Time- and concentration-dependent effects of resveratrol in HL-60 and HepG2 cells

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Abstract. Resveratrol, a phytochemical present in grapes, has been demonstrated to inhibit tumourigenesis in animal models. However, the specific mechanism by which resveratrol exerts its anticarcinogenic effect has yet to be elucidated. In the present study, the inhibitory effects of resveratrol on cell proliferation and apoptosis were evaluated in the human leukaemia cell line HL-60 and the human hepatoma derived cell line HepG2. We found that after a 2 h incubation period, resveratrol inhibited DNA synthesis in a concentration-dependent manner. The IC₅₀ value was 15 µM in both HL-60 and HepG2 cells. When the time of treatment was extended, an increase in IC₅₀ value was observed; for example, at 24 h the IC₅₀ value was 30 µM for HL-60 cells and 60 µM for HepG2 cells. Flow cytometry revealed that cells accumulated in different phases of the cell cycle depending on the resveratrol concentration. Furthermore, an increase in nuclear size and granularity was observed in the G₁ and S phases of HL-60 treated and HepG2-treated cells. Apoptosis was also stimulated by resveratrol in a concentration-dependent manner in HL-60 and HepG2 cells. In conclusion, resveratrol inhibits cell proliferation in a concentration- and time-dependent manner by interfering with different stages of the cell cycle. Furthermore, resveratrol treatment causes stimulation of apoptosis as well as an increase in nuclear size and granularity.

INTRODUCTION

Resveratrol (3,5,4'-trihydroxystilbene, the *trans*-isoform) is a phytoalexin naturally occurring in many plants, some of which are used for human consumption (Aggarwal *et al.* 2004). In 1976, the compound was identified in grapes (Langcake & Pryce 1976), where it is produced primarily in the skin in response to external stress factors such as injury, ultraviolet radiation and fungus infection (Soleas *et al.* 1997; Pervaiz 2003). Resveratrol is also found in high concentrations in one of the end products of grapes, red wine (Siemann & Creasy 1992; Stervbo *et al.* 2006).

The first report on cancer preventive effects of resveratrol was published by Jang *et al.* (1997). The authors evaluated the effect of resveratrol on tumourigenesis by a two-stage mouse skin cancer model and found a 98% reduction of skin tumours. Since this first study concerning the inhibitory effect of resveratrol on tumourigenesis, several reports have indicated that it may

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reduce tumour number (Carbo *et al.* 1999; Schneider *et al.* 2001; Kapadia *et al.* 2002) and tumour size (Caltagirone *et al.* 2000; Kimura & Okuda 2001; Liu *et al.* 2003; Chen *et al.* 2004; Wyke *et al.* 2004; Zhou *et al.* 2005) *in vivo*.

The mechanisms by which resveratrol exerts its anticarcinogenic effects are not well understood. *In vitro* studies have shown that it reduces cell proliferation and modulates the cell cycle in different cell lines, among which are HL-60 and HepG2 cells (Latruffe *et al.* 2002; Liang *et al.* 2003; Roberti *et al.* 2003; Ahmad *et al.* 2004; Fulda & Debatin 2004; Opipari *et al.* 2004; Castello & Tessitore 2005). Modulation of the cell cycle may be mediated by an increase in levels of the cyclin-dependent kinase inhibitor, cdkn1a and the stabilization of p53 (Hsieh *et al.* 1999; Kuo *et al.* 2002; Kim *et al.* 2003; Fulda & Debatin 2004). Resveratrol appears to stimulate caspase-dependent and caspase-independent apoptosis, as inhibition of caspase-3 results in only a reduction in resveratrol-stimulated cell death (Clement *et al.* 1998; Roman *et al.* 2002; Estrov *et al.* 2003; Roberti *et al.* 2003; Opipari *et al.* 2004). Thus, several cellular mechanisms can contribute to the chemopreventive effects of resveratrol.

The purpose of the present work was to study resveratrol's time- and concentration-dependent effects with respect to modulation of the cell cycle and stimulation of apoptosis in the two human cell lines, HL-60 and HepG2. Effects on the cell cycle were studied using [3H]dT and flow cytometry on propidium iodine (PI)-stained nuclei. Apoptosis was detected by alterations in nuclear morphology, as assessed by fluorescence microscopy, and binding of Annexin V. as well as DNA fragmentation, evaluated by flow cytometry.

MATERIALS AND METHODS

Chemicals and reagents

Resveratrol, camptothecin, 4',6-diamidino-2-phenylindole (DAPI), PI, Triton X-100, and dimethylsulfoxide (DMSO) were all obtained from Sigma (Copenhagen, Denmark). Roswell Park Memorial Institute medium (RPMI)-1640, L-glutamine, foetal calf serum (FCS) and Glutamax were from Invitrogen (Taastrup, Denmark). Phosphate buffer saline (PBS), penicillin-streptomycin-glutamine mix (10 000 units/ml penicillin, 10 000 µg/ml streptomycin and 29.2 mg/ml L-glutamine) and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (Taastrup, Denmark). Dulbecco's modified eagle's medium (DMEM) was obtained from Cambrex (Vallensbaek Strand, Denmark) and 4% paraformaldehyde in phosphate buffer, pH 7.4, was from Bie & Berntsen (Rodovre, Denmark).

Resveratrol was diluted in DMSO at millimolar concentrations, kept at -20 °C until use and stored for no more than 6 months. All cell culture flasks as well as 96-well microtitre plates and six-well multiwell dishes were from Nunc (Roskilde, Denmark), unless otherwise specified.

Cell lines

HL-60 cells, a human promyelocytic leukaemia cell line, were grown in suspension, and HepG2 cells, an epithelial human hepatoma-derived cell line, were obtained from the American Tissue Culture Collection. The HL-60 cells were cultured in RPMI-1640 supplemented with 200 mM L-glutamine and 10% heat-inactivated FCS. The HepG2 cells were cultured in DMEM supplemented with 1% penicillin-streptomycin-glutamine mix and 10% heat-inactivated FCS. All cells were maintained in a humidified 37 °C, 5% CO₂ incubator. When cell density reached approximately 80% confluence, the cells were subcultured. HL-60 cells were subcultured by diluting cells in fresh culture medium to approximately 2×10^5 cells/ml. HepG2 cells were detached by trypsin-EDTA and were seeded at 4×10^4 cells/cm². Cell lines were subcultured no more than 30 times.

Thymidine incorporation assay

Exponentially growing HL-60 cells were diluted in fresh culture medium to 6×10^4 cells/ml. The cell suspension was seeded in a 96-well microtitre plates, 100 μ l/well. Resveratrol solution or vehicle solution (0.1%) in culture medium were made in centrifuge tubes (Nalgene Labware, Albertslund, Denmark) in concentrations double that required for final concentration and were kept in the dark in a standard incubator. At different time points prior to cell harvest, 100 μ l resveratrol stock solution was passed to each well of the 96-well microtitre plate to a final volume of 200 μ l/well leading to the required final concentration.

Exponentially growing HepG2 cells were diluted in fresh culture medium to 3×10^4 cells/ml and were seeded in a 96-well microtitre plate, 200 μ l/well. All outer wells received 200 μ l PBS. The cells were allowed to adhere overnight. On the day of the experiment, resveratrol solution or vehicle solution (0.1%) in culture medium were made in centrifuge tubes (Nalgene Labware) at the required final concentrations and were kept in the dark in the incubator. At different time points prior to cell harvest, the medium was removed by gently inverting the plates and 200 μ l resveratrol solutions were added to each well. In all experiments, outer wells received 200 μ l PBS and the plates were left in the incubator for the desired incubation time.

Two hours before harvest, 20 μ l 6.25 μ Ci/ml [3 H]dT (Amersham Biosciences, Hillerød, Denmark) was added to each well, and the plates were left to incubate. Subsequently, the cells were transferred to filter plates (PerkinElmer, Allerød, Denmark) using a cell harvester (Packard, Greve, Denmark) and were left to dry overnight at room temperature. To each well on the dried filter plates, 30 μ l MicroScint O scintillation liquid (PerkinElmer) was added. The intensity of the radioactivity was measured in a TopCount NXT scintillation counter (Packard). The TopCount NXT was controlled by the TOPCOUNT NXT version 1.06 (Packard) external software on Windows 95.

Analysis by flow cytometry

The distribution of cells in the cell cycle as well as apoptosis was analysed by the CycleTEST Plus DNA Reagent Kit (BD Biosciences, Brøndby, Denmark). The assay is based on the method by Vindelov *et al.* (1983) where nuclei are isolated using a mixture of a non-ionic detergent and trypsin. The DNA is subsequently stained with DNA-binding dye, PI.

Exponentially growing HepG2 cells were seeded (1×10^6 cells in a T25 flask) one day prior to resveratrol treatment. On the day of treatment, the culture medium was replaced with culture medium containing resveratrol or vehicle solution (0.1%). HL-60 cells were diluted in fresh culture medium. Resveratrol or vehicle solution (0.1%) was added, and 1×10^6 cells were added to each well of a six-well multiwell dish. After a treatment period of 24 h, the cells were harvested and the assays were performed according to the manufacturer's protocol. Stained cells were analysed as described in succeeding discussions using the FL2-H parameter. The size and granularity of nuclei were investigated using the FSC-H (particle size) and SSC-H (particle complexity) parameters of the flow cytometer.

Evaluation of resveratrol-induced apoptosis was performed on HL-60 cells using the Annexin V-enhanced green fluorescent protein (V-EGFP) Apoptosis Detection Kit (Strattech Scientific, Suffolk, UK). Using DNA-binding dye PI, the cells with compromised membranes could be excluded and those treated with 10 μ M camptothecin for 4 h were used as positive controls.

Exponentially growing HL-60 cells were diluted in fresh culture medium and 1×10^6 cells were transferred to each well of a six-well multiwell dish. Resveratrol or vehicle solution was added at the appropriate concentration (0.1% vehicle). The cells were incubated for 18 h and were treated according to the manufacturer's protocol. Immediately prior to analysis, 5 μ l PI was added to each sample. Stained cells were analysed measuring Annexin-V and PI using FL1-H and FL3-H, respectively.

All the cells were analysed by flow cytometry using a FACSCalibur (Becton Dickinson, Brøndby, Denmark) equipped with a standard 488-nm laser, and at least 20 000 events were counted. The flow cytometer was controlled by CELLQUESTPRO version 4.0.2 (BD Biosciences) external software on Mac OS 9.

DAPI staining

To visualize changes in nuclear morphology, the cells were stained with DNA-binding dye DAPI and were observed using fluorescence microscopy. HL-60 cells were treated with 10- μ M camptothecin for 4 h and were used as positive controls. Exponentially growing HL-60 cells were diluted in fresh culture medium and 1×10^6 cells were transferred to each well of a six-well multiwell dish. Resveratrol or vehicle solution was added to obtain the appropriate concentration (0.1% vehicle). The cells were incubated for 18 h.

A stock solution of 1 mg/ml DAPI was made from DAPI dissolved in dH₂O. This solution was further diluted to 1 μ g/ml in PBS. Aliquots of 500 μ l cell suspensions (1×10^5 cells) were centrifuged (400 *g* at room temperature for 5 min) onto coated glass slides (Thermo Electron, Copenhagen, Denmark) using a Cytospin 4 Cyto centrifuge (Thermo Electron). The slides were left to dry completely for at least 30 min to allow full adhesion. A hydrophobic barrier was drawn around the dried cells using a DakoCytomation Pen (DakoCytomation, Glostrup, Denmark). The cells were fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4) at room temperature for 15 min and were washed with cold PBS in a Hellendahl-type staining jar (Bie & Berntsen) for approximately 3 min. The fixed cells were permeabilized with ice-cold 0.1% Triton X-100 at room temperature for 4 min and then washed twice with ice-cold PBS. Permeabilized cells were stained with a volume of 1 μ g/ml DAPI appropriate to fully cover the cells. The slides were left in the dark at room temperature for 5 min and were washed once with cold PBS. Coverslips were mounted using Vectashield mounting solution (Vector Laboratories, Rødovre, Denmark). Stained cells were visualized on a Leica DMR light microscope (Leica, Herlev, Denmark). Images of at least three random positions on the slide were captured with a Leica DC200 camera (Leica) using the Leica IM50 version 1.20 release 19 (Leica) external software on Windows 2000.

Software used

Cycle distribution was analysed by MODFITLT version 2.0 (Verity Software House) for Mac OS 9. Graphs were prepared by GRACE 5.1.x series and images were manipulated by GIMP version 2.2.x and were prepared for presentation with XFIG version 3.2.5 Alpha on an appropriate Linux system. WEASEL version 2.2.3, unlicensed (Walter and Eliza Hall Institute), was utilized for analysis of flow cytometric data. Image analysis was performed with IMAGEJ version 1.35d (Abràmoff *et al.* 2004). Both applications were run using the JAVA 2 RUNTIME ENVIRONMENT 5.0, build 5 for Linux.

Statistical analysis

Statistical analysis was performed using Student's two-tailed paired *t*-test.

RESULTS

Resveratrol induces a concentration-dependent inhibition of thymidine incorporation in HL-60 and HepG2 cells

Treatment of HL-60 cells with resveratrol increased the IC₅₀ value for incorporation of [3H]dT in a concentration- and time-dependent manner (Fig. 1a). IC₅₀ increased from 15 μ M resveratrol

Figure 1. Resveratrol induces a time- and concentration-dependent reduction in thymidine incorporation in HL-60 and HepG2 cells. Resveratrol was added to HL-60 (a) and HepG2 (b) cells at different time points, and all cells were harvested at the same time after the specified treatment times. Values are presented relative to untreated cells. The experiment was performed on three occasions and the results of a typical experiment are presented as mean $\pm$ SD, $n = 6$ (a) and $n = 6$ (b). Overlapping data points are annotated with the lowest common P value ($*P \leq 0.01$, $***P \leq 0.001$ compared to vehicle-treated cells).

at 12 h to 30 μM resveratrol at 24 h. Between 2, 4, 8 and 12 h, no significant differences in IC_{50} values were observed. At 48 h, the IC_{50} value remained identical to value at 24 h. This result shows that resveratrol inhibits [3H]dT incorporation in a concentration-dependent manner, and that the IC_{50} increases as a function of time up to 24 h.

A similar pattern was observed for the HepG2 cells. When treated with resveratrol for 2–48 h, an increase in IC_{50} values of [3H]dT incorporation in HepG2 cells was observed (Fig. 1b). The IC_{50} values ranged from 15 μM at 2 h to 50 μM at 12–24 h. However, at 48 h, the IC_{50} value decreased to 35 μM . This indicated that the modification of the IC_{50} value is a function of resveratrol concentration and incubation time when incubation time is less than 24 h. For incubation times greater than 24 h, IC_{50} is modified as a function of concentration and an inverse function of time in HepG2 cells.

The lowest concentration necessary for significant inhibition of incorporation of [3H]dT in HL-60 cells was 5 μM for up to 12 h of treatment, and 20 μM for 24 and 48 h of treatment (Fig. 1a). For HepG2 cells, the pattern was more complex (Fig. 1b). The lowest inhibitory concentration is 20 μM for 2 and 4 h of treatment, 40 μM for 8, 12 and 48 h of treatment and 80 μM for 24 h of treatment.

Cell cycle progression is altered by resveratrol in a concentration-dependent manner

To further explore the effects of resveratrol-induced inhibition of cell growth measured by [3H]dT incorporation, the cell cycle modulating properties of resveratrol were examined.

After treatment with resveratrol for 24 h, a clear concentration-dependent cell cycle accumulation was observed in HL-60 cells (Fig. 2a). At low concentrations (20 μM), the number of HL-60 cells increased in the S phase. At higher resveratrol concentrations (40 and 80 μM), there was a considerable accumulation of cells in the G_1 phase.

The effect on cell cycle progression in HepG2 cells (Fig. 2b) appeared to follow the same pattern as was observed with HL-60 cells, although at slightly higher concentrations. Incubation with 20 μM resveratrol for 24 h did not affect the cell cycle distribution whereas 40 μM increased the number of HepG2 cells in the S phase. At increased concentrations (80 μM), resveratrol also caused accumulation of cells in the S phase, but to a lesser extent than was observed for 40 μM .

These results demonstrated that resveratrol modulated the cell cycle *in vitro* and the effect on the cell cycle was concentration dependent.

Modulation of the size and granularity of cell nuclei

Based on the observed cell cycle modulating effects of resveratrol, we expected a slight increase in the size of the nucleus in cells treated with resveratrol at concentrations causing accumulation in the S phase. Indeed, modulation of cell cycle progression of HL-60 cells caused by resveratrol was demonstrated to correlate with a relative increase in the size of the nucleus of 20% at 20 μM resveratrol (Fig. 3a). Enlarged nuclei were observed in resveratrol-treated cells mainly in the G_1 and S phases, but cells in the $\text{G}_2 + \text{M}$ phase were also affected (data not shown). Increased resveratrol concentration reduced the size of the nucleus compared to control cells (8% decrease at 80 μM resveratrol for 24 h).

HL-60 cells treated with resveratrol also displayed an increased granularity of the cell nucleus (Fig. 3a). Cells in all phases of the cell cycle were affected by the treatment. The effect of resveratrol on the granularity of the nucleus appeared to be highest at 20 μM (Fig. 3a).

The size and granularity of HepG2 nuclei also increased when the cells were treated with resveratrol (Fig. 3b). Cells in all phases of the cell cycle appeared affected (data not shown). The peak effect appeared to be at 40 μM resveratrol (Fig. 3b).

Figure 2. Resveratrol induces cell cycle arrest in HL-60 and HepG2 cells. HL-60 (a) and HepG2 (b) cells were treated with resveratrol for 24 h. The nuclei were stained with PI and DNA content was examined by flow cytometry. All data are presented relative to untreated cells. The experiments were performed on three occasions and the results of a typical experiment are shown. Data are presented as mean $\pm$ SD, $n = 3$ (a) and $n = 1$ (b). Overlapping data points are annotated with the lowest common P value (** $P \leq 0.01$, *** $P \leq 0.001$ compared to vehicle treated cells).

Figure 3. Resveratrol stimulates an increase in the nuclear size and granularity in HL-60 and HepG2 cells. HL-60 (a) and HepG2 (b) cells were treated with resveratrol for 24 h, and the size and granularity of the nuclei was examined by flow cytometry. All data are presented relative to untreated cells. The experiments were performed on three occasions and the results of a typical experiment are shown. Data are presented as mean $\pm$ SD, $n = 3$ (a) and $n = 1$ (b) (* $P \leq 0.05$, ** $P \leq 0.01$ compared to vehicle-treated cells).

Figure 4. Resveratrol stimulates morphological changes of the cell nucleus of HL-60 in a concentration-dependent manner. Cells were treated with vehicle (a), 20 μM (b) or 40 μM (c) resveratrol for 18 h. After treatment, cells were stained with DAPI and the nuclei were visualized under a fluorescence microscope. Camptothecin 10 μM , 4 h incubation (d) was used as positive control. White arrowheads indicate morphological changes. The experiments were performed on four occasions and the results of a typical experiment are shown.

It was not possible to observe the resveratrol-induced alterations of nuclear size in HL-60 and HepG2 cells measuring the area of DAPI-stained nuclei visualized with fluorescent microscopy (data not shown).

Resveratrol stimulates apoptosis in HL-60 cells

The ability of resveratrol to modulate apoptosis was initially studied by examination of nuclear morphological changes associated with apoptosis. In a preliminary study, it was found that resveratrol treatment for 12–18 h in HL-60 cells resulted in optimal stimulation of apoptosis (data not shown). When treated with resveratrol for 18 h, there was an obvious concentration-dependent increase in the number of HL-60 nuclei with a condensed and fragmented morphology (Fig. 4). This demonstrated a concentration-dependent stimulation of apoptosis.

To further study the apoptosis-modulating effect in HL-60 cells, the binding of EGFP conjugated Annexin V was evaluated (Fig. 5a). Early apoptotic cells in this assay bind Annexin V but exclude the nuclear binding dye PI, that is, cells with lost membrane asymmetry but intact membranes.

HL-60 cells treated with resveratrol for 18 h showed a concentration-dependent increase in Annexin V-positive/PI-negative cells (Fig. 5a). Only a minor effect of 20 μM resveratrol on HL-60 cells was found, whereas higher concentrations greatly stimulated Annexin V binding. Treatment with 80 μM resveratrol resulted in 23% of the HL-60 cells being early apoptotic and 23% being late apoptotic or necrotic. The same degree of concentration-dependent apoptosis was observed in HepG2 cells (Fig. 5b).

Figure 5. Resveratrol stimulates apoptosis in a concentration-dependent manner in HL-60 and HepG2 cells. Cells were treated with resveratrol for 18 h (a) or 24 h (b). In (a), HL-60 cells were double stained with Annexin V and PI, and were examined by flow cytometry after resveratrol treatment. In (b), HepG2 cells were stained with PI and the sub-G₁ population was examined by flow cytometry. All data are presented relative to untreated cells. The experiments were performed on three occasions and results of a typical experiment are shown. They are presented as mean ± SD, *n* = 3 (a) and *n* = 1 (b). Overlapping data points are annotated with the lowest common *P* value (**P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001 compared to vehicle-treated cells).

DISCUSSION

The purpose of this study was to characterize the effect of resveratrol on DNA synthesis and cell cycle progression. In addition, the apoptosis-stimulating properties of resveratrol were also studied. Inhibition of cell proliferation and stimulation of apoptosis by resveratrol may be key responses in relation to the putative cancer chemopreventive effect of this product.

The concentration-dependent effect on cell growth measured as [3H]dT incorporation after 2 h was unexpected, and this very early effect indicates resveratrol's interference with cell replication machinery *in vitro*. To the best of our knowledge, this is the first report showing that such short-term treatment with resveratrol affects DNA-synthesis in HL-60 and HepG2 cells in culture. The observation has only been paralleled by a single observation using the human prostate cancer cell line LNCap (Kuwajerwala *et al.* 2002).

In HL-60 cells, the IC₅₀ value at 8, 24 and 48 h for inhibition of incorporation of thymidine in the DNA is in concordance with earlier reports (Surh *et al.* 1999; Roy *et al.* 2002; Horvath *et al.* 2005). The experiments presented here show that resveratrol inhibits thymidine incorporation in HepG2 cells. This finding is in contrast to studies by Goldberg *et al.* (1995) and Delmas *et al.* (2000), who did not observe an inhibitory effect of resveratrol. This difference may be caused by variations between experimental conditions.

The putative interference with cell replication machinery in HL-60 and HepG2 cells after such a short treatment time could be either a direct or an indirect inhibition of formation or movement of DNA polymerase. Resveratrol has been shown to interfere with DNA polymerase expression *in vitro* and *ex vivo* (Sun *et al.* 1998; Stivala *et al.* 2001) and inhibits the ribonucleotide reductase *in vitro* and *ex vivo* (Fontecave *et al.* 1998; Pozo-Guisado *et al.* 2002). Also, resveratrol has been shown to reduce intracellular levels of dNTPs in HL-60 cells (Horvath *et al.* 2005). The mechanism by which resveratrol inhibits DNA synthesis is not yet fully understood, but resveratrol is likely to interact with DNA polymerase (Stivala *et al.* 2001), whereas the assembly of replication complexes is not affected (Stivala *et al.* 2001).

Increase in IC₅₀ values of incorporated [3H]dT in both cell lines strongly suggests a role of cellular activity, such as metabolism or removal of resveratrol. Metabolism of resveratrol in cells has not been studied in detail and results diverge (Allen *et al.* 2001; Raucy 2003). Preliminary studies in our laboratory indicate that removal of resveratrol may indeed be the cause of increased IC₅₀ values, and further studies are required to fully investigate this phenomenon.

Increase in the size and granularity of the cell nuclei was observed in both cell lines. DAPI staining of nuclei indicates that this effect is not correlated to changes in the spatial arrangement of the DNA. An increase in nuclear size has previously been reported with two HIV protease inhibitors in adipose cells and with palmitate in cardiomyocytes (Kong & Rabkin 1999; Caron *et al.* 2003). To the best of our knowledge, no compound has been reported to stimulate nuclear enlargement in leukaemia and hepatoma cells, and further studies are needed to elucidate this novel property of resveratrol.

Nuclear granularity has only been investigated in a few cases, whereas increased cell granularity is a well known link to apoptosis. The peak of the effect of resveratrol appeared to be 20 and 40 µM for HL-60 and HepG2 cells, respectively. These concentrations correlate to resveratrol concentrations needed to stimulate accumulation in the S phase of the cell cycle of the respective cell lines. We therefore propose that S phase arrest stimulated by resveratrol correlates with morphological changes in the surface structure of the nuclei.

Resveratrol has previously been reported to stimulate accumulation of HepG2 cells in the S phase of the cell cycle (Delmas *et al.* 2000) and HL-60 cells in the G₁ or S phase (Ragione *et al.*

1998; Joe *et al.* 2002; Kang *et al.* 2003; Ahmad *et al.* 2004). A striking result of treating HL-60 and HepG2 cells with different concentrations of resveratrol is the concentration-dependent phase specific accumulation of cells (Fig. 3e,f). Treatment with 20 μM resveratrol for 24 h resulted in nearly a doubling of the number of cells in the S phase compared to untreated HL-60 cells. The same was observed for 40 μM in HepG2 cells. The apparent accumulation of cells in the S phase of the cell cycle may be attributed to apoptotic $G_2 + \text{M}$ phase cells. However, our preliminary studies show no indication of phase specific stimulation of apoptosis by resveratrol. Results presented here thus demonstrate that low concentrations of resveratrol prolong the S phase.

Treatment with 80 μM doubles the number of cells in the G_1 phase compared to HL-60 and HepG2 cells treated with 20 and 40 μM , respectively. This result clearly suggests that high concentrations of resveratrol stimulate accumulation of HL-60 cells in the G_1 phase and thus prevent cells from entering the S phase. This finding is in concordance with observations where resveratrol inhibited the production of polyamines by interfering with ornithine decarboxylase (Schneider *et al.* 2000), which catalyses the rate-limiting step in polyamine production.

The observed concentration-dependent cell cycle arrest in HL-60 and HepG2 cells is in agreement with results found in a range of other cell lines (Table 1). In general, Table 1 and our observations indicate that in experiments where a concentration-dependent effect on cell-cycle distribution by resveratrol are observed, lower resveratrol concentrations cause accumulation of cells in the S phase, whereas higher concentrations cause accumulation of the cells in the G_0/G_1 phase or $G_2 + \text{M}$ phases. This may indicate that resveratrol has more than one target for the inhibition of cell division modulated at different concentrations, as proposed in prostate cells by Kuwajerwala *et al.* (2002).

In HL-60 cells where 20 μM resveratrol-stimulated S-phase arrest, incorporation of [3H]dT was reduced only by 30%. In HepG2 cells, where 40 μM resveratrol showed maximal S-phase arrest, no significant decrease in incorporation of [3H]dT was observed. This suggests that accumulation in the S phase was caused by a slow down of progression through the cell cycle or an inhibition of S to G_2 phase transition as suggested by Latruffe *et al.* (2002). The effect of resveratrol on the dynamics of this prolongation of the cell cycle is, to the best of our knowledge, not well studied and deserves closer examination.

Change in nuclear morphology and loss of membrane asymmetry are two hallmarks of apoptosis, and these were used to study apoptosis-stimulating effects of resveratrol. The study of nuclear condensation and fragmentation, number of cells in the sub- G_1 fraction and exposure of phosphatidylserine identified a concentration-dependent effect of resveratrol. DAPI staining identified a possible pro-apoptotic effect of 20 μM resveratrol as seen by nuclear condensation. However, the same observation could not be made with the Annexin V binding assay, although higher concentrations resulted in a correlation between the results of DAPI staining and Annexin V binding. Nuclear condensation and exposure of phosphatidylserine are both controlled by caspase-3 activation (Hirata *et al.* 1998). Thus, the difference may be caused by a sequential process with loss of membrane asymmetry following nuclear condensation and fragmentation. In both cell lines, concentration-dependent stimulation of apoptosis is in concordance with previous reports (Kuo *et al.* 2002; Roberti *et al.* 2003).

In conclusion, short-term treatment with resveratrol results in concentration-dependent interference with DNA-synthesis and later cell proliferation. This early effect of resveratrol is previously unreported. Treatment of HL-60 and HepG2 cells with resveratrol for 24 h causes a complex pattern of cell phase accumulation. Low concentrations cause a slowdown in progression through the cell cycle, whereas higher concentrations induce a phase-specific cell cycle arrest. For the first time, resveratrol was shown to stimulate an increase in nuclear size of HL-60

Table 1. Resveratrol stimulates cell cycle arrest in several cell lines in a concentration-dependent manner

Model	Phase accumulation	Concentration (μM)	Treatment time (h)	Reference
Human embryonic fibroblasts	S	30	24	(Stivala <i>et al.</i> 2001)
	G ₁ /S	90	24	
MCF7* (mammary cells)	S	50	36	(Pozo-Guisado <i>et al.</i> 2002)
	G ₀ /G ₁	100, 150	36	
CEM-C7H2 (leukaemia cells)	S	10, 20	24	(Bernhard <i>et al.</i> 2000)
	G ₁ /S	40, 100	24	
LNCap (prostate cells)	S	10	24	(Kuwajerwala <i>et al.</i> 2002)
	G ₁	20, 25	24	
A2780 (ovarian cells)	S	50	24	(Opipari <i>et al.</i> 2004)
	G ₁	100	24	
HCT-116 (colonic cells)	S	50	24	(Wolter <i>et al.</i> 2001)
	G ₂ + M	100	24	
	almost none	200	24	
SW480 (colonic cells)	S	30, 50, 100	24/48	(Delmas <i>et al.</i> 2002)
	S	30	72	
	G ₂ + M	50, 100	72	
Caco2 (colonic cells)	S	25	16/24	(Schneider <i>et al.</i> 2000)
	G ₂ + M	25	40	
	none	25	48	
	S	25	64	
BPAE (endothelial cells)	S/G ₂ + M	50	72	(Hsieh <i>et al.</i> 1999)
	S	100	72	
CHL (Chinese hamster lung)	S	40	24	(Matsuoka <i>et al.</i> 2001)
	S	80	24	
	G ₁ /S	40	48	
	G ₁	80	48	

*Synchronized cells.

cells. An increase in granularity HL-60 and HepG2 nuclei was also observed. These increases in nuclear size and granularity are correlated to the phases of the cell cycle. Furthermore, resveratrol stimulates apoptosis in a concentration-dependent manner.

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