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ORIGINAL CONTRIBUTION

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The grape and wine polyphenol piceatannol is a potent inducer of apoptosis in human SK-Mel-28 melanoma cells

Summary *Background & aim* The resveratrol analogue piceatannol (3,5,3',4'-tetrahydroxy-*trans*-stilbene; PICE) is a polyphenol present in grapes and wine. PICE is a protein kinase inhibitor that modifies multiple cellular targets exerting immunosuppressive, antileukemic and antitumorigenic activities in several cell lines and animal models. The present work aims to evaluate the antimelanoma effect of PICE on human melanoma cells for the first time. To this purpose, the pro-apoptotic capacity, uptake and metabolism of PICE as well as its effect on cell cycle and cyclins A, E and B1 expression will be studied. *Methods* Human SK-Mel-28 melanoma cells were incubated with PICE (1–200 μM) for 72 hours. Cell cycle and viability were examined using flow cytometry analysis. Apoptosis was determined using the annexin V assay and also by fluorescence microscopy. Cyclins A, E and B1 were detected by Western blotting. Stability, cellular uptake and metabolism of PICE were evaluated using HPLC-DAD-MS-MS. *Results* The lowest PICE concentration assayed (1 μM) increased about 6-fold over the control the apoptotic population of melanoma cells (10.2 % at 8 hours which remained constant during 48 h). 100 μM PICE induced 13 % apoptosis at 8 h increasing up to 41.5 % at 48 h. The decrease in cell viability was highly correlated with the increase of apoptotic cells ($R = 0.996$; $P < 0.0001$) revealing that significant cytotoxic, unspecific effects did not occur in melanoma cells upon incubation with PICE. Cell cycle was arrested at G_2 phase which was supported by the down-regulation of cyclins A, E and B1. Two methyl-PICE derived metabolites, 3,5,4'-trihydroxy-3'-methoxy-*trans*-stilbene and 3,5,3'-trihydroxy-4'-methoxy-*trans*-stilbene (corresponding to 36 % of the initially PICE added) were excreted by cells to the medium. The same methyl-PICE derivatives were also found inside the cells (0.01 % of the initially PICE added; 0.0183 picograms/cell). *Conclusions* The antimelanoma properties of dietary piceatannol cannot be ruled out taking into account its fast and potent pro-apoptotic capacity at low concentration (1 μM).

Key words piceatannol – apoptosis – grape – wine – polyphenol – melanoma – cancer – SK-Mel-28 – cyclin – cell cycle – cell metabolite

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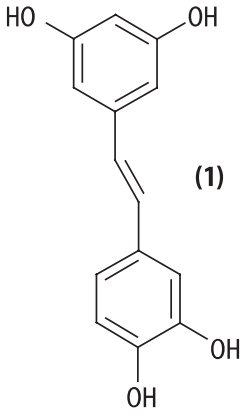
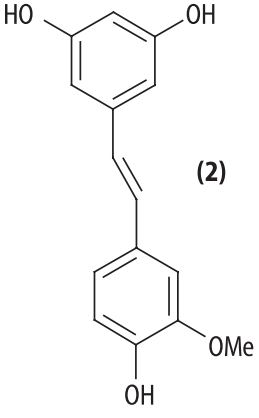
Introduction

The polyphenol piceatannol (3,5,3',4'-tetrahydroxy-*trans*-stilbene; PICE; Fig. 1, compound 1) has been used as an active ingredient in the traditional herbal medicine [1, 2] and identified as antileukemic agent from the seeds of *Euphorbia lagascae* [3]. In Western countries, piceatannol is mainly incorporated in the diet through the intake of grapes and red wine [4, 5].

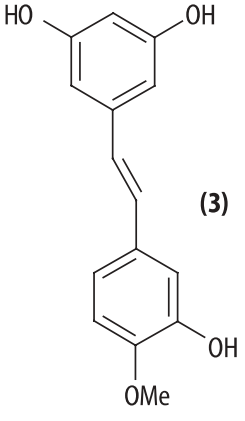
The health-promoting activities of the piceatannol analogue resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) have been widely established in a huge output of publications during the last few years [6–10]. However, the biological activity of PICE has been much less investigated. A previous report proposed that resveratrol could



3, 5, 3′, 4′ -tetrahydroxy-trans-stilbene
(*trans* -piceatannol; PICE)



3, 5, 4′-trihydroxy-3′-methoxy-trans-stilbene
(3′-methyl-PICE)



3, 5, 3′-trihydroxy-4′-methoxy-trans-stilbene
(4′-methyl-PICE)

Fig. 1 Piceatannol **[1]** and its methyl-derivatives: **[2]** 3′-methyl-piceatannol and **[3]** 4′-methyl-piceatannol

be converted to PICE in some tumours that overexpress the enzyme CYP1B1 with hydroxylase activity [11]. In this way, resveratrol would act as a pro-drug and the compound responsible for the biological activity against the tumour would be the resveratrol-hydroxylated derivative piceatannol.

PICE has been reported to be a protein-tyrosine kinase inhibitor [12] with immunosuppressive activity [13] which could be important for preventing graft rejection [14]. In addition, PICE possess anti-inflammatory properties by the suppression of activation of the nuclear transcription factor NF-κB through the inhibition of IκBα kinase and p65 phosphorylation [15]. Most of the previous studies about PICE have been focused in its protein-tyrosine kinase inhibitory properties. In fact, to our knowledge, only one previous work has described the potent pro-apoptotic capacity of PICE, in lymphoma cells and in primary leukemic lymphoblasts [16]. Another recent study has described the antiproliferative activity of PICE by inhibiting the progression of Caco-2 cells through the S phase of their cell cycle although no apoptosis assays were carried out [17].

Malignant melanoma is increasing at an alarming rate [18, 19]. The increased risk of melanoma is mainly related to UV exposure [20]. Melanoma can be cured by surgical excision if it is detected early. However, melanoma at later stages, with distant metastasis is currently incurable. Interferon-γ and interleukin-2 are used in melanoma therapy although with various degrees of success [21, 22]. Therefore, the search for new agents with a potential antimelanoma effect is encouraged.

Diet and melanoma incidence have been previously correlated [23, 24]. In this context, dietary bioactive compounds with potential antimelanoma activity at low (plasmatic) concentrations would be of great interest.

The aim of the present study is to evaluate the antimelanoma activity of piceatannol in the human melanoma cell line SK-Mel-28. Thus, the pro-apoptotic capacity and effect on cell cycle and cyclins A, E and B1 expression were studied. In addition, the uptake and metabolism of piceatannol by SK-Mel-28 melanoma cells were also investigated.

Materials and methods

Reagents

Piceatannol (3,5,3',4'-tetrahydroxy-*trans*-stilbene; PICE), minimum essential medium (MEM), glutamine, penicillin, streptomycin, sodium pyruvate, non-essential amino acids, fetal bovine serum, propidium iodide, dimethylsulfoxide (DMSO), sodium phosphate buffer pH 7 (PBS), Hoechst 33258 dye, staurosporine and RNase were purchased from Sigma-Aldrich (St. Louis, USA). Monoclonal cyclins E and B1 mouse anti-human antibodies

were purchased from BD Pharmingen (San Diego, USA). Monoclonal cyclin A mouse anti-human antibody was also obtained from Sigma.

Culture and treatment of melanoma cells

The SK-Mel-28 melanoma cell line was obtained from the ATCC (American Type Culture Collection; Manassas, USA). Cells were cultured (according to ATCC) in MEM containing 2 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 100 mM sodium pyruvate, non-essential amino acids and supplemented with 10 % fetal bovine serum (FBS). The cell line was maintained at 37 °C in a 5 % CO_2 atmosphere. The culture was periodically tested for *Mycoplasma* infection. Stock of PICE (20 mM) was prepared in DMSO and added to the culture media at concentrations of 1, 10, 25, 50 100 and 200 μM (depending on the assay). Control cells contained an equal amount of DMSO not exceeding 0.5 %. Media were replaced every 48 hours. Cells were used in the exponential growth phase (doubling time of 24 hours) in which the percentage of cells in each phase was constant up to 96 hours: G_0/G1, 72.5 ± 1.5 %; S, 19.2 ± 1.4 %; G_2M, 8.2 ± 1.6 %.

Piceatannol stability

Stability of PICE in MEM culture (at 37 °C and pH 7.5) was checked at 4, 8, 24 and 48 hours using a HPLC-DAD-MS-MS system equipped with both a DAD and mass detector in series from Agilent Technologies (Waldbronn, Germany). The mass detector was an ion-trap mass spectrometer equipped with an electrospray ionisation (ESI) system and controlled by software. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. Mass scan (MS) and daughter MS-MS spectra were measured from m/z 150 up to m/z 800. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas and the collision energy was set at 50 %. Mass spectrometry data were acquired in the positive ionisation mode. Culture media with piceatannol were filtered through a 0.45 μm membrane filter Millex-HV_{13} and aliquots of 20 μL were injected onto a reverse phase C_{18} LiChroCART column (25 × 0.4 cm, particle size 5 μm). The mobile phase was water:formic acid (99:1, v:v, Solvent A) and methanol (Solvent B) at a flow rate of 1 mL/min. The gradient started with 20 % B in A to reach 32 % B in A at 5 min, 40 % B in A at 10 min, 95 % B in A at 20 min. Chromatograms were recorded at 320 nm. HPLC-DAD-MS-MS experiments were repeated three times.

Cellular uptake

The uptake of PICE by SK-Mel-28 cells was carried out as described by Larrosa et al. [10]. Briefly, cells were incubated with 100 μM PICE for 48 h. After treatment, cells were washed with PBS, lysed by sonication and extracted with ethylacetate. Samples were analysed with the same HPLC-DAD-MS-MS equipment and assay conditions described above. Cellular uptake experiments were repeated three times.

Cell cycle distribution

Cells (1×10^6) were fixed in ice-cold ethanol:PBS [70:30] for 30 min at 4 °C, further re-suspended in PBS with 100 μg/mL RNase and 40 μg/mL propidium iodide and incubated at 37 °C for 30 min. DNA content (20,000 cells) was analysed using a FACScan instrument equipped with FACStation running Cell Quest software (Becton Dickinson). The analyses of cell cycle distribution were performed at least in triplicate for each treatment, including the control, at 24, 48 and 72 hours. Mean values of cell cycle distribution are shown. The coefficient of variation (CV), according to the FACStation Cell Quest software, was always less than 10 %.

Cell viability

Cells (1×10^5) were suspended in PBS with 5 μg/mL propidium iodide to stain dead cells. After 5 min, the percentage of viable cells (20,000 cells analysed) was calculated using the same flow cytometer described above. The determination of cell viability was carried out in triplicate. The coefficient of variation (CV), according to the FACStation Cell Quest software, was always less than 10 %. The analyses of cell viability were performed for each treatment, including the control, at 8, 24 and 48 hours. Mean values of cell viability are shown.

Western blotting

After 24, 48 and 72 hours of treatment, cells were washed twice with PBS and lysed in cold lysis buffer as described by Pozo-Guisado et al. [25]. Lysates were centrifuged at 15,000 g for 20 min at 4 °C and protein concentration was measured by the Bradford's method [26]. Protein (20 μg/lane for cyclins A and E and 40 μg/lane for cyclin B1) was separated by SDS-PAGE (10 % polyacrylamide) and transferred to Hybond™-P membranes (Amersham Biosciences, UK) by electroblotting. Membranes were incubated for two hours with the primary antibodies and one hour with the secondary antibody conjugated to horseradish peroxidase. Cyclins were detected

using an ECL™ detection system (Amersham Biosciences) according to manufacturer's instructions. For quantification, the density of the bands was detected with scanning densitometry, using a Syngene Genetools Analysis Software (Syngene, USA).

Annexin V assay

The annexin V/PI detection kit from Sigma was used. The perturbations in the cellular membrane during the early stages of apoptosis cause a redistribution of phosphatidylserine to the external side of the cell membrane. This provokes a flux of calcium which is required by the fluorescein-labeled annexin V to selectively bind to phosphatidylserine in order to identify cells undergoing apoptosis. Cells were also stained with propidium iodide to distinguish early and late apoptotic cells from necrotic cells. The protocol was that specified by the manufacturer with some modifications [10]. Staurosporine (0.1 μM) was assayed as standard inducer of apoptosis. The percentage of live, dead and apoptotic cells was determined. In the corresponding figure that illustrates the apoptosis (see Fig. 5), the viable cells (V) are located in the lower left corner (negative in both annexin V-FITC and propidium iodide). Early apoptotic cells (EA) are in the lower right corner (annexin V-FITC positive). Late apoptotic cells (LA) that show progressive cellular membrane and nuclear damage are in the upper right corner (positive in both annexin V-FITC and propidium iodide). Total percentage of apoptotic cells was considered as EA + LA [10]. The percentage of apoptotic cells was determined at 8, 24 and 48 hours.

Apoptosis assessment by fluorescence microscopy

Cells treated with 1–100 μM piceatannol for 8–48 h were fixed with methanol:glacial acetic acid [3:1] and stained with 50 mg/mL Hoechst 33258 dye at 37 °C for 20 min. Afterwards, the cells were examined under a Nikon Diaphot-TMD inverted microscope (Nikon, Japan). Hoechst 33258 is a DNA-binding fluorochrome dye which allows the study of the chromatin morphology. In contrast to normal cells, the nuclei of apoptotic cells have highly condensed chromatin that is uniformly stained by Hoechst 33258. This can take the form of crescents around the periphery of the nucleus, or the entire nucleus can appear to be one or a group of featureless, bright spherical beads.

Results and discussion

The number of human SK-Mel-28 melanoma cells decreased with increasing PICE concentrations and incubation time (Fig. 2). However, the decrease in cell viability was linear only with 100 μM piceatannol (PICE) because cell viability did not decrease after 8 hours of incubation with 1, 5 and 10 μM PICE (Fig. 2). The plateau was also reached after 24 hours with 50 μM PICE (Fig. 2). In addition, only slight differences in the cell viability decrease were observed with PICE treatments from 1 to 100 μM during the first 8 hours (Fig. 2). These results were not coincident with a recent report on the effect of other stilbenes of human SK-Mel-28 cells in which cell viability decreased linearly with increasing concentration (25–200 μM) and incubation time (24–96 h) [10]. However, the present results agree with a recent study on the antiproliferative activity of piceatannol on Caco-2 and HCT-116 cells in which no further decrease in cell viability was detected over 50 μM PICE [17].

According to FACS analysis, PICE induced G_2/M phase cell arrest concomitant with a decrease in G_0/G_1 and S phases in Sk-Mel-28 cells (Fig. 3) in contrast with the previously described S-phase arrest in Caco-2 cells [17]. It is not surprising that the same polyphenol can exert a different type of cycle arrest depending on both cell line and type of cancer as previously described. Although the information concerning the effect of PICE on cell cycle is scarce, however, the different effect of the same polyphenol depending on the cell line has been previously reported as in the case of the PICE analogue resveratrol (RES). Some studies have reported the RES-induced S-phase arrest in a number of cancer cell types such as Sk-Mel-28 [10], T47 breast carcinoma [27], CEM-C7H2 acute leukemia [28], etc., whereas other studies have reported the RES-mediated arrest in G_0/G_1 in human epidermoid carcinoma [29] and gastric adenocarcinoma cells [30]. A recent study has revealed the S-phase arrest of SK-Mel-28 cells by RES [10] whereas

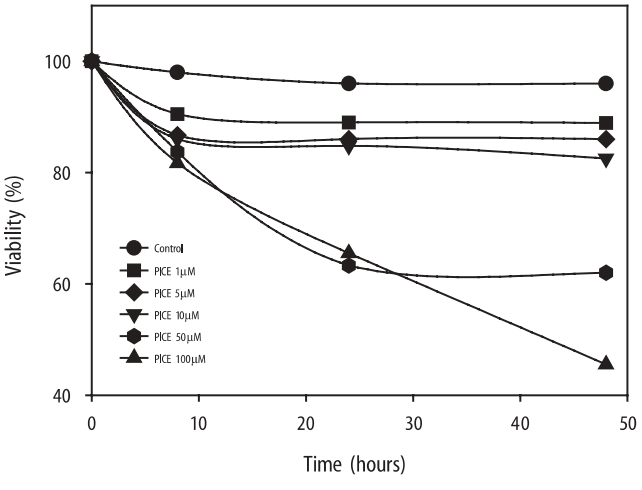


Fig. 2 Effect of PICE on cell viability of human SK-Mel-28 melanoma cells. Cell viability was determined by flow cytometry. Coefficient of variation (CV) was less than 10 %

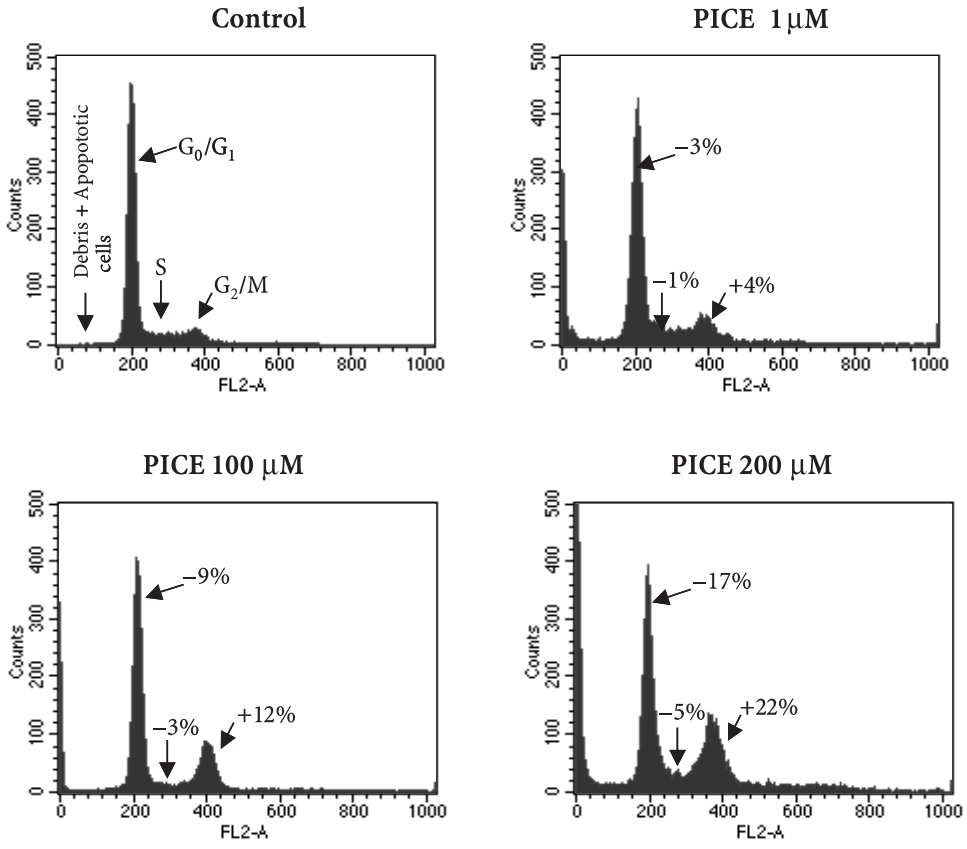


Fig. 3 Flow cytometry analysis of DNA distribution in control and PICE-treated SK-Mel-28 melanoma cells after 48 hours. Either the increase or decrease (%) of the corresponding phase with respect to the control is shown. CV was less than 10 %

the results presented here demonstrate that PICE arrested the same cells at G_2/M phase (Fig. 3). The presence of the extra hydroxyl group in the 3'-position of PICE with respect to RES appears to be critical in the activity of PICE. This was in accordance with the reported potent PICE-induced apoptosis in primary leukemic lymphoblasts whereas RES did not induce significant apoptosis in these cells [16]. Therefore, these results agree with the previously suggested important role regarding the number and position of hydroxyl groups as determinants in the anticancer and antioxidant activities of stilbenes [10, 31].

Cell cycle progression is regulated by the activity of cyclins, a family of proteins which activate the so-called cyclin-dependent-kinases (Cdks). Cyclin A is required for S-phase and passage through G_2, cyclin E activates Cdk2 protein near the start of S-phase and cyclin B1 is a critical regulator of mitosis. In general, uncontrolled expression of cyclins and/or Cdks leads to either tumorigenesis or cell cycle arrest [32]. Overall, the arrest of SK-Mel-28 melanoma cells in G_2/M was correlated with the downregulation in the expression of cyclins A, E and B1 upon increasing PICE concentrations and incubation time (Fig. 4). In a previous study, the arrest in the S-phase of Caco-2 cells was correlated with the downregulation of cyclins D and B1 whereas cyclins E and A were upregulated [17]. The same results were reported in the S-phase arrest of Caco-2 cells upon treatment with RES [8]. However, in the case of SK-Mel-28 cells, it has been previously published that the RES-induced S-phase arrest correlated with the upregulation of cyclins A, E and B1 [10] whereas the PICE-mediated G_2/M arrest of SK-Mel-28 melanoma cells was related with the downregulation of these cyclins (Fig. 4). It has been described that G_2 phase arrest is induced by DNA damage providing an opportunity to eliminate cells with irreversible damage by activating pathways leading to programmed cell death (apoptosis) if cellular damage cannot be properly repaired [33]. Recently, the downregulation of cyclin B1, as that observed in our study (Fig. 4), has been correlated with stable G_2 arrest [34]. However, whether the PICE-induced G_2 arrest in SK-Mel-28 melanoma cells was via a p53-dependent or -independent pathway was not evaluated in the present study and deserves further investigations.

Piceatannol was a potent inducer of apoptosis in human SK-Mel-28 melanoma cells as revealed by the annexin V assay (Fig. 5 A–D) and Hoechst dye staining (Fig. 5E). The lowest PICE concentration assayed (1 μM) was able to increase about 6-fold over the control the apoptotic population of cells after 48 hours (Fig. 5 A, B). The percentage of apoptotic melanoma cells increased with

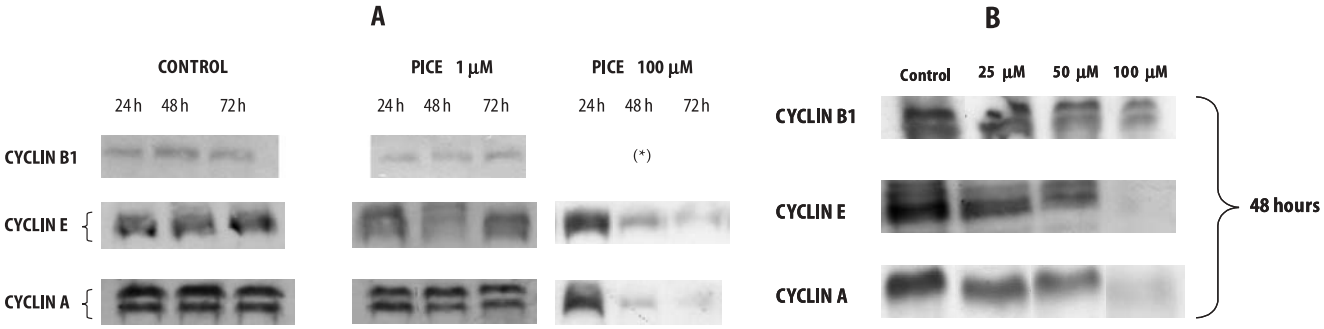


Fig. 4 Western blot analysis of cyclins B1, E and A in control and PICE-treated SK-Mel-28 melanoma cells. **A** PICE 1 and 100 μM at 24, 48 and 72 hours. (*) Not assayed. **B** PICE 25, 50 and 100 μM at 48 hours. Western blot experiments were repeated three times for each assay condition

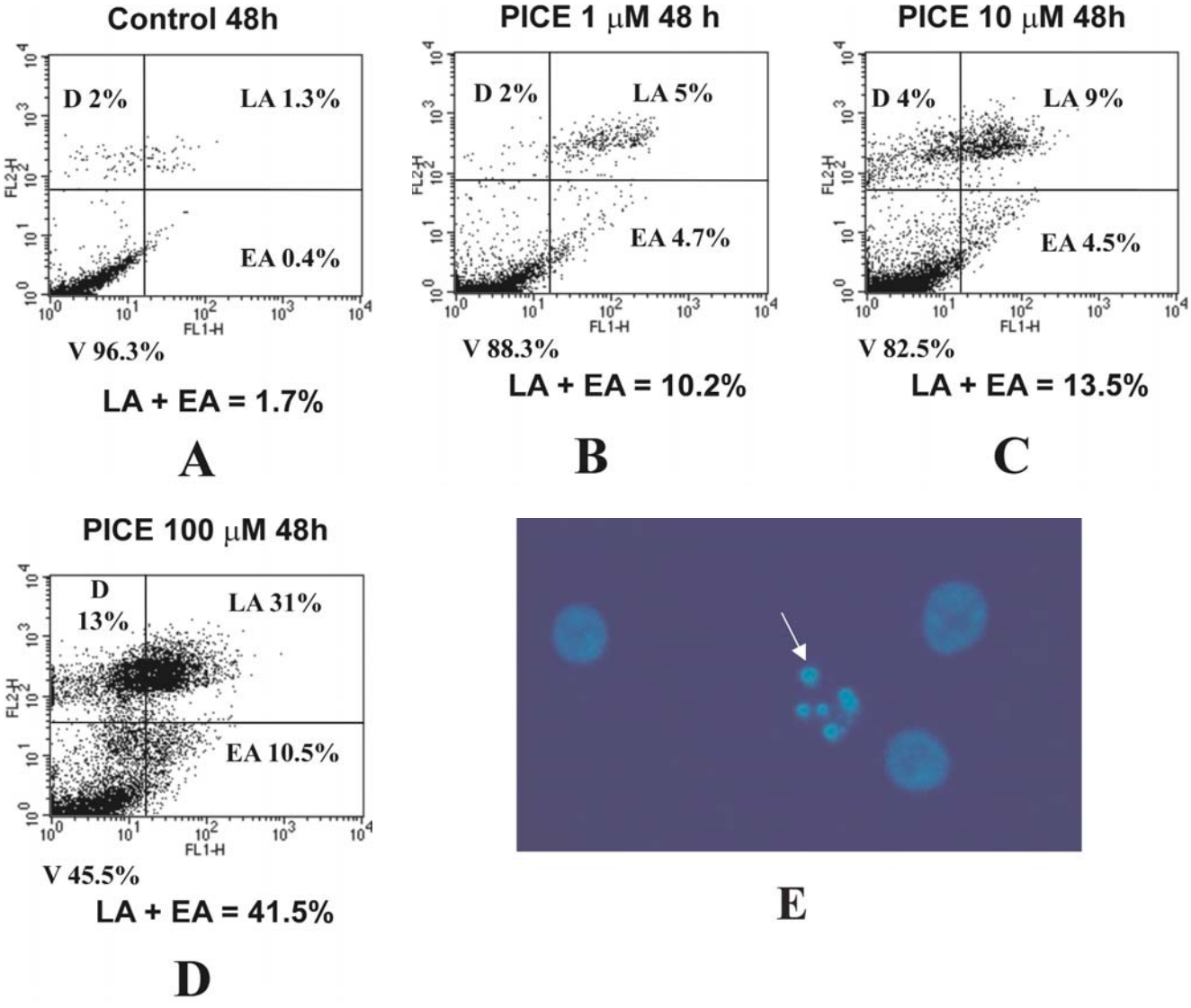


Fig. 5 **A–D** Apoptosis detection in SK-Mel-28 cells using the annexin V assay after 48 hours. *D* dead cells (upper left corner); *V* viable cells (lower left corner); *EA* early apoptotic cells (lower right corner); *LA* late apoptotic cells (upper right corner). The total percentage of apoptotic cells was considered as EA + LA. Staurosporine 0.1 μM (positive control) induced 25 % apoptosis after 48 h. Annexin V assays were performed in triplicate. **E** Apoptosis detection using the Hoechst 33258 dye. Cells were treated with 1 μM PICE for 8 hours. The arrow designates the nucleus of an apoptotic cell with the characteristic stained bright spherical beads. The Hoechst staining was carried out in three independent experiments

increasing PICE concentrations and incubation time but in a non-linear manner (Fig. 6), similar to the decrease pattern observed in cell viability (Fig. 2). In fact, the decrease in cell viability was strongly correlated with the increase of apoptotic cells ($R = 0.996$; $P < 0.0001$) which revealed that unspecific, cytotoxic effects of PICE on SK-Mel-28 melanoma cells were very low which was in accordance with the previously reported effect of PICE on the lymphoma BJAB cell line [16]. However, it should be also stressed that the correlation between viable and apoptotic cells was observed in the concrete assay conditions reported in the present study so that further extrapolation to other PICE concentrations and assay times should be taken with precaution.

To our knowledge, there are no previous studies describing the apoptosis of cancer cells in so low assay concentration (1 μM) since most of these studies assayed 10 μM or higher concentrations. This is of great interest since the current (basic) requirements to report anticarcinogenic potential *in vitro* strongly recommend a plausible ("rather realistic") concentration which could be, at least potentially, extrapolated *in vivo* [35]. Therefore, it should be a logical approach to assay low (plasmatic) concentrations in the screening of potential dietary anticancer molecules. Although 1 μM is still a rather high "plasmatic" concentration, at least, it is in the range of possible data regarding the bioavailability of polyphenols or polyphenol-derived metabolites [36].

A previous report revealed the high instability of PICE in the culture medium which called for the caution in the assay of *ortho*-diphenolic compounds [10]. In fact, 100 μM PICE disappeared in the medium following a decreasing uniexponential curve with $t_{1/2} = 4.5$ h (results not shown). Despite its rather high instability, PICE provoked the above-mentioned effects on melanoma cells.

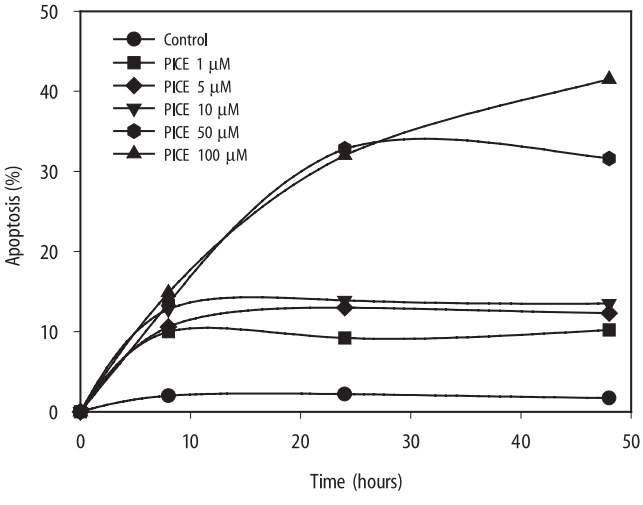


Fig. 6 Evolution of apoptotic SK-Mel-28 melanoma cells upon treatment with different PICE concentrations and incubation times (Apoptotic cells were determined using the annexin V assay)

To delve into this apparent controversy, the uptake and metabolism of PICE by human SK-Mel-28 melanoma cells was also investigated. After 48 h of treatment with 100 μM PICE, two metabolites were detected in both culture medium and cells lysate (Fig. 7). No oxidation products (piceatannol-quinone derivatives) were detected. After LC-MS-MS analysis (Fig. 8), the metabolites with [M + H] ion at 259 m/z were identified as 3,5,4'-trihydroxy-3'-methoxy-*trans*-stilbene (3'-methyl-PICE) and 3,5,3'-trihydroxy-4'-methoxy-*trans*-stilbene (4'-methyl-

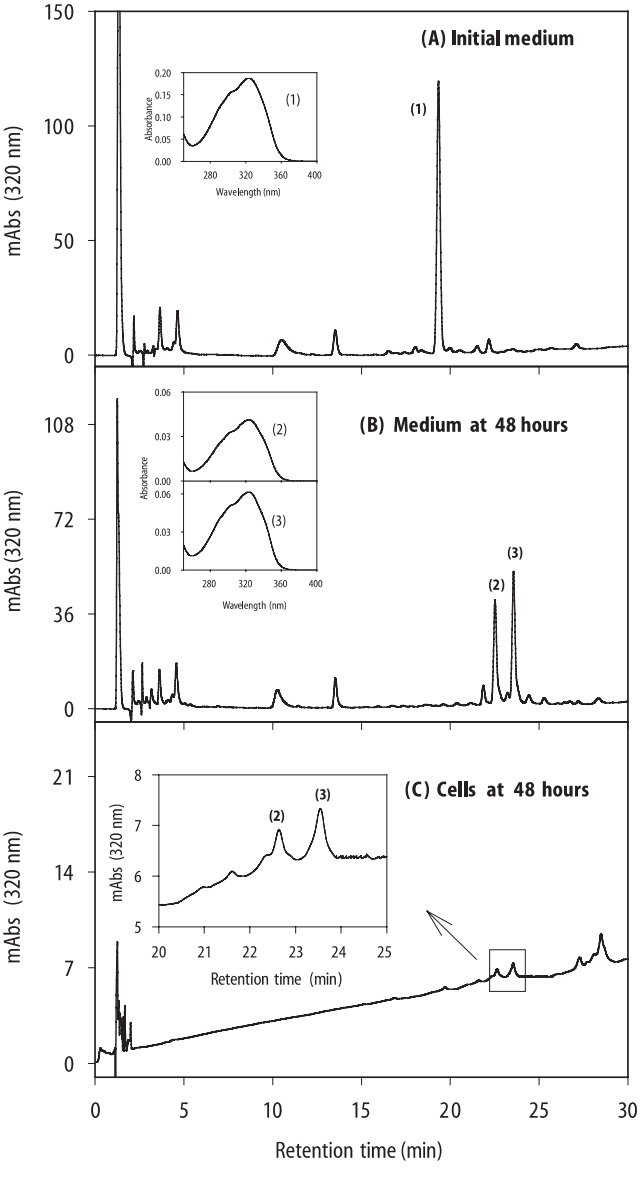


Fig. 7 **A** HPLC profile of initial culture medium containing 100 μM PICE (compound **1**; Fig. 1). The insert shows the UV spectrum of PICE. **B** HPLC profile of culture medium after 48 hours. **2** (3'-methyl-PICE, Fig. 1) and **3** (4'-methyl-PICE, Fig. 1) are PICE-derived metabolites excreted to the medium by SK-Mel-28 cells. The inserts show their corresponding UV spectra. **C** HPLC profile of lysed cells after treatment with 100 μM PICE for 48 hours. The insert amplifies the region of the chromatogram where compounds **2** and **3** are detected.

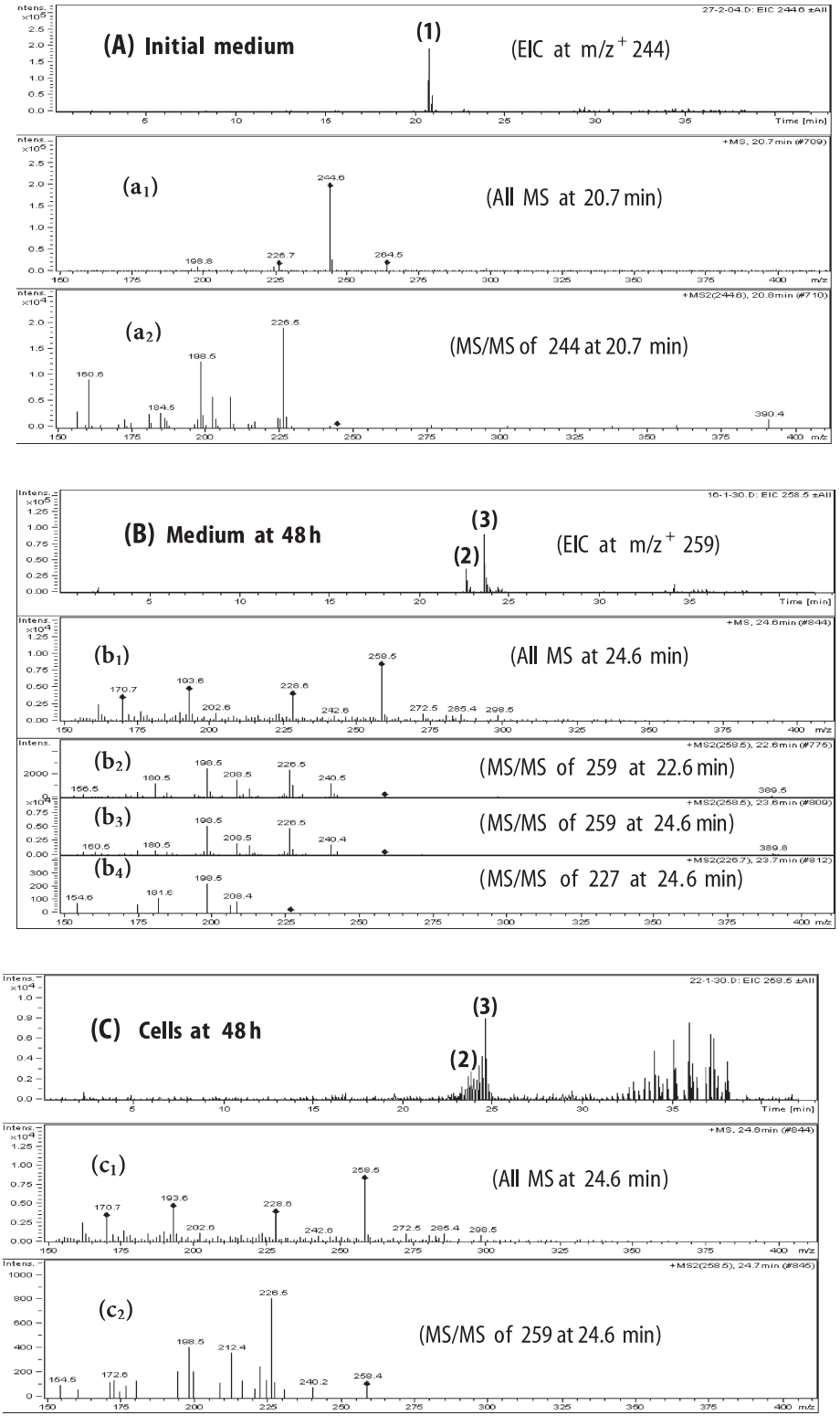


Fig. 8 MS-MS analysis of data shown in Fig. 7. **A** Initial culture medium containing 100 µM PICE. Extracted ion chromatogram (EIC) of ion 244 m/z+, corresponding to compound **1** (PICE). **(a_1)** All MS detected at the retention time of the EIC. **(a_2)** MS-MS daughter ions of 244 m/z+ showing the main (resveratrol) fragment at 227 m/z+. **B** MS-MS analysis of culture medium after 48 hours. EIC at 259 m/z+ (ion mass of methyl-piceatannol derivatives, **2** and **3**). **(b_1)** All MS detected at the retention time of EIC 259 m/z+. **(b_2** and **b_3)** MS-MS daughter ions of 259 m/z+. **(b_4)** MS-MS daughter ions of 227 m/z+ corresponding to resveratrol, the main daughter ion of both PICE and methyl-PICE derivatives. **C** MS-MS analysis of cells after 48 hours. EIC at 259 m/z+. **(c_1)** All MS at the retention time of EIC 259 m/z+. **(c_2)** MS-MS daughter ions of 259 m/z+

PICE) (Fig. 1, compounds 2,3). The reversed-phase HPLC behaviour of flavonoid isomers with either 3'-hydroxy-4'-methoxy (i. e., diosmetin, tamarixetin, catechin 4'-methyl ether, 5,3'-dihydroxy-6,7,4'-trimethoxyflavone) or the corresponding isomers with 3'-methoxy-4'-hydroxy substitutions (i. e., chrysoeriol, isorhamnetin, catechin 3'-methyl ether, 5,4'-dihydroxy-6,7,3'-trimethoxyflavone) shows that the 4'-methoxy isomers always elute with longer retention times than the corresponding 3'-methoxy isomers [37, 38]. This is most certainly due to the more acidic character of the hydroxyl present in the 4'-position than in the case of the 3'-position. A similar behaviour is observed with ferulic acid (caffeic acid 3'-methyl ether) and its *in vivo*-generated metabolite isoferulic acid (caffeic acid 4'-methyl ether) [39].

After 48 h, the methyl-PICE metabolites represented 0.01 % (0.0183 picograms/cell) and 36 % (88 µg) of the initially added PICE in the cells lysate and culture medium, respectively. These data were similar to those obtained for resveratrol (RES) in the same cell line [10] which meant that the extra hydroxyl group in the 3' position with respect to RES did not affect significantly the cellular uptake. In the case of RES no methyl-derivatives were observed because RES is not a substrate for COMT due to the lack of the *o*-diphenolic moiety.

The methylation of PICE in the 3' and 4' positions suggested the involvement of COMT (catechol-*O*-methyltransferase) in the metabolism of PICE by human SK-Mel-28 melanoma cells. The role of COMT (as detoxifying enzyme) in the metabolism of polyphenols has been described in different tissues, subcellular fractions and cell lines [40–42]. In the case of melanoma, the inhibition of COMT has been proposed as a tool to induce cytotoxicity in melanoma cells [43]. Therefore, it can be hypothesised that the melanoma cells tried to convert PICE to methyl-PICE derivatives to diminish the potential toxicity of the polyphenolic hydroxyl groups which have been reported to exert selectively great anticancer activity [31, 44]. Other polyphenols with *o*-diphenolic moiety assayed on SK-Mel-28 cells (quercetin, caffeic acid, dihydrocaffeic acid, hydroxytyrosol, etc.) also yielded the corresponding methyl-derivatives whereas their counterparts (without *o*-diphenolic moiety, i. e., kaempferol, *p*-coumaric acid, dihydro-*p*-coumaric acid, tyrosol, etc.) were not methylated which supported the COMT activity of melanoma cells (results not shown).

Taking into account the PICE instability in the medium and despite the kinetics of PICE uptake by cells not being evaluated, the fast action of PICE against melanoma cells can be assumed (Figs. 2, 5, 6). In fact, the plateau observed in the PICE pro-apoptotic activity (Fig. 6) could be related to both instability of PICE in the medium and detoxifying action of COMT in melanoma cells.

More investigations are needed to fully understand the underlying mechanism in the antimelanoma activity of piceatannol. In addition, there are no available data regarding the bioavailability of piceatannol in humans or animal models, which also deserves additional research. The present results support the previous data regarding the anticancer activity of piceatannol. In this context, the potent and fast pro-apoptotic capacity at low (possibly plasmatic) concentrations of piceatannol in melanoma cells could support the convenience of the intake of piceatannol-containing sources such as grapes and red wine as a healthy dietary habit.

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References

1. Inamori Y, Kato Y, Kubo M, Yasuda M, Baba K Kozawa M (1984) Physiological activities of 3,3'–4,5'-tetrahydroxystilbene isolated from the heartwood of Cassia garrettiana CRAIB. Chem Pharm Bull (Tokyo) 32:213–218
2. Matsuda H, Kageura T, Morikawa T, Toguchida I, Harima S, Yoshikawa M (2000) Effects of stilbene constituents from rhubarb on nitric oxide production in lipopolysaccharide-activated macrophages. Bioorg Med Chem Lett 10:323–327
3. Ferrigni NR, McLaughin JL, Powell RG, Smith CR (1984) Use of potato disc and brine shrimp bioassays to detect activity and isolate piceatannol as the antileukemic principle from the seeds of Euphorbia lagascae. J Nat Prod 47: 347–352
4. Cantos E, Espín JC, Tomás-Barberán FA (2002) Postharvest stilbene-enrichment of red and white table grape varieties using UV-C irradiation pulses. J Agric Food Chem 50:6322–6329
5. Cantos E, Espín JC, Fernández MJ, Oliva J, Tomás-Barberán FA (2003) Postharvest UV-C irradiated grapes as potential source for producing stilbene-enriched red wines. J Agric Food Chem 51:1208–1214
6. Della Ragione F, Cucciolla V, Borriello A, Della Pietra V, Racioppi L, Soldati G, Manna C, Galletti P, Zappia V (1998) Resveratrol arrests the cell division cycle at S/G2 phase transition. Biochem Biophys Res Commun 250:53–58
7. Gusman J, Malonne H, Atassi G (2001) A reappraisal of the potential chemopreventive and chemotherapeutic properties of resveratrol. Carcinogenesis 22: 1111–1117
8. Wolter F, Akoglu B, Clausnitzer A, Stein J (2001) Downregulation of the cyclin D1/Cdk4 complex occurs during resveratrol-induced cell cycle arrest in colon cancer cell lines. J Nutr 131: 2197–2203
9. Joe AK, Liu H, Suzui M, Vural ME, Xiao D, Weinstein IB (2002) Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. Clin Cancer Res 8: 893–903
10. Larrosa M, Tomás-Barberán FA, Espín JC (2003) Grape polyphenol resveratrol and the related molecule 4-hydroxystilbene induce growth inhibition, apoptosis, S-phase arrest and upregulation of cyclins A, E and B1 in human SK-Mel-28 melanoma cells. J Agric Food Chem 51:4576–4584

11. Potter GA, Patterson LH, Wanogho E, Perry PJ, Butler PC, Ijaz T, Ruparelia KC, Lamb JH, Farmer PB, Stanley LA, Burke MD (2002) The cancer preventative agent resveratrol is converted to the anticancer agent piceatannol by the cytochrome P450 enzyme CYP1B1. Br J Cancer 86:774–778
12. Geahlen RL, McLaughin JL (1989) Piceatannol (3,4,3',5'-tetrahydroxy-trans-stilbene) is a naturally occurring protein-tyrosine kinase inhibitor. Biochem Biophys Res Commun 165: 241–245
13. Seow CJ, Chue SC, Wong WS (2002) Piceatannol, a Syk-selective tyrosine kinase inhibitor, attenuated antigen challenge of guinea pig airways in vitro. Eur J Pharmacol 443:189–196
14. Fernández LA, Torrealba J, Yagci G, Ishido N, Tsuchida M, Tae Kim H, Dong Y, Oberley T, Fechner J, Colburn MJ, Shultz J, Kanmaz T, Hu H, Knechtle SJ, Hamawy MM (2002) Piceatannol in combination with low doses of cyclosporine A prolongs kidney allograft survival in a stringent rat transplantation model. Transplantation 74: 1609–1617
15. Ashikawa K, Mjumdar S, Banerjee S, Bharti AC, Shishodia S, Aggarwal BB (2002) Piceatannol inhibits TNF-induced NF-κB activation and NF-κB-mediated gene expression through suppression of IκBα kinase and p65 phosphorilation. J Immunol 169: 6490–6497
16. Wieder T, Prokop A, Bagci B, Essmann F, Bernicke D, Schulze-Osthoff K, Dörken B, Schmalz HG, Daniel PT, Henze G (2001) Piceatannol, a hydroxylated analog of the chemopreventive agent resveratrol, is a potent inducer of apoptosis in the lymphoma cell line BJAB and in primary, leukemic lymphoblasts. Leukemia 15:1735–1742
17. Wolter F, Clausnitzer A, Akoglu B, Stein J (2002) Piceatannol, a natural analog of resveratrol, inhibits progression through the S phase of the cell cycle in colorectal cancer cell lines. J Nutr 132: 298–302
18. Hall HI, Miller DR, Rogers JD, Bewerse B (1999) Update on the incidence and mortality from melanoma in the United States. J Am Acad Dermatol 40:25–42
19. Bastuji-Garin S, Diepgen TL (2002) Cutaneous malignant melanoma, sun exposure, and sunscreen use: epidemiological evidence. Br J Dermatol 146: 24–30
20. Gilchrest BA, Eller MS, Geller AC, Yaar M (1999) The pathogenesis of melanoma induced by ultraviolet radiation. New Engl J Med 340:1341–1348
21. Agarwala SS, Kirkwood JM (1998) Adjuvant interferon treatment for melanoma. Hematol Oncol Clin North Am 12:823–833
22. Keilholz U, Eggermont AM (2000) The emerging role of cytokines in the treatment of advanced melanoma. For the EORTC melanoma cooperative group. Oncology 58:89–95
23. Fu YM, Yu ZX, Ferrans VJ, Meadows GG (1997) Tyrosine and phenylalanine restriction induces G0/G1 cell cycle arrest in murine melanoma in vitro and in vivo. Nutr Cancer 29:104–113
24. Veierod MB, Thelle DS, Laake P (1997) Diet and risk of cutaneous malignant melanoma: a prospective study of 50,757 Norwegian men and women. Int J Cancer 71:600–604
25. Pozo-Guisado E, ÁAlvarez-Barrientos A, Mulero-Navarro S, Santiago-Josefat B, Fernández-Salguero PM (2002) The antiproliferative activity of resveratrol results in apoptosis in MCF-7 but not in MDA-MB-231 human breast cancer cells: cell-specific alteration of the cell cycle. Biochem Pharmacol 64: 1375–1386
26. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal Biochem 210:727–735
27. 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
28. Bernhard D, Tinhofer I, Tonko M, Hübl H, Ausserlechner MJ, Grell R, Kofler R, Csordas A (2000) Resveratrol causes arrest in the S-phase prior to Fas-independent apoptosis in CEM-C7H2 acute leukaemia cells. Cell Death Diff 7: 834–842
29. Ahmad N, Adhami VM, Afaq AF, Feyer DK, Mukhtar H (2001) Resveratrol causes WAF1/p21-mediated G1-phase arrest of cell cycle and induction of apoptosis in human epidermoid carcinoma A431 cells. Clin Cancer Res 7: 1466–1473
30. Atten MJ, Attar BM, Milson R, Holian O (2001) Resveratrol-induced inactivation of human gastric adenocarcinoma cells through a protein kinase C-mediated mechanism. Biochem Pharmacol 62:1423–1432
31. Stivala LA, Savio M, Carafoli F, Perusca P, Bianchi L, Maga G, Forti L, Pagnoni UM, Albini A, Prosperi E, Vannini V (2001) Specific structural determinants are responsible for the antioxidant activity and cell cycle effects of resveratrol. J Biol Chem 276:22586–22594
32. Le NTV, Richardson DR (2002) The role of iron in cell cycle progression and the proliferation of neoplastic cells. Biochim Biophys Acta 1603:31–46
33. Pietenpol JA, Stewart ZA (2002) Cell cycle checkpoint signalling: cell cycle arrest versus apoptosis. Toxicology 181–182:475–481
34. Clifford B, Beljin M, Stark GR, Taylor WR (2003) G2 arrest in response to topoisomerase II inhibitors: the role of p53. Cancer Res 63:4074–4081
35. Verhagen H, Aruoma OI, van Delft JHM, Dragsted LR, Ferguson LR, Knasmüller S, Pool-Zobel BL, Poulsen HE, Williamson G, Yannai S (2003) The 10 basic requirements for a scientific paper reporting antioxidant, antimutagenic or anticarcinogenic potential of test substances in in vitro experiments and animal studies in vivo. Food Chem Toxicol 41:603–610
36. Scalbert A, Williamson G (2000) Dietary intake and bioavailability of polyphenols. J Nutr 130:2073S–2085S
37. Manach C (2003) The use of HPLC with coulometric array detection in the analysis of flavonoids in complex materials. In: Santos-Buelga C, Williamson G (eds) Methods in Polyphenol Analysis. The Royal Society of Chemistry, Cambridge, pp 61–91
38. Bohn BA (1998) Introduction to flavonoids. Harwood Academic Publishers, Amsterdam, p 195
39. Rechner AR, Kuhnle G, Bremner P, Hubbard GP, Moore KP, Rice-Evans CA (2002) The metabolic fate of dietary polyphenols in humans. Free Radic Biol Med 33:220–235
40. Caruso D, Visioli F, Patelli R, Galli C, Galli G (2001) Urinary excretion of olive oil phenols and their metabolites in humans. Metabolism 50:1426–1428
41. Manna C, Galletti P, Maisto G, Cucciolla V, D'Angello S, Zapia V (2000) Transport mechanism and metabolism of olive oil hydroxytyrosol in Caco2-cells. FEBS Lett 470:341–344
42. Moridani MY, Scobie H, O'Brien PJ (2002) Metabolism of caffeic acid by isolated rat hepatocytes and subcellular fractions. Toxicol Lett 133:141–151
43. Smit NP, Pavel S (1995) Induction of cytotoxicity in melanoma cells through inhibition of catechol-O-methyltransferase. Biochem Pharmacol 50: 1955–1962
44. Morris GZ, Williams RL, Elliott MS, Beebe SJ (2002) Resveratrol induces apoptosis in LNCaP cells and requires hydroxyl groups to decrease viability in LNCaP and DU 145 cells. Prostate 52: 319–329