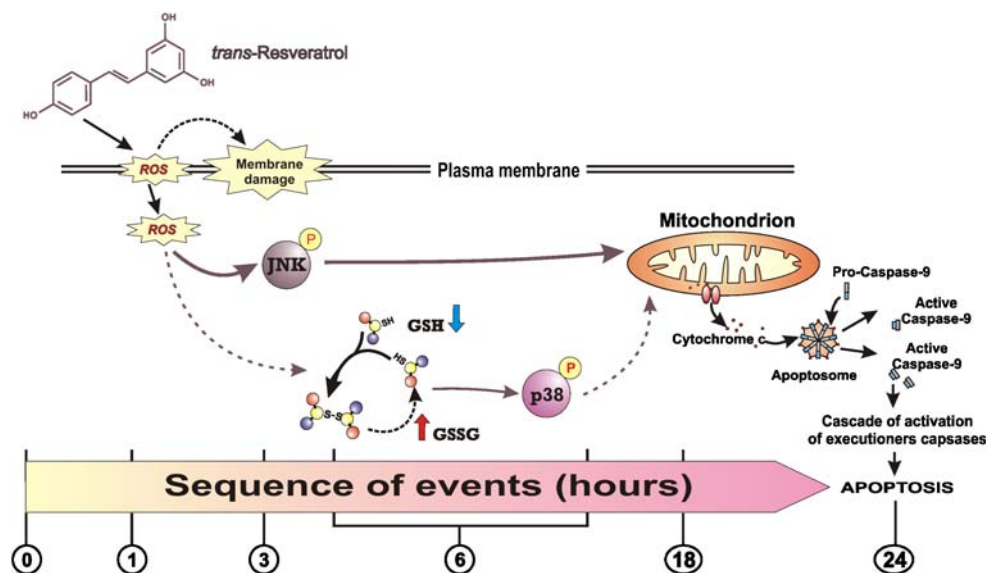
production of ROS, rather than reinforcing the antioxidant defense, and it has been hypothesized that this pro-oxidant function can play a pivotal role in resveratrol-induced apoptosis in tumor cells [19]. In fact, the generation of phenoxyl radicals of polyphenols by the peroxidase–H<sub>2</sub>O<sub>2</sub> system, which co-oxidizes cellular glutathione or NADH, accompanied by O<sub>2</sub> uptake to form ROS has been demonstrated [17, 18]. In addition, polyphenols have been proposed to induce oxidative stress indirectly by targeting mitochondrial electron transport chain, thereby generating a downstream flux of ROS [26, 48]. In agreement with previous works, which identified a role for oxyradicals in cytotoxicity of polyphenols [6, 46, 19], we observed an early increase of ROS levels after treatment with high doses of *trans*-resveratrol in human breast cancer cells MCF-7. The involvement of ROS has been proposed as upstream event occurring during treatment with high (micro-molar) concentrations of *trans*-resveratrol [1, 21], as well as with other polyphenols, including epigallocatechin-3-gallate [44, 53], woodfordin I [35] and quercetin [33, 64]. In our experimental system ROS burst occurs precociously (within the first hour) and the detrimental effects produced were confirmed by the increase in lipid peroxidation by-products, MDA and 4-HNE. On the other hand, no damage on protein in terms of carbonyls content was observed, implying that the hydrophobicity of *trans*-resveratrol renders the lipids the preferential targets of its pro-oxidant action. It is worth to notice that, even though the pro-oxidant effects observed in this study were referred to the highest dose of *trans*-resveratrol (50 µg/ml), we suggest that apoptosis observed at the lowest dose (6.25 µg/ml) is still mediated by ROS production and oxidative insults. However, since differentiated cells are equipped with high levels of both enzymatic and non-enzymatic antioxidants, we can presume that,

as previously demonstrated with other diet-derived pro-oxidant molecules [12], *trans*-resveratrol could be non toxic towards normal tissues.

We also found that *trans*-resveratrol induced a significant alteration of GSH/GSSG ratio in agreement with the knowledge that the phenoxyl radical form of polyphenols can produce ROS in association with a selective depletion of intracellular GSH, to form GSH-conjugates or GSSG. However, under our experimental conditions, GSH depletion was a late event, which allows speculating that it represents just a epiphenomenon rather than the pivotal redox change governing resveratrol-induced apoptosis.

The direct relationship between alteration of intracellular redox state and activation of MAP kinases-mediated phosphorylative pathways has been exhaustively demonstrated [15]. Moreover, a role for polyphenols in the activation of different MAP kinases members has been suggested as necessary event for downstream induction of apoptosis in cancer cells [31]. Here we reported that the oxidative stress deriving from treatments with *trans*-resveratrol is differently associated with MAP kinases activation. In particular, *trans*-resveratrol seems to preferentially induce the JNK/c-Jun-mediated phosphorylative cascade as the principal mediator of apoptosis. Moreover, if we consider the relationship with the oxidative alterations observed (ROS or GSH-dependent) we could hypothesize a selective activation of different MAP kinases, with JNK more responsive to the increase of ROS concentration and p38<sup>MAPK</sup> preferentially responsive to the alteration of GSH redox state. This different sensitivity of MAP kinases to ROS- or GSH-dependent intracellular redox changes is in line with our recent results suggesting that different types of oxidative stress could preferentially drive different phosphorylative pathways [10, 12, 13]. On

**Fig. 7** Scheme of MAPK-mediated pathways downstream of *trans*-resveratrol treatment



this basis, we can outline the results obtained as shown in Fig. 7. In particular *trans*-resveratrol activates JNK and p38<sup>MAPK</sup>, but its different capability to alter intracellular redox environment concur to make JNK activation the principal response, and p38<sup>MAPK</sup> a redundant kinase for the induction of cell death. Finally, further works should be done in animal models in order to determine the concentrations that allow *trans*-resveratrol to behave as chemotherapeutic other than antioxidant.

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## References

- Azmi AS, Bhat SH, Hanif S, Hadi SM (2006) Plant polyphenols mobilize endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage: a putative mechanism for anticancer properties. *FEBS Lett* 580:533–538
- Bhat KPL, Kosmeder JW, Pezzuto JM (2001) Biological effects of resveratrol. *Antioxid Redox Signal* 3:1041–1064
- Bohm C, Aaronson LM, Mann K, Pardini RS (1987) Inhibition of mitochondrial NADH oxidase, succinoxidase, and ATPase by naturally occurring flavonoids. *J Nat Prod* 50:427–433
- Briviba K, Pan L, Reckemmer G (2002) Red wine polyphenols inhibit the growth of colon carcinoma cells and modulate the activation pattern of mitogen-activated protein kinases. *J Nutr* 132:2814–2818
- Clement MV, Hirpara JL, Chawdhury SH, Pervaiz S (1998) Chemopreventive agent resveratrol, a natural product derived from grapes, triggers CD95 signaling-dependent apoptosis in human tumor cells. *Blood* 92:996–1002
- Chichirau A, Flueraru M, Chepelev LL, Wright JS, Willmore WG, Durst T, Hussain HH, Charron M (2005) Mechanism of cytotoxicity of catechols and a naphthalenediol in PC12-AC cells: the connection between extracellular autoxidation and molecular electronic structure. *Free Radic Biol Med* 38:344–355
- Delmas D, Rebe C, Lacour S, Filomenko R, Athias A, Gambert P, Cherkaoui-Malki M, Jannin B, Dubrez-Daloz L, Latruffe N, Solary E (2003) Resveratrol-induced apoptosis is associated with Fas redistribution in the rafts and the formation of a death-inducing signaling complex in colon cancer cells. *J Biol Chem* 278:41482–41490
- Dorrie J, Gerauer H, Wachter Y, Zunino SJ (2001) Resveratrol induces extensive apoptosis by depolarizing mitochondrial membranes and activating caspase-9 in acute lymphoblastic leukemia cells. *Cancer Res* 61:4731–4739
- Elbling L, Weiss RM, Teufelhofer O, Uhl M, Knasmueller S, Schulte-Hermann R, Berger W, Micksche M (2005) Green tea extract and (-)-epigallocatechin-3-gallate, the major tea catechin, exert oxidant but lack antioxidant activities. *FASEB J* 19:807–809
- Filomeni G, Aquilano K, Civitareale P, Rotilio G, Ciriolo MR (2005) Activation of c-Jun-N-terminal kinase is required for apoptosis triggered by glutathione disulfide in neuroblastoma cells. *Free Radic Biol Med* 39:345–354
- Filomeni G, Aquilano K, Rotilio G, Ciriolo MR (2005) Antiapoptotic response to GSH depletion: involvement of heat shock proteins and NF- $\kappa$ B activation. *Antioxid Redox Signal* 7:446–455
- Filomeni G, Aquilano K, Rotilio G, Ciriolo MR (2003) Reactive oxygen species-dependent c-Jun NH2-terminal kinase/c-Jun signaling cascade mediates neuroblastoma cell death induced by diallyl disulfide. *Cancer Res* 63:5940–5949
- Filomeni G, Aquilano K, Rotilio G, Ciriolo MR (2005) Glutathione-related systems and modulation of extracellular signal-regulated kinases are involved in resistance of AGS adenocarcinoma gastric cells to diallyl disulfide-induced apoptosis. *Cancer Res* 65:11735–11742
- Filomeni G, Rotilio G, Ciriolo MR (2002) Cell signalling and the glutathione redox system. *Biochem Pharmacol* 64:1057–1064
- Filomeni G, Rotilio G, Ciriolo MR (2005) Disulfide relays and phosphorylative cascades: partners in redox-mediated signalling pathways. *Cell Death Differ* 12:1555–1563
- Filomeni G, Rotilio G, Ciriolo MR (2003) Glutathione disulfide induces apoptosis in U937 cells by a redox-mediated p38 MAP kinase pathway. *FASEB J* 17:64–66
- Galati G, Chan T, Wu B, O'Brien PJ (1999) Glutathione-dependent generation of reactive oxygen species by the peroxidase-catalyzed redox cycling of flavonoids. *Chem Res Toxicol* 12:521–525
- Galati G, Sabzevari O, Wilson JX, O'Brien PJ (2002) Prooxidant activity and cellular effects of the phenoxyl radicals of dietary flavonoids and other polyphenolics. *Toxicology* 177:91–104
- Galati G, O'Brien PJ (2004) Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free Radic Biol Med* 37:287–303
- Garg AK, Buchholz TA, Aggarwal BB (2005) Chemosensitization and radiosensitization of tumors by plant polyphenols. *Antioxid Redox Signal* 7:1630–1647
- Hadi SM, Asad SF, Singh S, Ahmad A (2000) Putative mechanism for anticancer and apoptosis-inducing properties of plant-derived polyphenolic compounds. *IUBMB Life* 50:167–171
- Herman-Antosiewicz A, Singh SV (2005) Checkpoint kinase 1 regulates diallyl trisulfide-induced mitotic arrest in human prostate cancer cells. *Journal of Biol Chem* 280:28519–28528
- Hileman EO, Liu J, Albitar M, Keating MJ, Huang P (2003) Intrinsic oxidative stress in cancer cells: a biochemical basis for therapeutic selectivity. *Cancer Chemother Pharmacol* 53:209–219
- Hodnick WF, Kung FS, Roettger WJ, Bohmont CW, Pardini RS (1986) Inhibition of mitochondrial respiration and production of toxic oxygen radicals by flavonoids: a structure–activity study. *Biochem Pharmacol* 35:2345–2357
- Hodnick WF, Milosavljevic EB, Nelson JH, Pardini RS (1988) Electrochemistry of flavonoids: relationships between redox potentials, inhibition of mitochondrial respiration, and production of oxygen radicals by flavonoids. *Biochem Pharmacol* 37:2607–2611
- Hodnick WF, Ahmad S, Pardini RS (1998) Induction of oxidative stress by redox active flavonoids. *Adv Exp Med Biol* 439:131–150
- Hsieh TC, Wu JM (1999) Differential effects on growth, cell cycle arrest, and induction of apoptosis by resveratrol in human prostate cancer cell lines. *Exp Cell Res* 249:109–115
- Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* 275:218–220
- Kagawa S, Gu J, Honda T, McDonnell TJ, Swisher SG, Roth JA, Fang B (2001) Deficiency of caspase-3 in MCF7 cells blocks Bax-mediated nuclear fragmentation but not cell death. *Clin Cancer Res* 7:1474–1480
- Kim YA, Lee WH, Choi TH, Rhee SH, Park KY, Choi YH (2003) Involvement of p21WAF1/CIP1, pRB, Bax and NF- $\kappa$ B in induction of growth arrest and apoptosis by resveratrol in human lung carcinoma A549 cells. *Int J Oncol* 23:1143–1149
- Kong AN, Owuor E, Yu R, Hebbar V, Chen C, Hu R, Mandelkar S (2001) Induction of xenobiotic enzymes by the MAP kinase pathway and the antioxidant or electrophile response element (ARE/EpRE). *Drug Metab Rev* 33:255–271

32. Kong Q, Beel JA, Lillehei KO (2000) A threshold concept for cancer therapy. *Med Hypothesis* 55:29–35
33. Lee ER, Kang YJ, Kim JH, Lee HAT, Cho SG (2005) Modulation of apoptosis in HaCaT keratinocytes via differential regulation of ERK signaling pathway by flavonoids. *J Biol Chem* 280:31498–31507
34. Li Y, Liu J, Liu X, Xing K, Wang Y, Li F, Yao L (2006) Resveratrol-induced cell inhibition of growth and apoptosis in MCF7 human breast cancer cells are associated with modulation of phosphorylated Akt and caspase-9. *Appl Biochem Biotechnol* 135:181–192
35. Liu MJ, Wang Z, Li HX, Wu RC, Liu YZ, Wu QY (2004) Mitochondrial dysfunction as an early event in the process of apoptosis induced by woodfordin I in human leukemia K562 cells. *Toxicol Appl Pharmacol* 194:141–155
36. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin-phenol reagent. *J Biol Chem* 193:265–275
37. Manna SK, Mukhopadhyay A, Aggarwal BB (2000) Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappa B, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. *J Immunol* 164:6509–6519
38. Matsuzawa A, Ichijo H (2005) Stress-responsive protein kinases in redox-regulated apoptosis signaling. *Antioxid Redox Signal* 7:472–481
39. Matsuzawa A, Nishitoh H, Tobiume K, Takeda K, Ichijo H (2002) Physiological roles of ASK1-mediated signal transduction in oxidative stress- and endoplasmic reticulum stress-induced apoptosis: advanced findings from ASK1 knockout mice. *Antioxid Redox Signal* 4:415–425
40. Meunier S, Hanedanian M, Desage-El Murr M, Nowaczyk S, Le Gall T, Pin S, Renault JP, Boquet D, Creminon C, Mioskowski C, Taran F (2005) High-throughput evaluation of antioxidant and pro-oxidant activities of polyphenols with thymidine protection assays. *Chem Biochem* 6:1234–1241
41. Mgbonyebi OP, Russo J, Russo IH (1998) Antiproliferative effect of synthetic resveratrol on human breast epithelial cells. *Int J Oncol* 12:865–869
42. Michels G, Watjen W, Niering P, Steffan B, Thi QH, Chovolou Y, Kampkotter A, Bast A, Proksch P, Kahl R (2005) Pro-apoptotic effects of the flavonoid luteolin in rat H4IIE cells. *Toxicology* 206:337–348
43. Mohan J, Gandhi AA, Bhavya BC, Rashmi R, Karunakaran D, Indu R, Santhoshkumar TR (2006) Caspase-2 triggers Bax-Bak-dependent and -independent cell death in colon cancer cells treated with resveratrol. *J Biol Chem* 281:17599–17611
44. Nakazato T, Ito K, Ikeda Y, Kizaki M (2005) Green tea component, catechin, induces apoptosis of human malignant B cells via production of reactive oxygen species. *Clin Cancer Res* 11:6040–6049
45. Narayanan BA, Narayanan NK, Re GG, Nixon DW (2003) Differential expression of genes induced by resveratrol in LNCaP cells: p53-mediated molecular targets. *Int J Cancer* 104:204–212
46. Nemeikaite-Ceniene A, Imbrasaitė A, Sergediene E, Cenas N (2005) Quantitative structure-activity relationships in prooxidant cytotoxicity of polyphenols: role of potential of phenoxyl radical/phenol redox couple. *Arch Biochem Biophys* 441:182–190
47. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 139:271–279
48. Pardini RS (1995) Toxicity of oxygen from naturally occurring redox-active pro-oxidants. *Arch Insect Biochem Physiol* 29:101–118
49. Pelicano H, Carney D, Huang P (2004) ROS stress in cancer cells and therapeutic implications. *Drug Resist Updat* 7:97–110
50. Pellecchia M, Reed JC (2004) Inhibition of anti-apoptotic Bcl-2 family proteins by natural polyphenols: new avenues for cancer chemoprevention and chemotherapy. *Curr Pharm Des* 10:1387–1398
51. Podmore ID, Griffiths HR, Herbert KE, Mistry N, Mistry P, Lunec J (1998) Vitamin C exhibits pro-oxidant properties. *Nature* 392:559
52. Qanungo S, Das M, Haldar S, Basu A (2005) Epigallocatechin-3-gallate induces mitochondrial membrane depolarization and caspase-dependent apoptosis in pancreatic cancer cells. *Carcinogenesis* 26:958–967
53. Raza H, John A (2005) Green tea polyphenol epigallocatechin-3-gallate differentially modulates oxidative stress in PC12 cell compartments. *Toxicol Appl Pharmacol* 207:212–220
54. Rossi L, Aquilano A, Filomeni G, Lombardo MF, Rotilio G, Ciriolo MR (2004) Putative mechanisms of apoptosis related to redox unbalance. In: Ozben K, Chevion M (eds) *Frontiers in neurodegenerative disorders and aging: fundamental aspect, clinical perspectives and new insight*. IOS press, NATO Sciences Series, The Netherlands, pp 207–250
55. Rotilio G, Mavelli I, Rossi L, Ciriolo MR (1985) Biochemical mechanism of oxidative damage by redox-cycling drugs. *Environ Health Perspect* 64:259–264
56. Schneider Y, Vincent F, Duranton B, Badolo L, Gosse F, Bergmann C, Seiler N, Raul F (2000) Anti-proliferative effect of resveratrol, a natural component of grapes and wine, on human colonic cancer cells. *Cancer Lett* 158:85–91
57. Sexton E, Van Themsche C, LeBlanc K, Parent S, Lemoine P, Asselin E (2006) Resveratrol interferes with AKT activity and triggers apoptosis in human uterine cancer cells. *Mol Cancer* 175:45
58. She QB, Bode AM, Ma WY, Chen NY, Dong Z (2001) Resveratrol-induced activation of p53 and apoptosis is mediated by extracellular-signal-regulated protein kinases and p38 kinase. *Cancer Res* 61:1604–1610
59. Subbaramaiah K, Michaluart P, Chung WJ, Tanabe T, Telang N, Dannenberg AJ (1999) Resveratrol inhibits cyclooxygenase-2 transcription in human mammary epithelial cells. *Ann N Y Acad Sci* 889:214–223
60. Tinhofer I, Bernhard D, Senfter M, Anether G, Loeffler M, Kroemer G, Kofler R, Csordas A, Greil R (2001) Resveratrol, a tumor-suppressive compound from grapes, induces apoptosis via a novel mitochondrial pathway controlled by Bcl-2. *FASEB J* 15:1613–1615
61. Ueda S, Masutani H, Nakamura H, Tanaka T, Ueno M, Yodoi J (2002) Redox control of cell death. *Antioxid Redox Signal* 4:405–414
62. Woo JH, Lim JH, Kim YH, Suh SI, Min DS, Chang JS, Lee YH, Park JW, Kwon TK (2004) Resveratrol inhibits phorbol myristate acetate-induced matrix metalloproteinase-9 expression by inhibiting JNK and PKC delta signal transduction. *Oncogene* 23:1845–1853
63. Xiao D, Pinto JT, Soh JW, Deguchi A, Gundersen GG, Palazzo AF, Yoon JT, Shirin H, Weinstein IB (2003) Induction of apoptosis by the garlic-derived compound S-allylmercaptocysteine (SAMC) is associated with microtubule depolymerization and c-Jun NH2-terminal kinase 1 activation. *Cancer Res* 63:6825–6837
64. Yamashita N, Kawanishi S (2000) Distinct mechanisms of DNA damage in apoptosis induced by quercetin and luteolin. *Free Radic Res* 33:623–633
65. Yu R, Hebbar V, Kim DW, Mandlekar S, Pezzuto JM, Kong AN (2001) Resveratrol inhibits phorbol ester and UV-induced activator protein 1 activation by interfering with mitogen-activated protein kinase pathways. *Mol Pharmacol* 60:217–224