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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

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The effect of the naturally occurring polyphenol resveratrol (3,5,4′-trihydroxy-*trans*-stilbene; RES) on growth, cell cycle, and cyclins A, E, and B1 expression was investigated in the human SK-Mel-28 melanoma cell line. In addition, the structurally related compounds 4-hydroxy-*trans*-stilbene (4HST), piceatannol (3,5,3′,4′-tetrahydroxy-*trans*-stilbene (PICE), and 4-*trans*-stilbenemethanol (4STMe) were also assayed in order to investigate the requirements of stilbenes to exert activity against melanoma cells. Both RES and 4HST inhibited cell growth in a dose- and time-dependent manner and upregulated the expression of cyclins A, E, and B1 with subsequent irreversible arrest of melanoma cells in the S-phase, concomitant with a decrease in G_0/G_1 and G_2/M phases. In addition, potent apoptosis-mediated cell death was detected with the annexin V assay whereas no apoptosis was observed by flow cytometry, which encourages the assay of different methodologies to evaluate the effect of polyphenols on cell lines. The effect of PICE was not evaluated because of its instability in the reaction medium. No effect on cell cycle and cyclins expression was observed when 4STMe was assayed, which supported the critical requirement of the 4′-hydroxystyryl moiety to exert the above effects. In addition, this structural requirement also influenced the cellular uptake of stilbenes. The presence of two extra hydroxyl groups in RES increased its cytotoxicity whereas it diminished its efficiency to inhibit cell growth, upregulate cyclins expression, and arrest cell cycle in the S-phase with respect to 4HST. The present study suggests that the antimelanoma properties of dietary stilbenes, such as grape RES, cannot be ruled out, taking into account previous studies concerning the relationship between plasma and tissue concentrations and pharmacological activity of RES in animal models.

KEYWORDS: Apoptosis; grape polyphenol; melanoma; cancer; SK-Mel-28; resveratrol; 4-hydroxystilbene; cyclin; cell cycle; stilbenemethanol; flow cytometry; stilbene

INTRODUCTION

Melanoma is one of the types of cancer with an increasing incidence and mortality rate (*1*). The increased risk of melanoma is mainly related to UV exposure (*2*), which means that sun protection behaviors such as the use of sunscreen lotions are strongly recommended. However, it has been reported that the use of these lotions does not fully protect against melanoma and seems to increase the duration of recreational sun exposure (*3*). Nowadays, melanoma can be cured by surgical excision if it is detected early. However, melanoma with distant metastasis is currently incurable.

The polyphenolic compound resveratrol (3,5,4′-trihydroxy-*trans*-stilbene; RES), which naturally occurs in grapes and grape-derived foodstuffs such as red wine, has been claimed to exert many different potential health-promoting effects (*4*) due to its antioxidant (*5*), antiplatelet (*6*), antiinflammatory (*7*), estrogenic (*8*), cardio protective (*9*), and antitumor (*10*−*18*) activities. In fact, the huge output of studies concerning these potential health beneficial effects has given rise to highly demanded commercial RES-based nutraceuticals or stilbene-enriched functional foods (*19*, *20*).

The relationship between plasma and tissue concentrations and pharmacological activity of RES in animal models has been previously reported (*21*). This could indicate that long-term consumption of RES-containing foodstuffs could explain health benefits such as those involved in the so-called French Paradox, which suggests that moderate intake of red wine over a long time could prevent cardiovascular diseases (*22*).

Regarding the antitumor activities of RES, a number of studies have demonstrated its inhibitory effect on cell cycle progression. Most of these studies have reported to arrest cell cycle in the S/G_2 phase transition by modifying its regulation mechanism or by inhibiting the enzymes ribonucleotide reduc-

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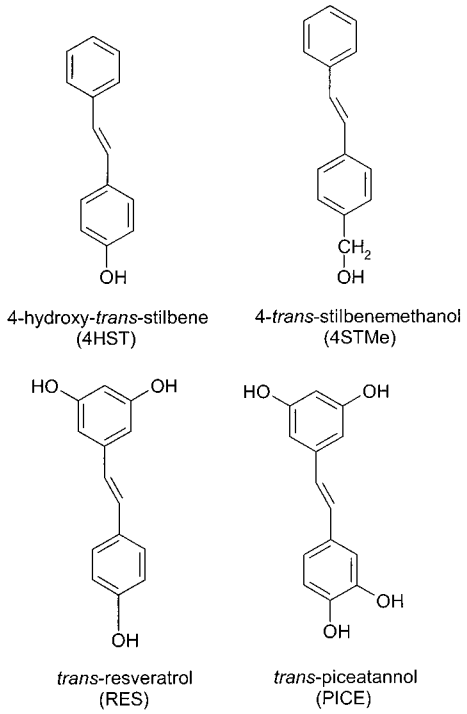

Figure 1. Stilbene structures assayed in the present study.

tase or DNA polymerase (*12*−*14*). Despite the high output of investigations regarding antitumor activities of RES in various cell lines corresponding to different types of cancer such as breast carcinoma, leukemia, colorectal cancer, etc. (*10*−*18*), however, only a few recent studies reported the effect of RES on murine melanoma cells (*23*, *24*). Despite a recent paper on the effect of RES on melanoma cells (*25*), to our knowledge, this is the first paper that describes the structure−activity relationship of RES and related stilbenes on a human melanoma cell line. In addition, it should be noted that the effect of phenolics on cells dramatically changes depending on the type of cancer and even on the cell line (*10*−*18*).

Although apparently the link between nutrition and melanoma could not be easily addressed, however, diet and melanoma incidence have been previously correlated (*26*, *27*). In this context, it is the aim of the present study to investigate the effect of RES and other related stilbenes (**Figure 1**), i.e., 4-hydroxy-*trans*-stilbene (4HST), 4-*trans*-stilbenemethanol (4STMe), and piceatannol (3,5,3′,4′-tetrahydroxy-*trans*-stilbene; PICE) on the human SK-Mel-28 melanoma cell line. To this purpose, the analysis of growth, viability, cycle, and expression pattern of the cyclins A, B1, and E in this human melanoma cell line will be studied.

MATERIALS AND METHODS

Reagents. 4HST was purchased from Acros Organics (Geel, Belgium). The stilbenes RES, PICE, and 4STMe were purchased from Sigma-Aldrich (St. Louis, MO). Minimum essential medium (MEM), glutamine, penicillin, streptomycin, sodium pyruvate, nonessential amino acids, fetal bovine serum, propidium iodide, dimethyl sulfoxide (DMSO), sodium phosphate buffer, pH 7 (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and RNase were also purchased from Sigma.

Monoclonal cyclins E and B1 mouse antihuman antibodies were purchased from BD Pharmingen (San Diego, CA). Monoclonal cyclin A mouse antihuman antibody and FITC-conjugated goat antimouse antibody were 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, VA). Cells were cultured in MEM containing 2 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 100 mM sodium piruvate, and nonessential amino acids and supplemented with 10% fetal bovine serum. The cell line was maintained at 37 °C in a 5% CO_2 atmosphere. The culture was periodically tested for *Mycoplasma* infection.

Stocks of the different stilbenes (previously dissolved in DMSO) were added to the culture media at concentrations of 25, 50, 100, and 300 μM for RES; 25, 50, and 100 μM for PICE; 25 and 50 μM for 4HST; and 25 μM for 4STMe. Stilbene compounds were protected from light to avoid conversion from trans to cis form. DMSO did not exceed 0.5% as the final concentration. This DMSO concentration did not affect either cell viability or cell cycle. Higher concentrations of 4HST and 4STMe could not be assayed due to solubility problems (higher DMSO concentrations interfered with cell viability). Other assayed solvents (methanol, dimethylformamide, etc.) did not improve stilbene solubility and also greatly decreased cell viability. Media were replaced every 48 h.

The stilbene content was analyzed in the media in the absence and in the presence of cells in order to investigate both the stilbene stability and the presence of possible excreted metabolites. Media were analyzed immediately after stilbene addition and after 48 h of treatment. Analysis of stilbene content in the media was checked daily using a high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS-MS) system equipped with both a diode array detector (DAD) and mass detector in series, which consisted of a HPLC binary pump, an autosampler, a degasser, and a photodiode array detector controlled by software from Agilent Technologies (Waldbronn, Germany). Culture media with stilbene content were filtered through a 0.45 μm membrane filter Millex-HV_{13} (Millipore Corporation, U.S.A.) and protected from light to avoid cis isomerization of stilbenes. Samples of 100 μL were loaded onto a reverse phase C_{18} LiChroCART column (25 cm × 0.4 cm, particle size 5 μm, Merck, Darmstadt, Germany). The mobile phase was water with 1% formic acid (solvent A) and HPLC grade 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, and 95% B in A at 25 min. UV chromatograms were recorded at 320 nm. Identification of stilbenes was achieved by comparison with authentic standards. The mass detector was an ion trap mass spectrometer (Agilent Technologies) equipped with an electrospray ionization 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 ionization mode. HPLC-DAD-MS-MS experiments were repeated three times.

Cellular Uptake. Sk-Mel-28 cells were grown up to confluence and were incubated for 48 h with 100 μM RES, 25 μM 4STMe, 100 μM PICE, and 50 μM 4HST. After treatment, cells (3×10^6) were trypsinized, transferred to plastic tubes, and washed three times with PBS and further lysed by sonication. Then, stilbenes were extracted twice with ethyl acetate. The organic phase was removed under a stream of nitrogen, resuspended in 150 μL of MeOH, and filtered through a 0.45 μm membrane filter Millex-HV_{13}. Afterward, samples (100 μL) were analyzed with the HPLC-DAD-MS-MS system described above following the same experimental procedure. Cellular uptake experiments were repeated three times.

Cell Viability (MTT Assay). The MTT colorimetric dye reduction assay is widely used to determine cell viability by measuring the formazan crystals at 570 nm due to mitochondrial dehydrogenase activity (*28*). The media were replaced before MTT addition in order to avoid any direct reduction of MTT by the stilbenes because interference of this method with phenolic compounds has been previously reported (*29*). The determinations were carried out in triplicate every 24, 48, 72, and 96 h. The mean values ± SD are shown.

Flow Cytometry: Number of Cells and Cell Cycle Distribution Analysis. Cells were fixed in ice-cold ethanol:PBS (70:30) for 30 min, further remixed with PBS with 100 μg/mL RNase and 40 μg/mL propidium iodide, and incubated at 37 °C for 30 min. DNA content

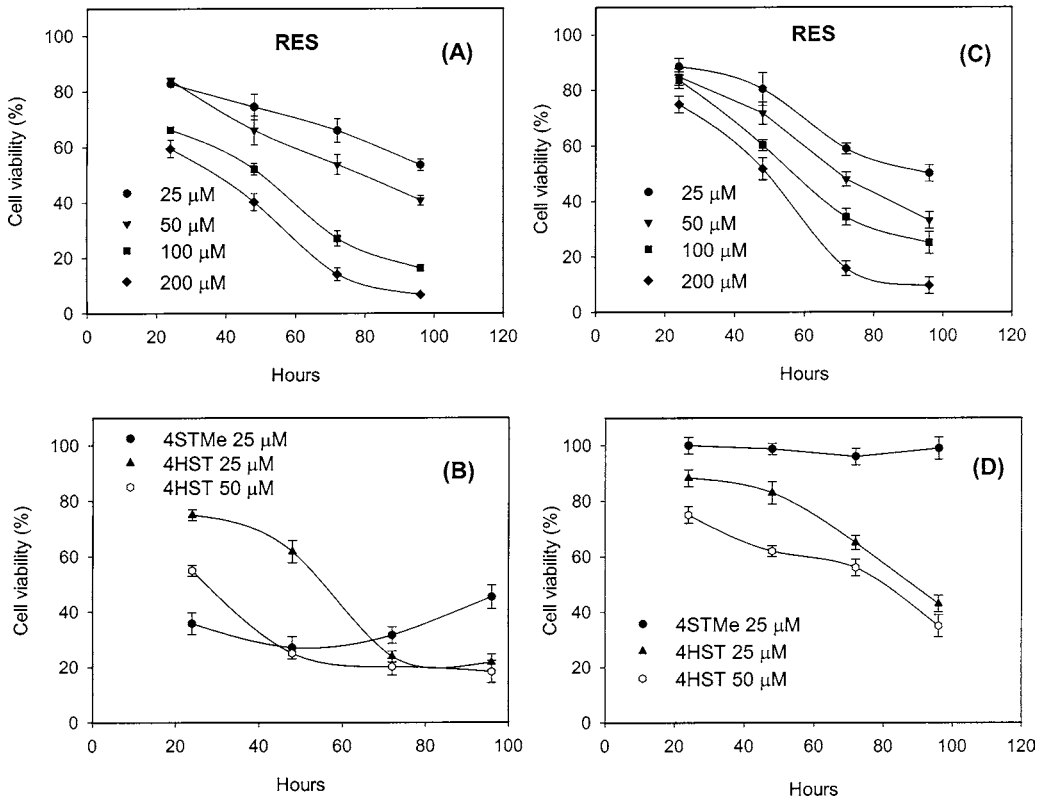

Figure 2. Effect of stilbenes on cell viability of human SK-Mel-28 melanoma cells. (**A**,**B**) Cell viability determined using the MTT method. (**C**,**D**) Cell viability determined by flow cytometry.

(20 000 cells) was analyzed using a FACScan instrument equipped with FACStation running Cell Quest software (Becton Dickinson). Flow cytometry analysis was performed at least in triplicate for each treatment. The mean value of cell cycle distribution is shown. The number of viable cells (another way to measure cell viability) was also determined. The mean value of viable cells is shown. The coefficient of variation in the determination of both cell cycle distribution and number of viable cells, according to the FACStation Cell Quest software, was always less than 10%.

Analysis of Cyclins Expression. Monoclonal cyclins E and B1 and A mouse antihuman antibodies together with FITC-conjugated goat antimouse antibody (as a secondary antibody) were used to detect cyclins expression by measuring fluorescence intensity using flow cytometry according to the method of Gong and others (*28*). Analysis of cyclins expression was performed in triplicate. Mean values ± SD are shown.

Annexin V Assay. Early stages of apoptosis are characterized by perturbations in the cellular membrane, which leads to 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. In addition, cells were also stained with propidium iodide to distinguish early and late apoptotic cells from necrotic cells. The annexin V/PI detection kit from Sigma was used. The protocol was that specified by the manufacturer. Cells (1×10^3) were resuspended in 100 μL of binding buffer and incubated in the dark with both annexin V and propidium iodide for 15 min. Then, a sample of 400 μL of binding buffer was added and 10^4 cells were analyzed with the flow cytometer specified above. Staurosporine (100 nM) was assayed as a standard inducer of apoptosis. The percentage of live, dead, and apoptotic cells was determined. In the corresponding figure that illustrates the apoptosis, 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). The total percentage of apoptotic cells was considered as EA + LA.

RESULTS

The stilbenes RES and 4HST decreased the number of human SK-Mel-28 melanoma cells in a dose- and time-dependent manner (**Figure 2A**,**B**). In the case of 4STMe, the effect was not time-dependent since the decrease in cell viability determined with MTT method was approximately the same along the 96 h of assay (**Figure 2B**). The values of cell viability in the presence of both RES and 4HST, determined by flow cytometry, were in accordance with the results obtained with the MTT method (**Figure 2C**,**D**). However, cell viability measured by using the flow cytometer did not decrease in the presence of 4STMe showing similar values to those observed for control cells (≈98% viability) (**Figure 2D**) and thus an apparent contradiction arose from the comparison of both methods to determine cell viability.

The stilbene PICE (**Figure 1**) was rendered unstable only 4 h after its addition to the culture medium without an apparent effect on cell cycle after 48 h of assay (results not shown). The rest of the stilbenes were rather stable in the medium with around 5% (or less) of conversion from trans to cis forms after 48 h of assay (results not shown). The isomerization was detected despite protecting the stilbenes from light, which is the main important factor to promote this trans to cis conversion. However, this isomerization can also be favored by neutral pH and relatively high temperature (37 °C).

After 48 h of treatment, 1.55% of the initially added 4HST (50 μM) was taken up by cells (**Table 1**) reaching the value of 0.51×10^{-12} g 4HST/cell (17.3×10^{-12} M in each cell). The stilbene 4STMe was less available for cells since 0.21% of initial 4STMe concentration (25 μM) was detected in the cells (0.036×10^{-12} g/cell; 1.15×10^{-12} M in each cell) (**Table 1**). However, RES was the least available stilbene because only 0.016% of initial RES concentration (100 μM) was detected in

Table 1. Uptake of Stilbenes by SK-Mel-28 Human Melanoma Cells[a]

	initial medium		medium at 48 h		cell at 48 h	
stilbene	concn (μM)	amount (μg)	concn (μM)	amount (μg)	% of initially added[b]	amount (pg/cell)
RES	100	228	88.6	202	0.016	0.012
4HST	50	98	36.9	72.4	1.55	0.51
4STMe	25	52.5	20.7	43.6	0.21	0.036

[a] Cells (3×10^6) were cultured for 48 h in the presence of the above stilbenes. [b] Percentage of stilbene detected inside the cell with respect to the initial amount of stilbene present in the medium.

the cells (0.012×10^{-12} g/cell; 0.43×10^{-12} M inside each cell) (**Table 1**).

Both RES and 4HST induced S-phase cell cycle arrest of human SK-Mel-28 melanoma cells concomitant with a decrease in G_0/G_1 and G_2/M phases (**Figure 3**). The effect of both RES and 4HST on melanoma cells was irreversible. The removal of the stilbenes assayed here from the medium after 48 h did not restore the normal cell cycle, which remained arrested in the S-phase despite the absence of both RES and 4HST (results not shown). In contrast, 4STMe did not cause any effect on cell cycle (**Figure 3**).

The analysis of SK-Mel-28 melanoma cell cycle by flow cytometry did not reveal an apoptotic subG_1 peak after 96 h of treatment (**Figure 4**) with concentrations up to 300 μM RES (results not shown) or 50 μM 4HST (**Figure 4**) in triplicate experiments. However, the annexin V assay revealed that both RES and 4HST were potent inducers of apoptosis in SK-Mel-28 melanoma cells (**Figure 5**). In fact, after 48 h, 21.5 and 20.8% of the cell population was found in EA plus LA apoptosis when treated with 100 μM RES and 50 μM 4HST, respectively (**Figure 5**).

An induction in the expression of cyclins A, E, and B1 was observed after treatment with both RES and 4HST (**Figure 6**). However, 4STMe treatment did not change the cyclin expression pattern, which corroborated that this stilbene lacked an effect on human SK-Mel-28 melanoma cells (**Figure 6**). The involvement of cyclin accumulation on the arrest of human SK-Mel-28 melanoma cells in the S-phase was also checked (**Figure 7**). It should be noted that in this cell line, a slight increase of cyclin B1 expression was significantly correlated with the accumulation of cells in S-phase ($R = 0.98$, $P < 0.0001$; **Figure 7A**). In the case of cyclin A, a threshold expression up to $\approx$50% did not have a significant effect on the cell cycle arrest in the S-phase (**Figure 7B**). However, a higher accumulation (about 1.2-fold over the control) sharply increased the progression of cell cycle to this phase. On the other hand, values higher than 63% were not correlated with the accumulation in the S-phase. This indicated that the effective expression range of cyclin A as a mediator to enter the cell in the S-phase in SK-Mel-28 melanoma cells was rather narrow. In the case of cyclin E, approximately the same threshold expression observed for cyclin A was also detected (**Figure 7C**). Higher expression values of cyclin E (about 1.7-fold over the control) were always positively correlated with the accumulation of melanoma cells in the S-phase in the SK-Mel-28 melanoma cells.

DISCUSSION

Despite the huge output of investigation concerning the antitumor effects of the grape-derived polyphenol RES, its effect on human melanoma cell lines has been scarcely approached (*25*). The present study demonstrates the antiproliferative effect

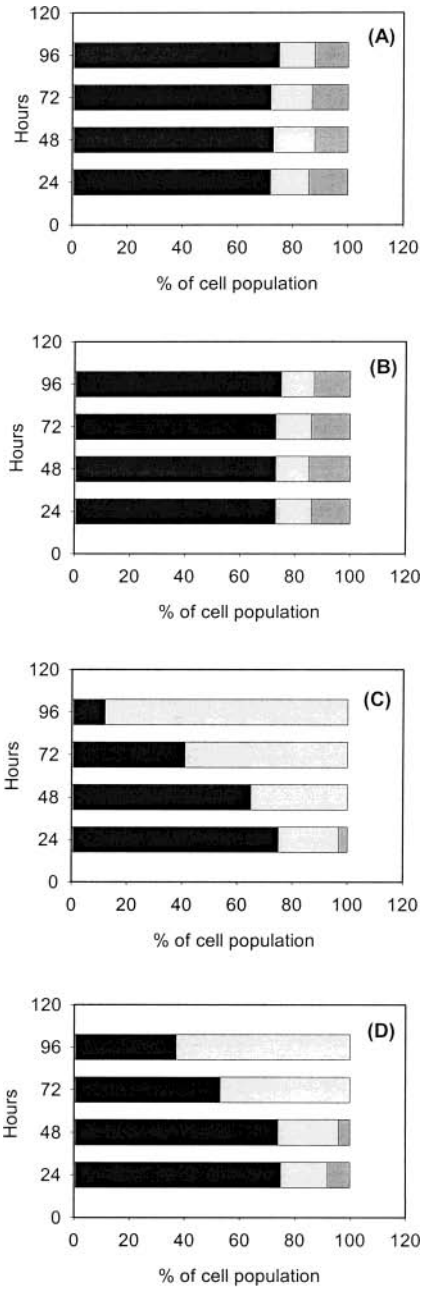

Figure 3. Cell cycle distribution of control and stilbene-treated SK-Mel-28 melanoma cells. (**A**) Control; (**B**) 4STMe, 25 μM; (**C**) 4HST, 50 μM; (**D**) RES, 100 μM. Phases: light gray, S; black, G_0/G_1; medium gray, G_2/M.

of RES and the related molecule 4HST. The MTT tetrazolium assay has been reported to interfere with phenolics (*29*) so that the possible effect of phenolics on the viability of cancer cells assayed with this method should be corroborated with additional assays (such as flow cytometry) to discard possible artifacts. In fact, cell viability determined using the MTT method was approximately coincident with that determined by flow cytometry (**Figure 2A**,**C**). However, contradictory results were obtained when the apparent effect of both 4STMe and 4HST on cell viability was compared using MTT and flow cytometry methods (**Figure 2B**,**D**). This contradiction was more remarkable in the case of 4STMe, which showed a high inhibition of cell viability using the MTT method whereas no effect was observed using flow cytometry. Therefore, these results support previous papers concerning the interference of the MTT method with some polyphenols (*29*).

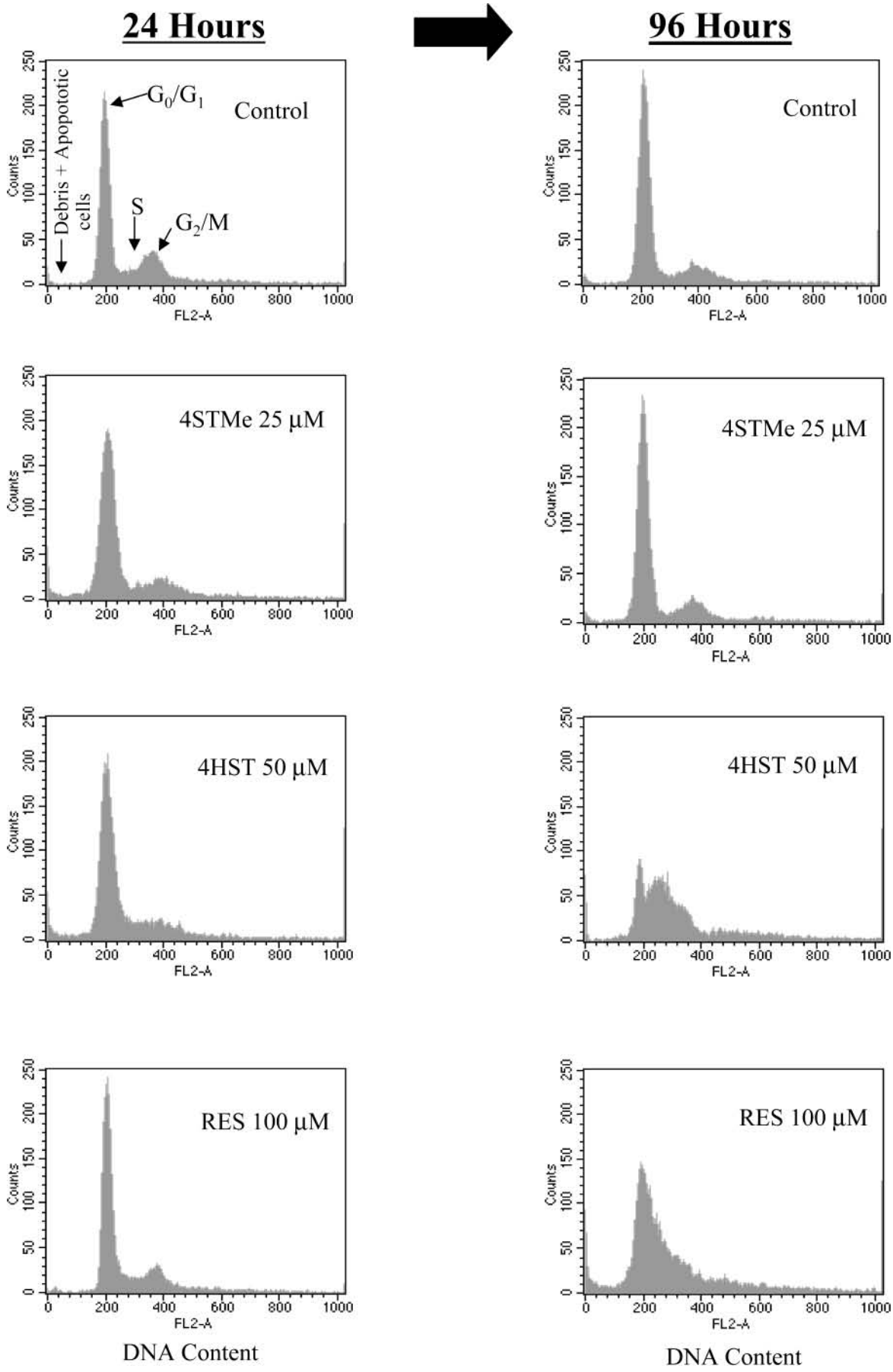

Figure 4. Flow cytometry analysis of DNA distribution in control and stilbene-treated human SK-Mel-28 melanoma cells at 24 and 96 h of assay.

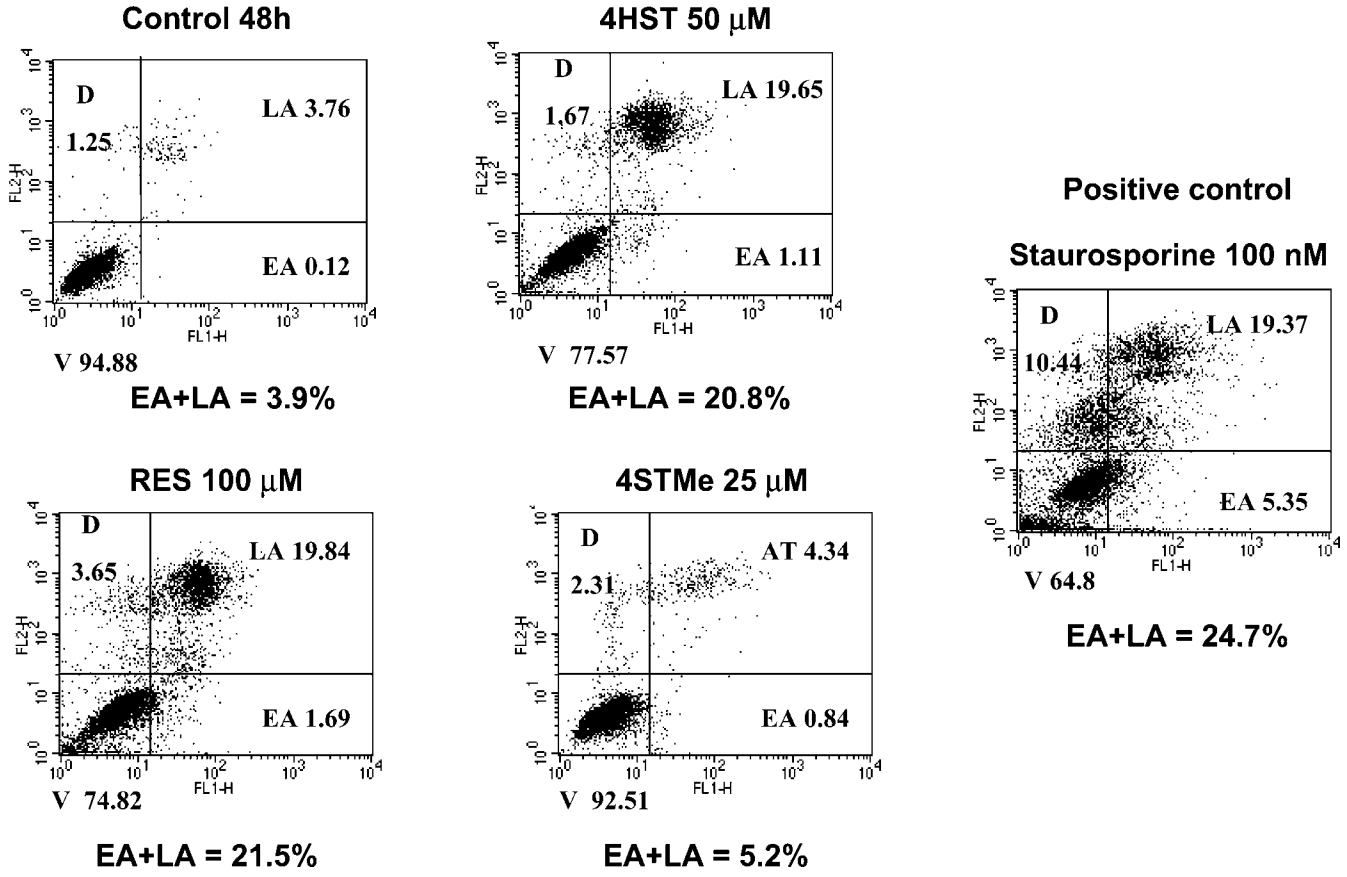

Figure 5. Apoptosis detection in SK-Mel-28 cells using the annexin V assay after 48 h. D (upper left corner); V (lower left corner); LA (upper right corner); EA (lower right corner). The total percentage of apoptotic cells was considered as EA + LA.

The high instability of phenolics such as PICE in standard culture media should be noted. This instability is not surprising due to the ortho-diphenolic structure present in the molecule (**Figure 1**). Both the incubation temperature (37 °C) and the pH (≈7) are critical in the stability of this type of compound. In this context, the present study calls for caution in the assay of compounds with *o*-diphenolic moiety. Another important concern to be taken into account is the isomerization of stilbenes from trans to cis form. This possibility is not usually checked in the studies regarding RES bioactivity in cell culture media. Although this isomerization is mainly induced by light, aqueous media at neutral pH and 37 °C also promote it. This is important because the lack of effect of *cis*-RES on cell cycle and proliferation of cancer cells has been reported in a recent study (*30*). This means that the medium should be changed every (at least) 48 h in order to diminish this trans to cis conversion when this type of molecule is assayed.

The present study also reports the different stilbene uptake by Sk-Mel-28 cells after 48 h of treatment (**Table 1**). It is of note that cellular uptake was inversely correlated with variations on the most simple 4′-hydroxystyryl moiety (**Figure 1**), i.e., 4HST > 4STMe > RES (0.51, 0.036, and 0.012 pg per cell, respectively). Apparently, the presence of the $-CH_2$ group in the case of 4STMe and two extra hydroxyl groups in the case of RES was critical to hamper the entrance of stilbene into the cell. Taking into account the initial stilbene content added to the medium and the intracellular stilbene content, 4HST was 97- and 7.5-fold more available than RES and 4STMe, respectively. The same intracellular concentration observed here for 4STMe (0.036 pg per cell) has been previously reported for the flavonoid epigallocatechin gallate (EGCG) after 48 h of treatment in Caco-2 cells with EGCG 100 μM (*31*). The stilbenes assayed here remained rather stable in the medium during the 48 h of assay (**Table 1**). The stilbenes detected inside the cells matched in the retention time, UV spectrum, and *m*/*z* ion of those initially added in the medium. This meant that apparently, no stilbene-derived metabolites were detected inside SK-Mel-28 cells, which could indicate that the effects observed were directly provoked by the unmodified stilbene precursor and not by cell-generated metabolites. However, this deserves more research so that the metabolism of these molecules by melanoma cells should not be ruled out.

Many previous studies described the RES-mediated arrest in the S-phase of different cancer cell lines such as HL60 (*15*), T47 breast carcinoma (*16*), CEM-C7H2 acute leukemia (*17*), etc., which was in accordance with the results obtained in the present study. However, other studies have reported arrest of cycle in G_0/G_1 in human epidermoid carcinoma (*32*) and gastric adenocarcinoma cells (*33*). In addition, the effect of RES on cells has been previously reported to be either reversible (*11*) or irreversible (*32*) as in the case of our study, which corroborates the different actions that RES can exert depending on both cell line and type of cancer.

The induction of apoptosis by RES has been previously published (*14*, *17*). The RES concentrations required to induce apoptosis have been reported to greatly depend on the cell line, ranging, for example, from 20 μM in leukemia cells (*17*) to 200 μM in Caco-2 cells (*12*). However, once again, the effects of RES on cell cycle progression can vary in different experimental systems (*14*) because the absence of apoptosis in the presence of RES (*10*) and even antiapoptotic effects (*18*) have also been described. In the present study, apoptosis was

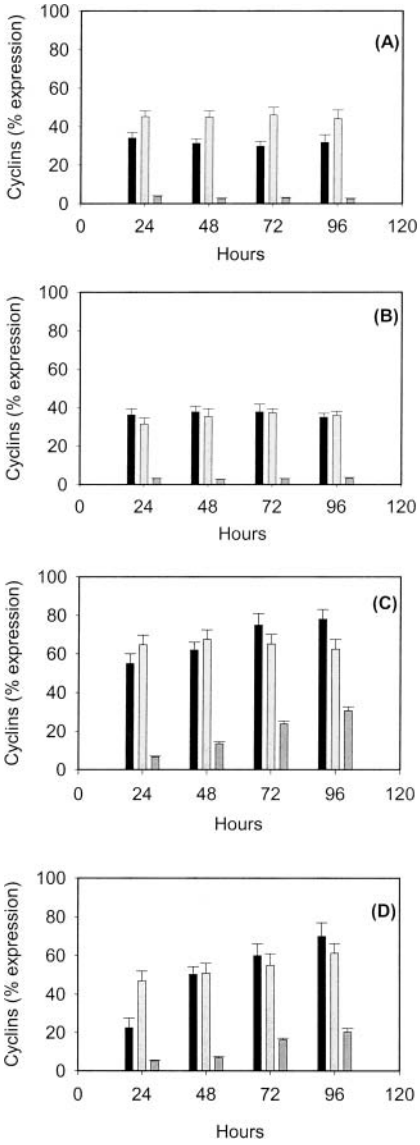

Figure 6. Cyclin expression of control and stilbene-treated SK-Mel-28 melanoma cells. (**A**) Control; (**B**) 4STMe, 25 μM; (**C**) 4HST, 50 μM; (**D**) RES, 100 μM. Cyclins: light gray, E; black, A; medium gray, B1.

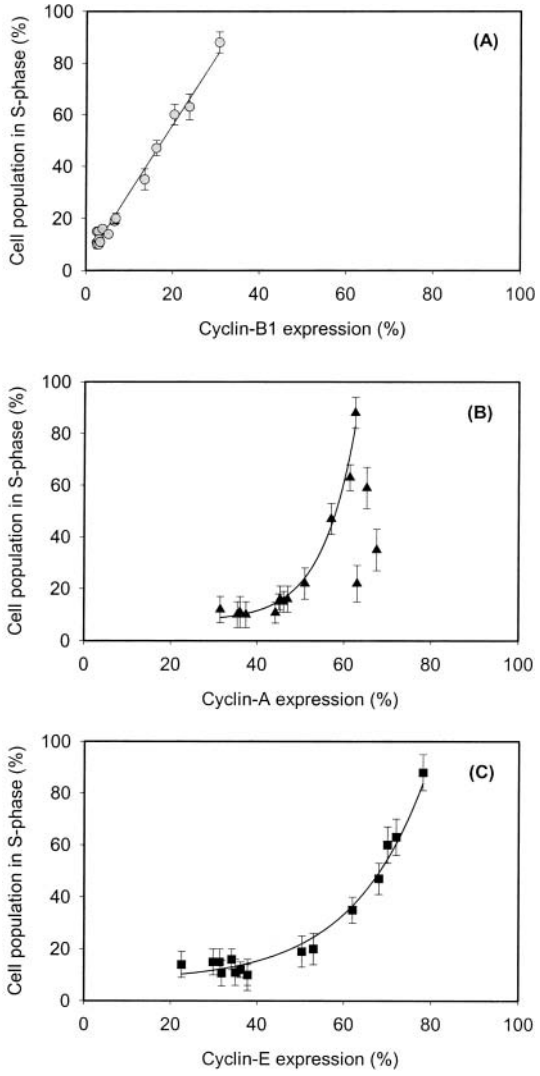

Figure 7. Effect of cyclins induction on S-phase arrest in SK-Mel-28 melanoma cells. (**A**) Cyclin B1; (**B**) cyclin A; (**C**) cyclin E.

not detected when the cell cycle was analyzed by flow cytometry since no subG$_1$ (apoptotic) peak was observed (**Figure 4**). However, the annexin V assay revealed that both RES and 4HST were potent inducers of apoptosis in SK-Mel-28 melanoma cells (**Figure 5**). As the annexin V assay is more specific and sensitive to determine apoptotic cells than flow cytometry, the induction of apoptosis by both RES and 4HST should be accepted. Therefore, and once again, the use of different assay methods to evaluate the effects of polyphenols on cell lines is strongly encouraged.

In contrast with the cell cycle arrest in the S-phase mediated by both RES and 4HST, the stilbene 4STMe did not exert any effect, which was in accordance with a previous study on colon cancer cell lines (*12*) (**Figure 4**). This result supported the validity of the cell viability assay determined by flow cytometry in contrast with the apparent high effect detected with the MTT tetrazolium assay (**Figure 2B**,**D**). To fully discard the lack of effect of 4STMe on SK-Mel-28 melanoma cells, higher 4STMe concentrations should be assayed although this was not approached in the present study due to solubility problems. However, under similar assay concentrations (25 μM), it should be stressed that RES and 4HST did affect both cell cycle and cell viability (results not shown).

To shed some light on the molecular mechanism involved in the arrest of cell cycle in the S-phase, the expression of the cyclins A, E, and B1 was determined. Cell cycle progression is regulated by the activity of cyclins, a family of proteins that 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 the S-phase, whereas cyclin B1 is a critical regulator of mitosis. In general, uncontrolled expression of cyclins and/or Cdks leads to either tumorogenesis or cell cycle arrest (*34*). As in the effect on cell growth and cell cycle, the studies concerning the effect of RES on cyclins expression also differ depending on the cell line. Our study is coincident with previous studies, which described the RES-mediated upregulation of the above cyclins and the accumulation of cells in the S-phase in other human cancer cell lines such as HL60 (*15*), Caco-2 (*12*), and histiocytic lymphoma U937 (*11*). In the human SK-Mel-28 melanoma cell line, the slight accumulation of cyclin B1 was critical to promote cell cycle arrest in the S-phase whereas higher cyclin A and E accumulation was required to exert similar arrest in the S-phase.

In light of the results obtained in the present study, one of the main conclusions is that the presence of the hydroxyl group

directly bonded to the carbon at the 4′-position of the stilbene structure is essential to exert the effects reported here (**Figure 1**). Variations on the simplest 4HST (**Figure 1**) also hampered the cellular uptake. The presence of a $-CH_2$ group between the aromatic ring and the hydroxyl group seemed to dramatically affect the potential activity of the molecule as in the case of 4STMe, which showed no effects on cells at the assayed concentration (**Figure 1**) despite entering into the cell with a 13-fold higher relative efficiency than that of RES. Therefore, the presence of the hydroxyl group in the 4′-position appears to be essential (*30*) since stilbene molecules with this structural requirement (RES and 4HST) induced apoptosis, upregulated cyclins A, E, and B1, and arrested cell cycle in the S-phase of human SK-Mel-28 melanoma cells. Moreover, the differences observed between both molecules are probably due to the *m*-diphenolic structure of RES, which is absent in 4HST (**Figure 1**). These extra hydroxyl groups proved to hamper the entrance of RES into the cell and decreased the capacity to inhibit melanoma cell growth (**Figure 2A**,**B**), to arrest them in the S-phase (**Figures 3** and **4**), and to upregulate cyclins A, E, and B1 (**Figure 6**). However, the presence of these extra hydroxyl groups in RES with respect to 4HST decreased cell numbers (**Figure 2C**,**D**). This is in agreement with a recent paper, which also described the requirement of hydroxyl groups to decrease viability in LNCaP and DU 145 cells since the RES derivative tri-methoxy-RES did not show any effect on the above cancer cells (*35*).

With all of the caution called for in drawing conclusions from in vitro cell line experiments regarding the responses of cancer cells in vivo, the dietary component RES and structurally analogous compounds that share the 4′-hydroxystyryl moiety based on their selective actions (upregulation of cyclins A, E, and B1, irreversible cell arrest in the S-phase, and cell death) could be claimed to be of relevance in chemoprevention of malignant melanoma. It should also be stressed that the present study has been conducted with SK-Mel-28 melanoma cells and a further extrapolation to other melanoma cell lines should also be made with caution. Additional studies are required to fully characterize the molecular mechanism by which RES and related stilbenes exert their effect on human melanoma cells. In addition, toxicity and bioavailability assays are also required to validate these molecules as possible effective antimelanoma drugs in vivo.

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