Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 2047–2056
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Induction of apoptosis in two human leukemia cell lines as well as differentiation in human promyelocytic cells by cyanidin-3-*O*-β-glucopyranoside

Carmela Fimognari[a,*], Fausto Berti[a], Michael Nüsse[b], Giorgio Cantelli-Forti[a], Patrizia Hrelia[a]

[a]*Department of Pharmacology, University of Bologna, Via Irnerio 48, I-40126 Bologna, Italy*
[b]*GSF-Flow Cytometry Group, D-85758 Neuherberg, Germany*

Received 26 August 2003; accepted 10 February 2004

Abstract

Little is known about the potentially chemopreventive mechanisms of anthocyanins apart from their antioxidant activity. We investigated the in vitro capacity of the anthocyanin cyanidin-3-*O*-β-glucopyranoside (Cy-g) to induce apoptosis in T-lymphoblastoid, as well as apoptosis and differentiation in HL-60 promyelocytic cells. Although Cy-g-induced apoptosis (as well as necrosis) in the two systems, HL-60 cells were much less sensitive than T-lymphoblastoid cells. Moreover, treatment of HL-60 cells with Cy-g caused differentiation into macrophage-like cells and granulocytes. Concerning the mechanism of action, the induction of apoptosis in Jurkat T cells can be explained by a modulation of p53 and bax protein expression. At the molecular level, the induction of apoptosis and cytodifferentiation in HL-60 cells involved different proteins, thus suggesting that the effects of Cy-g on apoptosis and cytodifferentiation induction are two distinct events. These interesting biological properties should encourage further investigation into the chemopreventive and/or chemotherapeutic potential of Cy-g.

Keywords: Chemoprevention; Anthocyanins; Cytodifferentiation; Apoptosis; Human T-lymphoblastoid cells; Promyelocytic leukemia cells

1. Introduction

Uncontrolled imbalances between cell proliferation and physiological cell death (apoptosis) and between cell proliferation and differentiation can lead to development of malignant clones. Differentiation of malignant/premalignant cells into more mature or normal-like cells, and apoptosis during multi-step carcinogenesis are theoretically amenable to preventive cancer intervention. Thus, compounds capable of inducing differentiation or apoptosis are candidate cancer chemopreventive and/or chemotherapeutic agents [1,2].

Hundreds of compounds, selected drugs, and naturally occurring compounds have been identified as potential chemopreventive agents. Anthocyanins are the largest group of pigments present in many berries, in dark grapes, cabbages and other pigmented foods, plants, and vegetables. They can be found to be absorbed unmodified from the diet [3] and to be incorporated in cell cultures, both in the plasma membrane and in the cytosol [4]. They exert antioxidant activities, which could help explain their anti-atherosclerotic [5], anti-carcinogenic [6], and anti-inflammatory [7] properties. Cyanidin-3-*O*-β-glucopyranoside (Cy-g), the main anthocyanin present in juice of pigmented oranges, has been reported as one of the most effective antioxidants [8–11]. It acts as a real antioxidant, and not as a simple metal-chelating compound, because of its peculiar redox potential [12]. Its antioxidant activity is greater than that of resveratrol and ascorbic acid [12], two of most active natural antioxidants. However, little is

Abbreviations: Cy-g, cyanidin-3-*O*-β-glucopyranoside; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; IC_{50}, inhibitory concentration causing cell toxicity by 50% following one cell-cycle exposure; NBT, nitroblue tetrazolium; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; RPMI, Roswell Park Memorial Institute; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

* Corresponding author. Tel.: +39-051-2095636; fax: +39-051-2095624.
E-mail address: carmela.fimognari@unibo.it (C. Fimognari).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.02.021

currently known regarding the potentially chemopreventive mechanisms of anthocyanins apart from their antioxidant activity.

The present study was designed to expand our knowledge of the potential chemopreventive (and chemotherapeutic) properties of Cy-g. We investigated the potential activity of Cy-g in parallel on two human leukemia cell lines: human lymphoblastoid Jurkat T cells and HL-60 promyelocytic cells. These cell lines represent two different models of leukemia: Jurkat is a non-tumorigenic T-lymphoblastoid cell line, whereas HL-60 is a tumorigenic promyelocytic cell line, that consists predominantly of promyelocytes and can be induced to terminally differentiated toward granulocytes and/or macrophages. Moreover, Jurkat and HL-60 cells harbor defective [13] and no p53 [14], respectively. These characteristics could modulate the responsiveness of Jurkat and HL-60 cells to potential chemopreventive agents, such as Cy-g, as the status of the tumor suppressor *p53* has emerged as a key determinant in this context.

2. Materials and methods

2.1. Chemicals

Cy-g (CAS registry number 7084-24-4) was kindly offered by Professor G. Galvano (Department of Chemical Sciences, University of Catania, Italy). Structure formula is reported in Fig. 1. The purity of Cy-g was more than 97%. Cy-g was dissolved in sterile water acidified (pH 3) with HCl 0.1 N. The solutions were stocked in a dark camera at 0–4 °C. The concentrations of Cy-g used were from 3.1 to 200.0 μg/ml of cell suspension. The treatment of cultures with Cy-g did not modify the pH of cultures (data not shown).

2.2. Cells and in vitro culture conditions

The human promyelocytic leukemia cell line HL-60 was obtained from the Istituto Zooprofilattico of Brescia, Italy. Cells were grown in Roswell Park Memorial Institute (RPMI) supplemented with 20% heat-inactivated fetal bovine serum (Sigma), 1% penicillin/streptomycin solution (Sigma), and 1% glutamine solution 200 mM (Sigma).

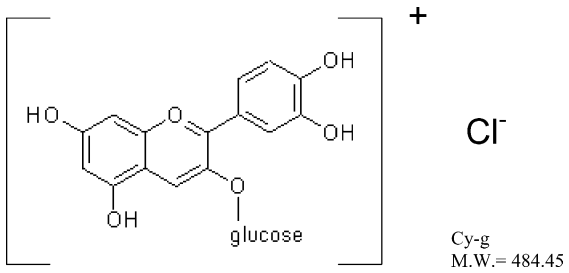


Fig. 1. Cy-g's structure formula.

To reduce spontaneous differentiation, cells were never allowed to exceed the concentration of 1.0×10^6 cells/ml.

Jurkat T-leukemia cells were generously provided by Dr. Michael Nüsse (GSG-Flow Cytometry Group, Neuherberg, Germany). Cells were grown in RPMI supplemented with 10% heat-inactivated fetal bovine serum and 1% glutamine 200 mM with addiction of 1% penicillin/streptomycin solution. To maintain exponential growth, the cultures were divided every third day by dilution to a concentration of 1.0×10^5 cells/ml.

2.3. Cytotoxicity test

Viability was determined using trypan blue dye-exclusion test, which distinguishes viable and non-viable cells. Jurkat and HL-60 cells were treated with various concentrations of Cy-g for 24 and 30 h, respectively (i.e. the exposure times correspond to the length of the cell-cycle). Cell concentrations were measured by counting trypan blue-excluding cells. Results were calculated as viable cells in Cy-g-treated cultures relative to control. Inhibitory concentration causing cell toxicity by 50% following one cell-cycle exposure (i.e. IC_{50}) was calculated by interpolation from dose–response curves.

2.4. Flow cytometry

Flow cytometry was performed using a FACStar$^+$ flow cytometer (Becton Dickinson) equipped with an argon laser (Innova 90; Coherent Radiation) operating at 488 nm (500 mW) for excitation of propidium iodide and fluorescein isothiocyanate.

2.5. Evaluation of apoptosis and necrosis by flow cytometry

Cells of exponentially growing cultures were collected at a time corresponding to one cell-cycle (24 h for Jurkat T cells, 30 h for HL-60 cells). Since apoptosis death is a feature of normal terminally differentiated cells [15], an additional time-point (8 h) was chosen for HL-60 cells in order to discriminate between apoptosis as a primary effect of Cy-g and apoptosis as a consequence of terminal differentiation by Cy-g. Aliquots of 0.5×10^6 cells were centrifuged ($100 \times g$) for 5 min and washed with PBS. The cell pellet was resuspended in 100 μl of labeling solution (ANNEXIN-V-FLUOS; Boehringer Mannheim) containing 2 μl Annexin V labeling reagent and 0.1 μg propidium iodide (Sigma) and incubated for 10–15 min, as per manufacturer's instructions. Immediately after adding 0.1 ml of incubation buffer (10 mM HEPES/NaOH, 140 mM NaCl, 5 mM $CaCl_2$), green (Annexin V–fluorescein isothiocyanate), and red (propidium iodide uptake) fluorescence of individual cells were measured with a FACStar$^+$ flow cytometer, using 488 nm excitation and a 530 nm bandpass filter for fluorescein isothiocyanate detection and a filter

>590 nm for propidium iodide detection. Hence, it is possible to detect non-apoptotic live cells (Annexin $V^{negative}$–propidium $iodide^{negative}$), early apoptotic cells (Annexin $V^{positive}$–propidium $iodide^{negative}$), late apoptotic or necrotic cells (Annexin $V^{positive}$–propidium $iodide^{positive}$), and late necrotic cells (Annexin $V^{negative}$–propidium $iodide^{positive}$) [16]. For each sample, 10,000 events were registered. Electronic compensation was required to exclude overlapping of the two emission spectra.

2.6. Analysis of cell differentiation

To test the potency of compounds to induce cell differentiation, HL-60 cells in log phase were treated with different concentration of Cy-g. After incubation, cells were analyzed to determine the percentage exhibiting enzymatic markers or morphologic features of differentiated cells, as judged by three assays described below. Nitroblue tetrazolium (NBT)-reducing activity is indicative of cell differentiation along both granulocytic and monocytic lineage [2]; differentiation into macrophage-like phenotype was assayed by the ability of cells to markedly adhere to plastic with prominent pseudopodia formation [17]; non-specific/specific acid esterase activity suggests differentiation along monocytic/macrophage lineage and monocytic/granulocytic lineage, respectively [2].

2.6.1. Ability to produce oxidative burst, evaluated using the NBT reduction assay

This assay was used to evaluate the ability of sample-treated HL-60 cells to produce superoxide when challenged with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Sigma). The assay was performed as reported by Cafino [18]. Cells were treated for 72 h with Cy-g. A 1:1 (v/v) mixture of cell suspension (10^6 cells) and freshly prepared TPA/NBT solution (PBS containing 0.2% NBT and 200 ng/ml TPA) was incubated for 30 min at 37 °C. The reaction was stopped by placing the tubes on ice for 5 min. Cell were then smeared on glass slides. Positive cells reduce NBT yielding intracellular black–blue formazan deposits and this was determined by microscope examination (200× total magnification) of 500 cells. The results were expressed as percentage of positive over total cells. DMSO 1% (v/v) was used as positive control.

2.6.2. Adherence to the plastic substrate

Dish-anchored cells are easily distinguished from the undifferentiated suspension cells. Cells (approximately 1.0×10^6) were grown in RPMI media supplemented with 20% fetal bovine serum. After 72 h, the medium was removed and remaining non-adherent cells were gently washed away with PBS. The number of adherent cells was counted on a light microscope. Results were reported as number of attached cells [19]. TPA 100 nM was used as positive control.

2.6.3. Non-specific/specific esterase activity

Assays for α-naphthyl acetate esterase (non-specific acid esterase) and naphthyl AS-D chloroacetate esterase (specific acid esterase) were performed using cytochemical kits from Sigma (91-A and 91-C) [20]. Differentiated cells were assessed by microscopic examination of a minimum of 200 cells (in duplicate) for each experiment.

2.7. Analysis of cell differentiation in the presence of kinase inhibitors

To study the involvement of protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3-K) in the differentiation of cells exposed to Cy-g, we investigated the effects of the PKC inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) (Sigma) and the PI3-K inhibitor LY-294002 (Sigma) on NBT reduction stimulated with Cy-g.

H-7 and LY-294003 were dissolved in sterile water and methanol, respectively. Cells were incubated for 40 min in the presence of different concentrations of H-7 or LY-294002 at 37 °C and 5% CO_2 and then treated with Cy-g 200 μg/ml for 72 h. The NBT reduction assay's procedure was followed as previously described. Results were expressed as percentage of positive over total cells. The percentage of differentiated cells induced by Cy-g 200 μg/ml was indicated as 100%.

2.8. Flow cytometric evaluation of p53, bcl-2, bax, and c-myc proteins

Detection by flow cytometry of different proteins involved in cell-cycle regulation and in the apoptotic process offers rapid and objective quantification of protein levels [21–23]. Under the same exposure conditions described above, 1×10^6 cells were fixed and permeabilized by a commercially available kit, namely Leucoperm solution A and B (Serotec). They were then incubated with 10 μl of each antibody, i.e. fluorescein isothiocyanate p53 (35 μg/ml; Novocastra) against phosphorylated and non-phosphorylated protein, fluorescein isothiocyanate bcl-2 (200 μg/ml; Serotec) against phosphorylated and non-phosphorylated protein, fluorescein isothiocyanate c-myc (200 μg/ml; Santa Cruz Biotechnology), and bax (200 μg/ml; Santa Cruz Biotechnology) or isotype-matched negative control (Serotec). The cells were washed and incubated with 10 μl of fluorescein isothiocyanate-labeled secondary antibody (5 μg/ml; Serotec) for the bax-stained cells. Finally, the cells were washed and resuspended in 0.2 ml of 1% paraformaldehyde. The cells were then analyzed to quantitate fluorescein isothiocyanate binding by flow cytometry. From each sample 10,000 cells were analyzed and non-specific binding was excluded by gating around those cells which were labeled by the isotype negative control antibodies.

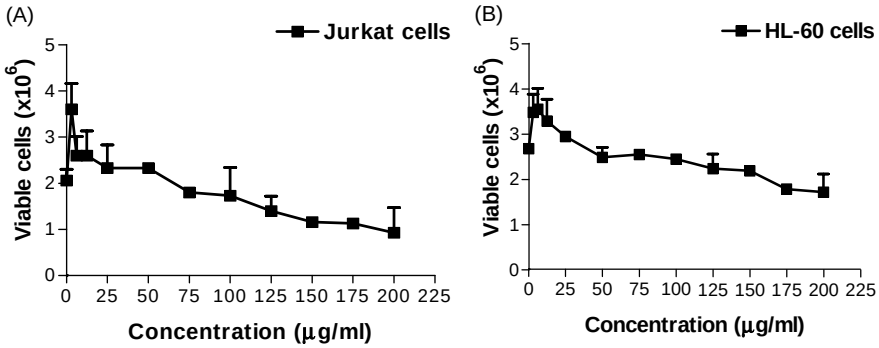


Fig. 2. The effect of Cy-g on viability of Jurkat cells (A) and HL-60 cells (B). The time of treatment was 24 h for Jurkat cells and 30 h for HL-60 cells. Cells were removed from drug-treated and untreated control cultures after one cell-cycle. The data presented is averaged from three independent experiments with error bars denoting standard errors.

2.9. Statistical analysis

All data are the mean ± S.E. of at least three experiments. Statistical significance of the results was evaluated using ANOVA, and Bonferroni or Dunnet *t*-test as post test. All *P* values were two-sided. All statistical analyses were performed in GraphPad Instat version 3.00 for Windows 95, GraphPad software, San Diego, CA, USA.

3. Results

3.1. Viability of Jurkat T-lymphoblastoid cells and HL-60 promyelocytic cells after treatment with Cy-g

Fig. 2 shows the growth curves of Jurkat cells (Fig. 2A) and HL-60 cells (Fig. 2B) after treatment with different concentrations of Cy-g. In both cell systems, a gradual, but not significant, dose-dependent decrease in the number of viable cells was recorded. The IC_{50} for Jurkat cells (measured by the number of viable cells in cultures after the addition of Cy-g, and calculated by interpolation from the dose–response curve) was seen at a concentration of 174.9 μg/ml. Only a slight decrease in the number of viable cells was observed for HL-60, where the IC_{50} value did not fall within the range of concentrations tested. In fact, Cy-g treatment at 200.0 μg/ml produced a 37% reduction in viable HL-60 cells (1.7×10^6 versus 2.7×10^6 in untreated HL-60 cells).

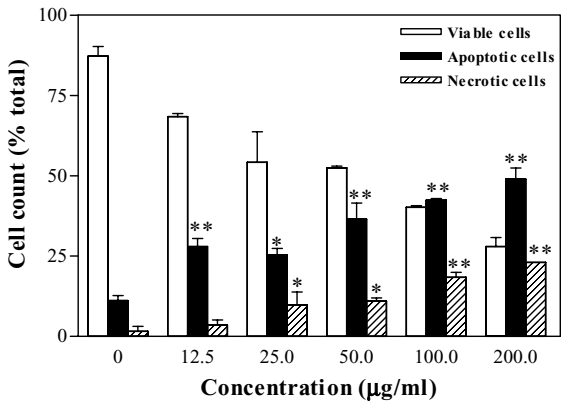
It is interesting to note that an increase of viable cells was observed at the lower Cy-g concentrations in both cell lines.

3.2. Induction of apoptosis and necrosis of Jurkat T-lymphoblastoid cells and HL-60 promyelocytic cells after treatment with Cy-g

Cy-g treatment induced apoptosis in both cell systems. However, different concentrations of Cy-g were necessary to trigger apoptosis. In Jurkat cells (Fig. 3), even the lowest concentration tested (12.5 μg/ml) was sufficient to increase the number of cells displaying features of apoptosis (i.e. Annexin $V^{positive}$ and propidium $iodide^{negative}$: 28.0% versus 11.2% in untreated controls; $P < 0.01$). After treatment with higher doses, the fraction of apoptotic cells continued to increase, reaching 49.0% ($P < 0.01$ with respect to controls) at 200.0 μg/ml.

When HL-60 cells were exposed to Cy-g for 8 h, we recorded a dose-dependent increase in the fraction of apoptotic cells, which reached an about two-fold increase with respect to the control, only at the highest concentration tested (at 200 μg/ml: 18% versus 8% in controls) (Fig. 4A). When the treatment was prolonged until 30 h, we recorded a statistically significant increase in the apoptotic cell fraction starting from 50 μg/ml Cy-g (Fig. 4B). This fraction increased to 32.5% at 100 μg/ml and 36.0% at 200.0 μg/ml ($P < 0.01$). HL-60 cells were, therefore, more resistant to Cy-g-induced apoptosis than Jurkat cells.

Membrane permeabilization, revealed by propidium iodide uptake (Annexin $V^{positive}$–propidium $iodide^{positive}$)



*P<0.05, **P<0.01 with respect to controls (Dunnet test)

Fig. 3. Fraction of viable, apoptotic and necrotic cells, as detected in Jurkat cells treated with Cy-g at the indicated doses for 24 h. Cells were removed from treated and untreated cultures and stained with Annexin V–fluorescein isothiocyanate and propidium iodide. The data presented is averaged from three independent experiments with error bars denoting standard errors.

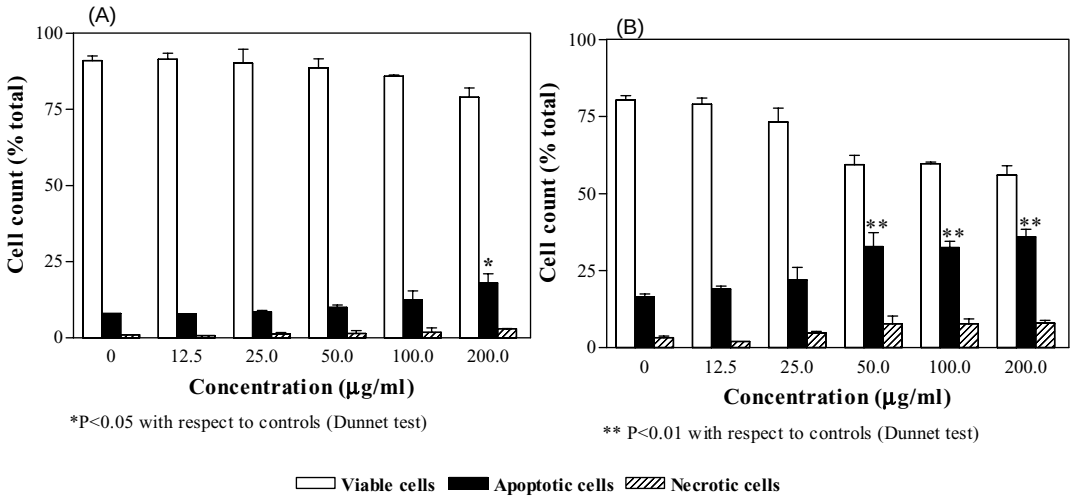


Fig. 4. Fraction of viable, apoptotic and necrotic cells, as detected in HL-60 cells treated with Cy-g at the indicated doses for 8 h (A) and 30 h (B). Cells were removed from treated and untreated cultures and stained with Annexin V–fluorescein isothiocyanate and propidium iodide. The data presented is averaged from three independent experiments with error bars denoting standard errors.

indicated that Cy-g was active in Jurkat cells but not in HL-60 cells. In Jurkat cells (Fig. 3), induction of necrosis started at the concentration of 25.0 μg/ml Cy-g (9.8 % versus 1.6% in controls, $P < 0.05$). By comparison, in HL-60 cells (Fig. 4A and B), no significant induction of necrosis was reached in the range of the concentrations tested (at 200.0 μg/ml for 30 h: 8.0% versus 3.3% in controls, $P =$ ns).

3.3. Induction of differentiation of HL-60 promyelocytic cells treated with Cy-g

To explore the ability of Cy-g to induce differentiation, HL-60 cells were treated with various doses of Cy-g (12.5–200.0 μg/ml). As indicated in Fig. 5A, Cy-g-induced dose-dependent differentiation of HL-60 cells, as determined by NBT reduction, a functional marker for granulocyte/monocyte differentiation. The highest effect was recorded after treatment with 200 μg/ml Cy-g, where the fraction of NBT-positive cells reached 32.4% ($P < 0.01$ with respect to controls), thus comparable to the fraction of NBT-positive cells recorded for the positive control (DMSO). Moreover, Cy-g treatment (Fig. 5B) increased the number of adherent cells by over 15-fold ($P < 0.01$), suggesting that Cy-g induces differentiation into monocyte/macrophage-like phenotype. Cy-g also induced naphtol AS-D chloroacetate activity in HL-60 cells, that represents a marker for granulocytic differentiation (Fig. 5C). At the concentration of 50 μg/ml, the naphtol AS-D chloroacetate esterase positive cells were around 38%. This percentage slightly rose to 44 and 48% at Cy-g 100 and 200 μg/ml, respectively. It is important to note that undifferentiated HL-60 cells constitute a positive control for the naphtol AS-D chloroacetate esterase, thus explaining the high percentage of positive cells in the control (Fig. 5C).

Finally, Cy-g-induced a significant dose-related increase in the number of α-naphthyl acetate esterase positive cells, that indicates a differentiation toward the monocytic/macrophagic lineage (Fig. 5D). The highest effect was recorded at the concentration of 200 μg/ml Cy-g, where the fraction of positive cells reached a percentage of 57.5.

3.4. Reduction of HL-60 cell differentiation after incubation with PKC or PI3-K inhibitors and Cy-g

Previous studies have provided evidence that activation of PKC and PI3-K is necessary for the differentiation of HL-60 cells [24,25]. To determine any relationship between the effect of Cy-g on cellular differentiation and PKC and/or PI3-K activation, HL-60 cells were treated with specific inhibitors, H-7 and LY-294002, followed by incubation with Cy-g at the concentration where the highest NBT reduction was recorded (200 μg/ml). Afterwards, the degree of cellular differentiation was assessed by the NBT reduction assay.

Cy-g significantly increased the percentage of differentiated cells with respect to the control (data not shown). As illustrated in Fig. 6A and B, both H-7 and LY-294002 significantly inhibited HL-60 cell differentiation induced by Cy-g. A dose-dependent reduction in the percentage of differentiated cells was observed beginning with the lowest concentration tested (5 μM for H-7 and 0.5 μM for LY-294002) where about 16 and 50% of total cells were able to produce oxidative burst, respectively. The reduction in the percentage of NBT-positive cells reached the maximum value at the highest concentration of each inhibitor tested (20 μM for H-7 and 5 μM for LY-294002), where only 6.5 and 7% of total cells were able to reduce NBT salts, respectively.

The PKC and PI3-K inhibitors were not toxic to the HL-60 cells at the used concentrations in the experiments,

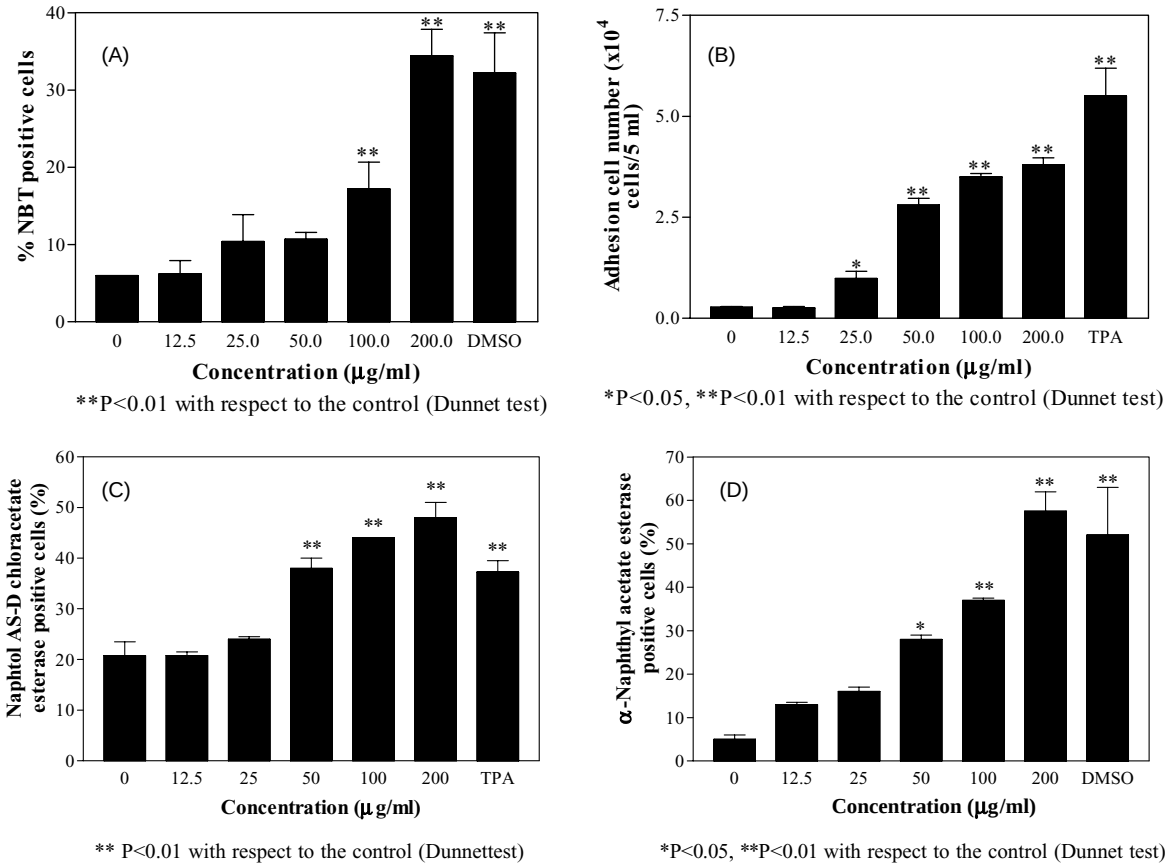


Fig. 5. Dose-dependent effects of Cy-g on HL-60 cell differentiation as evaluated by NBT-reducing ability (A), cell adhesion (B), naphtol AS-D chloroacetate esterase activity (C), and α-naphthyl acetate esterase activity (D). Data are mean ± S.E. of three independent experiments.

as demonstrated by the trypan blue exclusion assay (data not shown).

3.5. Expression of p53, bax, bcl-2, and c-myc in Jurkat and HL-60 cells treated with Cy-g

In order to delineate the events leading to apoptosis and/or cytodifferentiation elicited by Cy-g, we analyzed the expression of different proteins, that are known to be involved in cell growth and differentiation, and apoptosis. Flow cytometric measurements were performed at the time points and the concentrations where it was recorded an induction of apoptosis. Apoptotic cells were gated—based on their modified forward- and side-angle light-scatter characteristics—and proteins were then quantified.

Jurkat cells were treated for 24 h with Cy-g 200 μg/ml. Fig. 7 shows histograms of p53, bcl-2, bax, and c-myc protein levels in apoptotic cells following incubation with

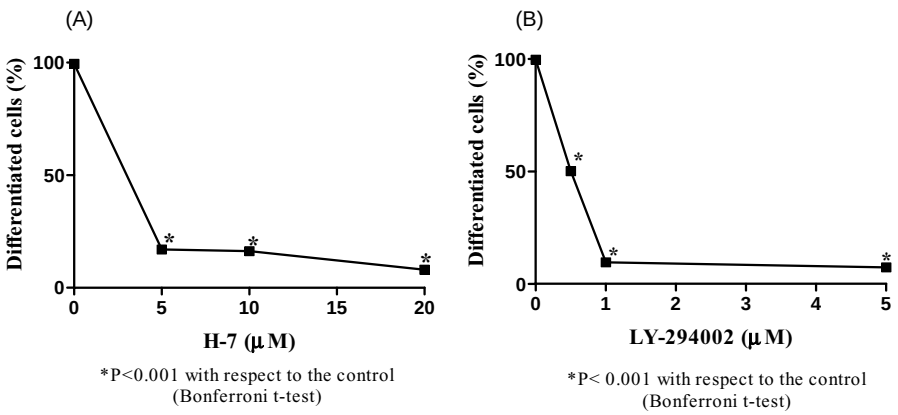


Fig. 6. Effect of PKC (A) and PI3-K (B) inhibitors on HL-60 cell differentiation induced by Cy-g 200 μg/ml. HL-60 cells were treated with varying concentrations of PKC inhibitors (H-7) or PI3-K (LY-294002) for 40 min, followed by incubation with Cy-g 200 μg/ml for 72 h. Data are mean ± S.E. of three independent experiments.

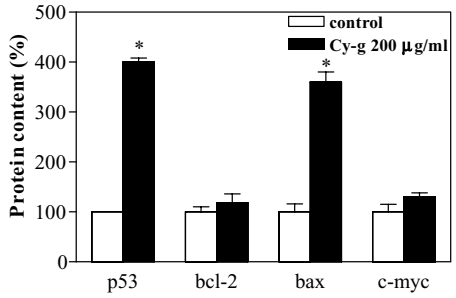


Fig. 7. p53, bcl-2, bax, and c-myc protein contents following 24 h culture of Jurkat cells in the absence or presence, respectively, of Cy-g (200 μg/ml). Proteins were measured by flow cytometry. The results are expressed as the percentage of the protein content in untreated cells (mean ± S.E. of three independent experiments).

and without Cy-g. Treated cells showed a marked increase in p53 and bax protein levels (4- and 3.6-fold decrease with respect to the control, respectively), whereas bcl-2 and c-myc levels were substantially unchanged.

The analysis of protein levels in HL-60 cells was performed after 8 (Fig. 8A) and 30 h (Fig. 8B) of treatment with Cy-g 200 μg/ml. As expected, we were not able to detect p53 expression in HL-60 cells after treatment with Cy-g, since HL-60 are known to lack a functional p53 pathway [14]. However, when HL-60 cells were exposed to Cy-g, an apoptotic cell fraction was observed by FACS analysis (Annexin $V^{positive}$–propidium $iodide^{negative}$ cells, see Fig. 4), showing that the ability of this compound to induce apoptosis in this cell line was not requiring a functional p53.

The analysis of bcl-2, bax, and c-myc protein levels at 8 and 30 h gave us some interesting information. After 8 h of treatment, we recorded a marked decrease by about three-fold only in the bcl-2 levels, while c-myc and bax were not affected by Cy-g. However, when the samples were analyzed after 30 h of treatment, we detected a 4.5-fold decrease in the levels of c-myc paralleled by a significant decrease in the bcl-2 levels. The levels of bax remained unchanged.

4. Discussion

Inducing of apoptosis or differentiation are two promising cancer prevention strategies, as well as therapy. Recent findings have shown that defects in the cell death pathway are not only important for the origin of cancer, but also may markedly influence the response to chemotherapeutic treatment [26]. On the other hand, most cancer cells exhibit a defect in their capacity to mature into non-replicating adult cells, thereby existing in a highly proliferating state, which results in outgrowing their normal cellular counterparts. The induction of terminal differentiation represents an alternative approach to the treatment of cancer by conventional anti-neoplastic agents, by generating cells with limited or no replicative capacity and ultimately undergo apoptosis [2].

We, therefore, investigated the ability of Cy-g of inducing apoptosis and cytodifferentiation in leukemic cell lines. Although Cy-g-induced apoptosis (as well as necrosis) both in Jurkat and in HL-60 cells, these latter were much less sensitive to these effects than Jurkat cells.

In Jurkat cells, the IC_{50} was estimated at a concentration of 175 μg/ml Cy-g, whereas in HL-60 cells this value was not reached in the range of tested concentrations. Furthermore, a significant induction of apoptosis was detected in Jurkat cells even at the lowest concentration tested (12.5 μg/ml). In HL-60 cells, 200 μg/ml for 8 h or 50 μg/ml of Cy-g for 30 h was required to trigger a significant increase in apoptosis. Moreover, the highest amount of apoptosis induced in Jurkat cells was about two times greater than that recorded in human promyelocytic cells. It should be noted that almost all tested doses of Cy-g caused necrosis in Jurkat cells, whereas no significant incidence of necrosis was recorded for HL-60 cells until the highest concentrations tested. This observation suggests that Cy-g does also exert toxic effects in Jurkat cells.

The lower concentrations of Cy-g caused an increase in the number of viable cells in both cell lines. This seems to be an actual phenomenon because the effect was reproducible. This apparently paradoxical finding is open to

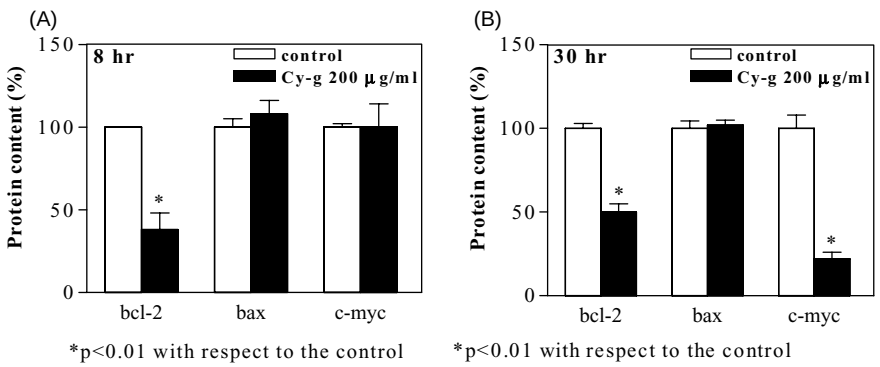


Fig. 8. bcl-2, bax and c-myc protein contents following 8 h (A) and 30 h (B) culture of HL-60 cells in the absence or presence, respectively, of Cy-g (200 μg/ml). Proteins were measured by flow cytometry. The results are expressed as the percentage of the protein content in untreated cells (mean ± S.E. of four independent experiments).

question. One possibility is that Cy-g at lower concentrations has no general cytotoxicity activity. However, it was shown for many compounds that at low level of disruption or toxicity many biological systems display an overcompensation response, which results in the apparent low-dose stimulation component of the response curve. At higher doses with greater initial toxicity, the system often display a more limited capacity for a compensatory response, usually insufficient to return to control values [27–30]. These findings have important clinical implications.

The present study also demonstrated that Cy-g is an effective inducer of differentiation in vitro, able to commit leukemia cells toward terminal maturation. Three assays, NBT reduction, non-specific/specific acid esterase activity, and the adhesion of cells to the substrate correlate with each other, and suggest the lineage of differentiation. Cy-g treatment increased NBT reduction activity (a marker for differentiation to monocytes/granulocytes), markedly increased monocytic and, also if at a lesser degree, granulocyte esterase activities, and caused a marked adhesion of cells to the substrate (a marker for differentiation to macrophage-like cells) [31]. These results indicate that Cy-g induces differentiation mostly toward macrophage lineage.

A differentiating agent can exhibit different mechanisms. PI3-K is an enzyme that participates in a myriad of cellular processes and its activity has been linked to cell growth and transformation, differentiation, motility, insulin action, and cell survival. This enzymatic activity can phosphorylate the 3′-hydroxyl group on the inositol ring of phosphatidylinositol (PtdIns) or same group of PtdIns 4-phosphate or PtdIns 4,5-biphosphate and generate three distinct membrane lipids, the phosphatidylinositol (3) phosphate. They act as intracellular mediators themselves and subsequently activate many effector enzymes (e.g. protein kinase, phospholipase, and G-protein) [18]. PKC is a family of phospholipid-dependent serine-threonine protein kinases and is a good candidate for mediating PI3-K signaling. PKC plays an important role in signal transduction pathways that regulate growth and differentiation in a variety of cells types [17]. In our study, inhibitors for PKC and PI3-K significantly decreased HL-60 cell differentiation induced by Cy-g, suggesting that PKC and PI3-K may be involved in Cy-g-mediated differentiation. However, H7 not only acts as a PKC inhibitor but also as a protein kinase A and protein kinase G inhibitor. Thus, the role of PKA and PKG on Cy-g-induced cytodifferentiation is open to speculation.

On the whole, our results indicate that Cy-g is able to stimulate the differentiation of HL-60 cells. This consideration is of relevance since differentiated cells contain amounts of survival-maintaining molecules lower than their immature progenitors, whose great immaturity is likely to account for their resistance to apoptosis [32].

Treatment of HL-60 cultures with Cy-g caused apoptosis, that may represent an event only partly dependent on cytodifferentiation in our experimental conditions. In fact, after 8 h of treatment with Cy-g, we had already recorded an increase in the apoptotic cell fraction. The analysis of the apoptosis- and differentiation-related proteins offered some explanations. The measurements of protein contents after 8 h of HL-60 treatment with Cy-g indicated a strong decrease in the content of bcl-2, whereas the levels of bax and c-myc were not affected. This might suggest that Cy-g induces apoptosis through a modulation of bcl-2 protein. Phosphorylation of bcl-2 at serine residues can be regulated by treatment with growth factors, as well as activators and inhibitors of PKC, although the functional significance of this observation remain unclear [33]. Since we demonstrated that PKC is involved in the effects of Cy-g, we can not exclude the possibility that Cy-g could affect the bcl-2 protein at a post-transcriptional level, for example, by altering its phosphorylation. After 30 h of treatment, we still recorded a significant decrease in bcl-2 content, but also a strong decrease in the levels of c-myc. This latter information, together with the onset of phosphatidylserine exposure 8 h after exposure to Cy-g—a time-point when no major changes in c-myc levels were observed—suggests that c-myc downregulation is a secondary event, possibly associated with an anti-proliferative effect of Cy-g, as a consequence of terminal maturation. On the whole, our results indicated that Cy-g-induced differentiation is accompanied by cytostasis and c-myc downregulation.

It is noteworthy that HL-60 cells are p53 null [14]. Thus, the induction of apoptosis in this system indicates that Cy-g may exert its effects independently of the *p53* gene. However, the analysis of protein levels in Jurkat cells showed that Cy-g-induced apoptosis is associated with significant changes in p53 and bax proteins, suggesting that alterations in the levels of these proteins are directly responsible for the death signal delivered by Cy-g. On the whole, our results do not allow us to exclude a p53-dependent pathway in the apoptotic effect of Cy-g. If the apoptotic pathway of Cy-g is p53-dependent, this could represent a possible explanation for the less sensitivity of HL-60 cells to the effects by Cy-g than Jurkat cells.

Cy-g is well known for its antioxidant properties, which have been demonstrated in several cell systems [12,34–36]. A link might exist between the ability of Cy-g to protect against reactive oxygen species damage and the pro-apoptotic effects observed in our study. Cancer cells constitutively produce high amounts of H_2O_2 [37] and the concept of ‘‘persistent oxidative stress in cancer’’ was formulated [38]. Despite the relatively greater damage to key cellular components such as DNA, cancer cells tolerate the damage, survive, and continue to growth and proliferate as if the oxidative stress was actually conducive to their vitality [37]. If the H_2O_2 functions as a secondary messenger in triggering signaling events leading to the activation of cancer cell proliferation genes [37], antioxidant compounds, such as Cy-g, might induce apoptosis in

cancer cells by scavenging H_2O_2, thereby depriving them of an essential molecule needed for their existence.

Our in vitro findings indicate that Cy-g does possess interesting biological properties that should encourage further investigation as regards both its chemopreventive and chemotherapeutic potential. Many natural products or synthetic compounds can induce differentiation of HL-60 cells. The potential activity of these agents can be evaluated by the percentage of cells that differentiate and the concentration at which these agents induce maximum differentiation. As an example, the polar–planar drugs DMSO, acetamide, piperidine and triethylene glycole induce cytodifferentiation at concentrations of about 10^{-1} to 10^{-2} M [39], thus higher than those used in this study. However, some questions may be raised about the concentrations and the exposure times used in in vitro experiments, which may not be representative of an in vivo situation. The bioavailability of anthocyanins is still questionable. Most of the studies have been performed by using extracts or juices obtained from different fruits, where the presence of other compounds might modify the bioavailability of single anthocyanins. However, some information are currently available. Netzel and coworkers [40] compared pharmacokinetic parameters and the bioavailability of several dietary anthocyanins following consumption of red grape juice in nine healthy volunteers. They were given a single oral dose of 283.5 mg total anthocyanins and the relative bioavailability of cyanidine-3-glucoside was calculated to be 65.7%. Moreover, it was demonstrated that, after the ingestion of 153 mg of anthocyanins, only 0.020–0.050% of the oral doses was excreted in the urine [41]. Taken together, these observations indicate that the concentrations used in the present study can be achievable in vivo. If Cy-g is to be applied in clinical settings as a suitable cancer chemopreventive agent, the compound should be administered in such a way as to maintain a steady state of drug in the body, also considering the growth enhancement seen at low doses.

In any case, any definitive conclusion on the potential chemopreventive effect of Cy-g would be premature. The knowledge that relapsed acute promyelocytic leukemia patients are resistant to further treatment with retinoic acid even when their leukemia cells respond to retinoic acid in vitro [42] provides an interesting illustration of the limitations of in vitro studies.

Acknowledgments

We thank Professor G. Galvano (Department of Chemical Sciences, University of Catania, Italy) for providing Cy-g. This work was jointly supported by the University of Bologna to the project ‘‘Sviluppo di farmaci di origine naturale ad azione antiossidante per prevenzione e trattamento di patologie epatobiliari ed intestinali’’ and by MIUR ex 60% and FIRB grants.

References

[1] Hong WK, Sporn MB. Recent advances in chemoprevention of cancer. Science 1997;278:1073–7.
[2] Suh N, Luyengi L, Fong HH, Kinghorn AD, Pezzuto JM. Discovery of natural product chemopreventive agents utilizing HL-60 cell differentiation as a model. Anticancer Res 1995;15:233–9.
[3] Cao G, Muccitelli HU, Sanchez-Moreno C, Prior RL. Anthocyanins are absorbed in glycated forms in elderly women: a pharmacokinetic study. Am J Clin Nutr 2001;73:920–6.
[4] Youdim KA, Martin A, Joseph JA. Incorporation of the elderberry anthocyanins by endothelial cells increases protection against oxidative stress. Free Radic Biol Med 2000;29:51–60.
[5] Ghiselli A, Nardini M, Baldi A, Scaccini C. Antioxidant activity of different phenolic fractions separated from an Italian red wine. J Agric Food Chem 1998;46:361–7.
[6] Bomser J, Madhavi DL, Singletary K, Smith MA. In vitro anticancer activity of fruit extracts from *Vaccinium* species. Planta Med 1996;62:212–6.
[7] Lietti A, Cristoni A, Picci M. Studies on *Vaccinium myrtillus* anthocyanosides. I. Vasoprotective and antiinflammatory activity. Arzneimittelforschung 1996;26:829–32.
[8] Wang H, Cao G, Prior RL. Oxygen radical absorbing capacity of anthocyanins. J Agric Food Chem 1997;45:304–9.
[9] Tsuda T, Shiga K, Ohshima K, Kawakishi S, Osawa T. Inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigments isolated from *Phaseolus vulgaris* L. Biochem Pharmacol 1996;52:1033–9.
[10] Tsuda T, Horio F, Osawa T. Dietary cyanidin 3-*O*-beta-D-glucoside increases ex vivo oxidation resistance of serum in rats. Lipids 1998;33:583–8.
[11] Tsuda T, Horio F, Kitoh J, Osawa T. Protective effects of dietary cyanidin 3-*O*-beta-D-glucoside on liver ischemia-reperfusion injury in rats. Arch Biochem Biophys 1999;368:361–6.
[12] Amorini AM, Fazzina G, Lazzarino G, Tavazzi B, Di Pierro D, Santucci R, et al. Activity and mechanism of the antioxidant properties of cyanidin-3-*O*-beta-glucopyranoside. Free Radic Res 2001;35:953–66.
[13] Iwamoto KS, Mizuno T, Ito T, Tsuyama N, Kyoizumi S, Seyama T. Gain-of-function p53 mutations enhance alteration of the T-cell receptor following X-irradiation, independently of the cell cycle and cell survival. Cancer Res 1996;56:3862–5.
[14] Danova M, Giordano M, Mazzini G, Riccardi A. Expression of p53 protein during the cell cycle measured by flow cytometry in human leukemia. Leuk Res 1990;14:417–22.
[15] Martin SJ, Bradley JG, Cotter TG. HL-60 cells induced to differentiate towards neutrophils subsequently die via apoptosis. Clin Exp Immunol 1990;79:448–53.
[16] Peña FJ, Johannisson A, Wallgren M, Rodríguez-Martínez H. Assessment of fresh and frozen-thawed boar semen using an Annexin-V assay: a new method of evaluating sperm membrane integrity. Theriogenology 2003;60:677–89.
[17] Kim MS, Lim WK, Cha JG, An NH, Yoo SJ, Park JH, et al. The activation of PI 3-K and PKC zeta in PMA-induced differentiation of HL-60 cells. Cancer Lett 2001;171:79–85.
[18] Cafino JJ. Microtiter assay useful for screening of cell differentiation agents. J Natl Cancer Inst 1988;80:962–6.
[19] Ahmed N, Williams JF, Weidemann M. The human promyelocytic HL-60 cell line: a model of myeloid cell differentiation using dimethylsulphoxide, phorbol ester and butyrate. Biochem Int 1991; 23:591–602.
[20] Zhou JY, Norman AW, Lubbert M, Collins ED, Uskokovic MR, Koeffler HP. Novel vitamin D analogs that modulate leukemic cell growth and differentiation with little effect on either intestinal calcium absorption or bone mobilization. Blood 1989;74:82–93.
[21] Liu X, Zhu XZ. Roles of p53, c-Myc, Bcl-2, Bax and caspases in glutamate-induced neuronal apoptosis and the possible neuroprotective

mechanism of basic fibroblast growth factor. Mol Brain Res 1999;71: 210–6.

[22] Viallard JF, Lacombe F, Dupouy M, Ferry H, Belloc F, Reiffers J. Flow cytometry study of human cyclin B1 and cyclin E expression in leukemic cell lines: cell cycle kinetics and cell localization. Exp Cell Res 1999;247:208–19.

[23] Zhao S, Tsuchida T, Kawakami K, Shi C, Kawamoto K. Effect of As_2O_3 on cell cycle progression and cyclins D1 and B1 expression in two glioblastoma cell lines differing in p53 status. Int J Oncol 2002;21:49–55.

[24] Pan Q, Granger J, O'Connell TD, Somerman MJ, Simpson RU. Promotion of HL-60 cell differentiation by 1,25-dihydroxyvitamin D3 regulation of protein kinase C levels and activity. Biochem Pharmacol 1997;54:909–15.

[25] Bertagnolo V, Neri LM, Marchisio M, Mischiati C, Capitani S. Phosphoinositide 3-kinase activity is essential for all-*trans*-retinoic acid-induced granulocytic differentiation of HL-60 cells. Cancer Res 1999;59:542–6.

[26] Raff M. Social control on cell survival and cell death. Nature 1993; 356:397–400.

[27] Foekens JA, Sieuwerts AM, Stuurman-Smeets EM, Dorssers LC, Berns EM, Klijn JG. Pleiotropic actions of suramin on the proliferation of human breast-cancer cells in vitro. Int J Cancer 1992;51: 439–44.

[28] Fong CJ, Sutkowski DM, Braun EJ, Bauer KD, Sherwood ER, Lee C, et al. Effect of retinoic acid on the proliferation and secretory activity of androgen-responsive prostatic carcinoma cells. J Urol 1993;149: 1190–4.

[29] Abe T, Gotoh S, Higashi K. Attenuation by glutathione of hsp72 gene expression induced by cadmium in cisplatin-resistant human ovarian cancer cells. Biochem Pharmacol 1999;58:69–76.

[30] Hsu JT, Jean TC, Chan MA, Ying C. Differential display screening for specific gene expression induced by dietary nonsteroidal estrogen. Mol Reprod Dev 1999;52:141–8.

[31] Yam LT, Li CY, Crosby WH. Cytochemical identification of monocytes and granulocytes. Am J Clin Pathol 1971;55:283–90.

[32] Pae HO, Seo WG, Kim NY, Oh GS, Kim GE, Kim YH, et al. Induction of granulocytic differentiation in acute promyelocytic leukemia cells (HL-60) by water-soluble chitosan oligomer. Leuk Res 2001;25: 339–46.

[33] Ruvolo PP, Deng X, Carr BK, May WS. A functional role for mitochondrial protein kinase Calpha in Bcl2 phosphorylation and suppression of apoptosis. J Biol Chem 1998;273:25436–42.

[34] Amorini AM, Lazzarino G, Galvano F, Fazzina G, Tavazzi B, Galvano G. Cyanidin-3-*O*-beta-glucopyranoside protects myocardium and erythrocytes from oxygen radical-mediated damages. Free Radic Res 2003;37:453–60.

[35] Ramirez-Tortosa C, Andersen OM, Gardner PT, Morrice PC, Wood SG, Duthie SJ, et al. Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats. Free Radic Biol Med 2001;31:1033–7.

[36] Kim MY, Iwai K, Onodera A, Matsue H. Identification and antiradical properties of anthocyanins in fruits of Viburnum dilatatum thunb. J Agric Food Chem 2003;51:6173–7.

[37] Loo G. Redox-sensitive mechanisms of phytochemical-mediated inhibition of cancer cell proliferation (review). J Nutr Biochem 2003;14:64–73.

[38] Toyokuni S, Okamoto K, Yodoi J, Hiai H. Persistent oxidative stress in cancer. FEBS Lett 1995;358:1–3.

[39] Kong G, Lee SJ, Kim HJ, Surh YJ, Kim ND. Induction of granulocytic differentiation in acute promyelocytic leukemia cells (HL-60) by 2-(allylthio) pyrazine. Cancer Lett 1999;144:1–8.

[40] Frank T, Netzel M, Strass G, Bitsch R, Bitsch I. Bioavailability of anthocyanidin-3-glucosides following consumption of red wine and red grape juice. Can J Physiol Pharmacol 2003;81:423–35.

[41] Netzel M, Strass G, Janssen M, Bitsch I, Bitsch R. Bioactive anthocyanins detected in human urine after ingestion of blackcurrant juice. J Environ Pathol Toxicol Oncol 2001;20:89–95.

[42] Brackman D, Lund-Johansen F, Aarskog D. Expression of cell surface antigens during the differentiation of HL-60 cells induced by 1,25-dihydroxyvitamin D3, retinoic acid and DMSO. Leuk Res 1995;19: 57–64.