

# Inhibition of Cell Cycle Progression by Hydroxytyrosol Is Associated with Upregulation of Cyclin-Dependent Protein Kinase Inhibitors p21<sup>WAF1/Cip1</sup> and p27<sup>Kip1</sup> and with Induction of Differentiation in HL60 Cells<sup>1</sup>

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## Abstract

Recent evidence indicates that the cancer preventive activity of olive oil can be mediated by the presence of minor components, such as antioxidant phenolic compounds. However, their mechanisms of action remain largely unknown. In this study, we investigated the *in vitro* effects of one of the main olive oil phenols, hydroxytyrosol [3,4-dihydroxyphenylethanol (3,4-DHPEA)], on proliferation, cell cycle progression, apoptosis, and differentiation of HL60 human promyelocytic leukemia cells. 3,4-DHPEA showed a potent inhibitory activity on DNA synthesis, as evidenced by a 92% reduction of [<sup>3</sup>H]-thymidine incorporation at 100  $\mu$ mol/L, and an induced apoptosis, as evidenced by the release of cytosolic nucleosomes and flow cytometry. This phenol, 3,4-DHPEA, was also able to inhibit the progression of the cell cycle in synchronized HL60 cells, which accumulated in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle after 25 h of treatment. Furthermore, 3,4-DHPEA induced differentiation on HL60 cells with a maximum effect (22% of cells) at 100  $\mu$ mol/L after 72 h of treatment. Among the different proteins involved in the regulation of the cell cycle, 3,4-DHPEA reduced the level of cyclin-dependent kinase (CDK) 6 and increased that of cyclin D3. With regard to the CDK inhibitors, p15 was not altered by 3,4-DHPEA treatment, whereas the expression of p21<sup>WAF1/Cip1</sup> and p27<sup>Kip1</sup> was increased at both protein and mRNA levels. To our knowledge, these results provide the first evidence that 3,4-DHPEA may effect the expression of genes involved in the regulation of tumor cell proliferation and differentiation. *J. Nutr.* 138: 42–48, 2008.

## Introduction

In several epidemiological and animal studies, researchers have suggested that virgin olive oil may have a protective effect against cancer. Out of 22 case-control studies performed within the Mediterranean area, only 2 did not have a statistically relevant association between olive oil intake and cancer (1,2). All the other studies showed an inverse correlation between olive oil consumption and cancer in different sites, like the breast (3–6), prostate (7,8), oral cavity (9), and colon (10). In a follow-up approach, olive oil consumption was correlated to a better prognosis for laryngeal cancer (11) and a reduced incidence of breast cancer (12). Furthermore, it was found that olive oil intake is associated with a reduced number of DNA adducts in

peripheral leukocytes (13). Recently, it was hypothesized that the cancer preventive capacity of olive oil could be mediated, at least in part, by the presence of potent antioxidant phenolic compounds that were shown to possess several biological activities. Direct evidence in favor of this hypothesis came from a multicenter study, which showed that only in the Spanish center did a clear inverse association exist between breast cancer and the content of oleic acid in the adipose tissue (OR = 0.4). In the northern European countries, where consumption of olive oil is notoriously low, this correlation was not found (OR = 1.27), therefore suggesting that olive oil may exert its beneficial effects, beyond the oleic acid content, through the presence of minor compounds (14). In addition, an intervention study showed that supplying postmenopausal women with virgin olive oil containing a high amount of phenolic compounds caused a decrease of oxidative damage to lymphocytes DNA (15).

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Among the different phenols known to be present in olive oil, hydroxytyrosol [3,4-dihydroxyphenylethanol (3,4-DHPEA)],<sup>2</sup> an ortho-diphenol derived from the hydrolysis of oleuropein, recently received particular attention because it may inhibit both initiation and promotion steps of carcinogenesis *in vitro*. Indeed, 3,4-DHPEA possess a clear scavenging activity toward different free radicals (16), and it is able to avoid several injuries caused by reactive oxygen species, such as hydrogen peroxide induced DNA strand breaks in Jurkat (17) and prostate (18) cancer cells, DNA base modification, and tyrosine nitration induced by peroxyxynitrite (19). In addition, both a complex methanolic extract of the virgin olive oil, containing several phenolic compounds, and purified 3,4-DHPEA are able to induce apoptosis in different tumor cell lines, as demonstrated by previous studies conducted in our laboratory (20) and by other investigators (21). Furthermore, it was also shown that 3,4-DHPEA inhibits the proliferation of HL60 human promyelocytic leukemia cells and alters the cell cycle progression, inducing an accumulation of cells in the G<sub>0</sub>/G<sub>1</sub> phase (22). However, it should be noted that the subcellular events contributing to the 3,4-DHPEA antiproliferative and cell cycle effects on tumor cells remain completely unknown.

The cell cycle is regulated through the sequential activation and inactivation of cyclin-dependent protein kinases (CDK) that control specific steps of the cycle progression, such as G<sub>1</sub>-S and G<sub>2</sub>-M transitions. CDK activation requires the binding to different cyclins (A, B, E, and D), which are timely expressed during the course of the cell cycle (23). CDK activity is also regulated by a diverse family of proteins termed CDK inhibitors (CKDi) that bind and inactivate CDK-cyclin complexes. Two classes of CKDi are known, 1 is the CIP/KIP family, which includes p21 (WAF1/Cip1), p27 (Kip1), and p57 (Kip2), and the other is the INK family, which includes p15 (INK4B), p16 (INK4A), p18 (INK4C), and p19 (INK4D) (24). A central role in the cell cycle progression is played by the retinoblastoma family of proteins, also called "pocket proteins" [retinoblastoma protein (pRb), p107, and p130], which are critical target substrates and are phosphorylated by the CDK-cyclin complexes. The phosphorylation of pocket proteins controls gene expression through the release of the E2F transcription factor, which can transactivate genes whose products are important for S-phase entry (25).

There is a general agreement that the control of differentiation is tightly coupled to proliferation and some important molecules involved in the control of cell cycle progression, such as p21<sup>WAF1/Cip1</sup> and p27<sup>Kip1</sup>, which are also implicated in the regulation of differentiation and apoptosis (26). It is therefore reasonable to assume that the cells that exit the cell cycle may undergo a differentiation process. The differentiation inducing ability of several compounds has been effectively measured in HL60 cells. In fact, these cells have been widely used as a model system from which to investigate the mechanism of leukocyte differentiation *in vitro* because they are able to differentiate into both monocytic and granulocytic lineage, depending on the stimulus used (27).

On these bases, therefore, we aimed to evaluate the effect of 3,4-DHPEA on the cycle progression and differentiation of HL60 cells and to determine whether 3,4-DHPEA is able to modify the expression of the main proteins involved in the regulation of the cell cycle, apoptosis, and differentiation.

## Materials and Methods

**Materials.** 3,4-DHPEA was prepared and used as previously described (28,22). Human promyelocytic leukemia cells (HL60), obtained from the American Type Culture Collection, were cultured in RPMI 1640 medium as previously described (22).

**Cell proliferation and apoptosis assays.** Cell proliferation was determined by measuring the incorporation of [<sup>3</sup>H]-thymidine into DNA. HL60 cells were seeded at a density of 0.25 × 10<sup>9</sup>/L in a culture medium on 24-well plates (Celbio S.r.l.) and incubated for 24 h in the presence of different concentrations of 3,4-DHPEA (25, 50, 75, and 100 μmol/L) at 37°C and 5% CO<sub>2</sub>. The cells were then treated with 1 mCi/L of [<sup>3</sup>H]-thymidine (Amersham) for 2 h, harvested by centrifugation (400 × g; 7 min) and washed 3 times with cold (4°C) PBS containing 10% of trichloroacetic acid. The cell pellet was then dissolved in 0.5 mL of a water solution containing 10% of trichloroacetic acid and 2 N perchloric acid and incubated at 60°C for 30 min. The cell extract was clarified by centrifugation, the supernatant was diluted with 5 mL of scintillation cocktail, and radioactivity was measured in a scintillation counter.

Apoptosis was determined by the release of nucleosomes into the cytoplasm after treatment of the HL60 cells with increasing concentration of 3,4-DHPEA for 24 h. The nucleosomes were measured using the "Cell Death Detection ELISA<sup>PLUS</sup>" (Roche Diagnostics GmbH), following the kit instruction manual. Alternatively, the percentage of apoptotic cells was determined by flow cytometry and fluorescence microscopy, as previously described (22).

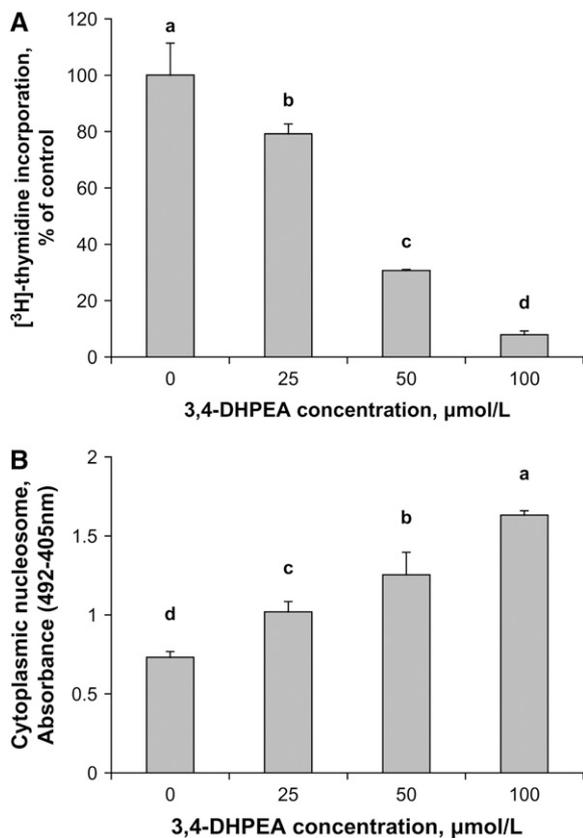
**Cell synchronization and cycle analysis.** HL60 cells were synchronized to the G<sub>1</sub>/S boundary by thymidine double block, as described by Kozaki et al. (29). Briefly, 6 h after seeding (0.25 × 10<sup>9</sup>/L), the cells were incubated with 2 mmol/L thymidine for 16 h, washed twice with fresh medium without thymidine, incubated for 8 h, and treated again with 2 mmol/L thymidine for another 16 h. The cells were then released from the cycle block by removal of thymidine and incubated in RPMI medium, with or without 100 μmol/L 3,4-DHPEA. At each established experimental time, the distribution of the cell cycle phases was determined by propidium iodide staining and flow cytometry, as previously described (22).

**Differentiation assay.** The differentiation of HL60 cells was evaluated by the nitroblue tetrazolium (NBT) reduction assay as previously described (20).

**Western blot analysis.** HL60 cells were washed twice with PBS and the cell pellet was suspended on ice in a lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP40, 0.5% sodium deoxycholate, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulphonyl fluoride, 1 mg/L each of aprotinin, leupeptin, and pepstatin, pH 7.5). After 30 min at 4°C, the extracts were centrifuged at 10,000 × g for 10 min, and the supernatants were used to determine the protein content by the Bio-Rad DC protein assay using bovine serum albumin as a standard. Samples containing 50 μg of total protein were resolved by a 12.5% SDS-PAGE, gel transferred onto a nitrocellulose membrane by electroblotting, and probed with anti-p15, anti-p21, anti-p27, anti-CDK6, anti-β actin (Cell Signaling Technology), anti-Cyclin D3, anti-cyclin B1, anti-cyclin A, and anti-cyclin E (Abcam) antibodies. After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, the blots were developed using enhanced chemiluminescence.

**RT-PCR.** Total RNA was isolated from 5–10 × 10<sup>6</sup> cells using the TRIzol reagent and digested with DNase I (Invitrogen S.R.L.), according to the manufacturer's recommended procedures. From each sample, 3–5 μg of RNA was reverse-transcribed into cDNA in a total volume of 20 μL, using a SuperScript II RNase H<sup>-</sup> Reverse Transcriptase, 1 mmol/L deoxyribonucleoside triphosphates and 250 ng of random primers (Invitrogen). An aliquot of cDNA (2 μL) was subjected to PCR in a total volume of 20 μL, using 2 U of Platinum Taq DNA polymerase, 2 mmol/L deoxyribonucleoside triphosphates, and 1 μmol/L of sense and antisense primers. The sequence of the primers was as follows: p21 sense 5'-TTAGGGCTTCCTCTTGAGAAGAT-3' and antisense 5'-ATGTCAG

<sup>2</sup> Abbreviations used: 3,4-DHPEA, hydroxytyrosol or 3,4-dihydroxyphenyl-ethanol; ATRA, all-trans retinoic acid; CDK, cyclin-dependent protein kinases; CKDi, CDK inhibitors; DMSO, dimethylsulfoxide; NBT, nitroblue tetrazolium; PMA, phorbol 12-myristate 13-acetate; pRb, retinoblastoma protein.



**FIGURE 1** Effect of increasing concentrations of 3,4-DHPEA on DNA synthesis assessed by the incorporation of [<sup>3</sup>H]-thymidine (A) and apoptosis evaluated by the release of cytoplasmic nucleosomes (B) of HL60 cells after treatment for 24 h. Values are means  $\pm$  SD,  $n = 3$ ; means without a common letter differ,  $P < 0.05$ .

AAC CGGCTGGGGATGTC-3'; p27 sense 5'-CCTCTTCGGCCCGGT GGAC-3' and antisense 5'-TTTGGGAACCGTCTGAAAC-3';  $\beta$ -actin sense 5'-AACAGAGGCATCCTCACCT-3' and antisense 5'-TACAT GGCTGGGGTGTGAA-3'. PCR cycles were as follows: 45 s at 94°C for denaturation, 45 s at 60°C ( $\beta$ -actin), and 55°C (p21, p27) for annealing, 1 min at 72°C for polymerization, 32–38 cycles. The PCR

products were analyzed by 1.8% agarose gel electrophoresis and visualized by ethidium bromide staining.

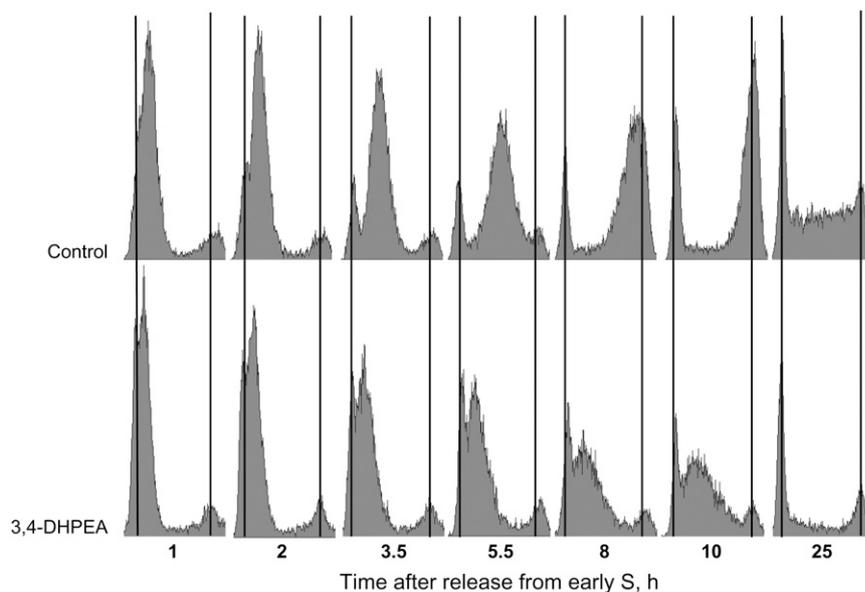
**Data analysis.** All tests were run in triplicate for each experimental condition and each experiment was repeated at least 3 times; the results are reported as the means  $\pm$  SD. Significant differences among treatments were assessed using a 1-way ANOVA. When a significant ( $P < 0.05$ ) treatment effect was detected, the mean values were compared using Tukey's post-hoc comparisons and Dunnett's test.

## Results

The treatment of HL60 cells for 24 h with 3,4-DHPEA dose-dependently reduced the DNA synthesis (Fig. 1A), resulting in a 92% inhibition of [<sup>3</sup>H]-thymidine incorporation at the highest concentration tested (100  $\mu\text{mol/L}$ ) and induced apoptosis in HL60 cells, as evidenced by the release of cytoplasmic nucleosomes (Fig. 1B).

On the basis of previous results, which showed that treatment of HL60 cells with 3,4-DHPEA caused an increase of cells in the  $G_0/G_1$  phase of the cycle (22), and considering that 3,4-DHPEA exhibited a strong inhibitory effect on DNA synthesis, further experiments were carried out to ascertain if 3,4-DHPEA could interfere with the  $G_1$ -S transition or simply affect the S progression. With this purpose, HL60 cells were synchronized in a  $G_1$ -S boundary by the thymidine double block and then released in fresh medium with or without 100  $\mu\text{mol/L}$  of 3,4-DHPEA. The progression of the cell cycle was followed for 25 h after release, and the percentage of cells in the different phases were analyzed by ethidium bromide staining and flow cytometry (Fig. 2). It was evident that the cells incubated in the control medium progressed quickly through the S-phase, which was completed within 8 h. In contrast, in the 3,4-DHPEA treated cells, the S-phase was dramatically prolonged, as evidenced by the significant increase in the percentage of cells in  $G_0/G_1$  and the decrease of those in the S-phase after 3.5 h from release (Table 1). This effect was further demonstrated by the reduced number of 3,4-DHPEA treated cells reaching the  $G_2/M$  phase and accumulating in the S-phase when compared with the control cells after 10 h from release (Table 1). After 25 h from release (Table 1), the control cells were similarly distributed between  $G_0/G_1$  and S-phases; in contrast, the 3,4-DHPEA treated cells

**FIGURE 2** Effect of 100  $\mu\text{mol/L}$  of 3,4-DHPEA on the cell cycle progression of synchronized HL60 cells. After synchronization, the cells were released either in fresh medium alone (Control) or in medium containing 3,4-DHPEA; the cell cycle was followed over time by flow cytometry.



**TABLE 1** Effect of 3,4-DHPEA (100  $\mu\text{mol/L}$ ) on cell cycle progression and apoptosis of synchronized HL60 cells<sup>1</sup>

Time after release, h	Treatment	G <sub>0</sub> /G <sub>1</sub>	S, %	G <sub>2</sub> /M	Apoptosis
3.5	Control	16.4 $\pm$ 4.3 <sup>c</sup>	68.2 $\pm$ 8.7 <sup>a</sup>	15.4 $\pm$ 5.5 <sup>b</sup>	5.7 $\pm$ 1.2 <sup>c</sup>
	3,4-DHPEA	41.7 $\pm$ 9.3 <sup>b</sup>	44.4 $\pm$ 11.7 <sup>b</sup>	13.9 $\pm$ 1.5 <sup>b</sup>	5.3 $\pm$ 0.6 <sup>c</sup>
10	Control	35.8 $\pm$ 9.0 <sup>b</sup>	10.6 $\pm$ 3.1 <sup>c</sup>	53.6 $\pm$ 8.4 <sup>a</sup>	5.7 $\pm$ 1.2 <sup>c</sup>
	3,4-DHPEA	32.1 $\pm$ 6.0 <sup>b</sup>	49.0 $\pm$ 7.8 <sup>b</sup>	18.9 $\pm$ 9.5 <sup>b</sup>	26.7 $\pm$ 1.5 <sup>b</sup>
25	Control	36.4 $\pm$ 7.6 <sup>b</sup>	39.2 $\pm$ 5.6 <sup>b</sup>	24.4 $\pm$ 5.9 <sup>b</sup>	6.1 $\pm$ 9.6 <sup>c</sup>
	3,4-DHPEA	55.8 $\pm$ 9.5 <sup>a</sup>	17.9 $\pm$ 8.6 <sup>c</sup>	26.3 $\pm$ 7.6 <sup>b</sup>	54.4 $\pm$ 11 <sup>a</sup>

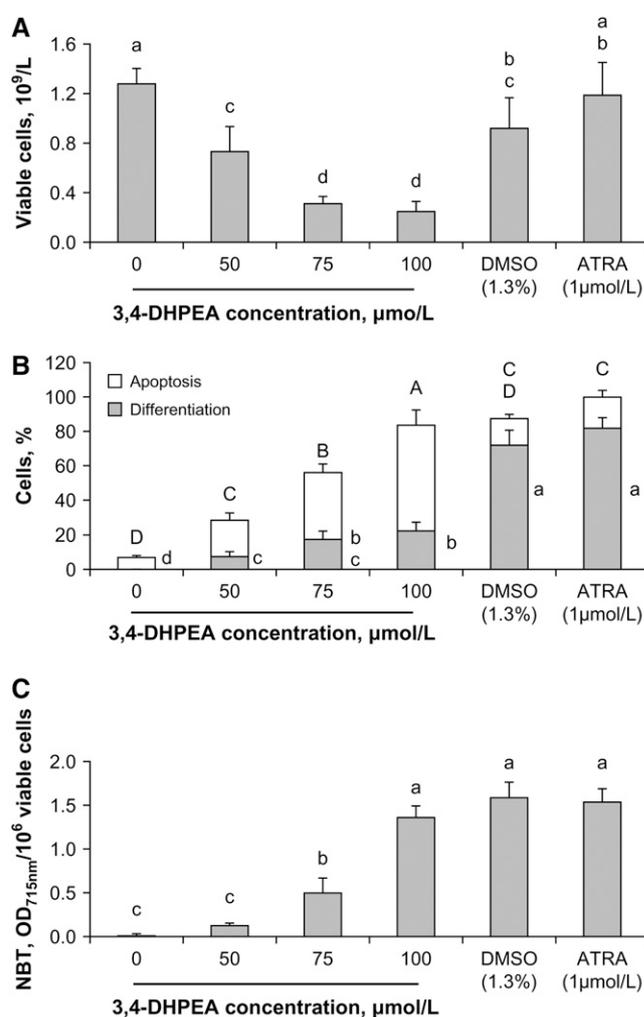
<sup>1</sup> Values are means  $\pm$  SD,  $n = 3$ . Means in a column without a common letter differ,  $P < 0.05$ .

accumulated in G<sub>0</sub>/G<sub>1</sub>, whereas the percentage of cells in the S-phase was clearly reduced ( $P < 0.05$ ). Furthermore, the percentage of apoptotic cells quantified by the fluorescence microscopy, which was very low after 3.5 h (Table 1), was increased by 3,4-DHPEA treatment at 10 and 25 h from release. These results clearly indicate that 3,4-DHPEA is able to inhibit both the progression of S-phase and the transition from G<sub>0</sub>/G<sub>1</sub> to S. In addition, they suggest that the cells in S-phase are more prone to undergo apoptosis than those in G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M, as demonstrated by the emptying of the S-phase and the compensatory increment of apoptosis.

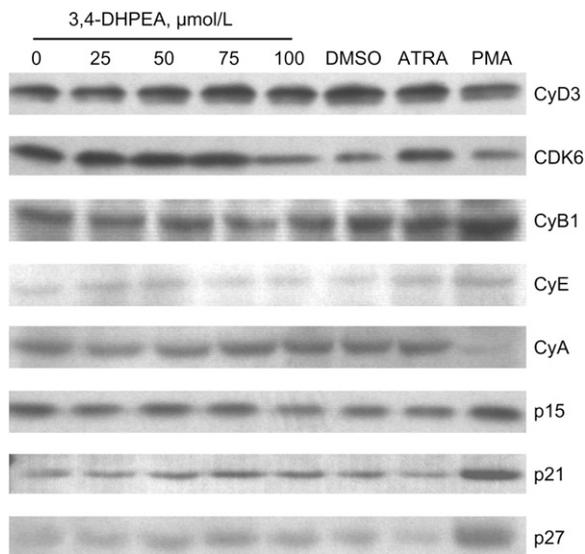
Because in many cases proliferation and cell cycle progression are tightly correlated to a terminal differentiation program, the effect of 3,4-DHPEA on the differentiation state of HL60 cells was also investigated. Both dimethylsulfoxide (DMSO) and all-trans retinoic acid (ATRA), 2 well-known compounds that are able to induce differentiation of HL60 cells toward the granulocytic lineage, were used as positive controls. The cells were treated with an increasing concentration of 3,4-DHPEA, DMSO, and ATRA, harvested after 72 h, counted for viability and apoptosis, and then subjected to the NBT reduction assay (Fig. 3). The most pronounced effect was exhibited by 3,4-DHPEA on both growth inhibition (Fig. 3A) and apoptosis induction (Fig. 3B), when compared with that obtained with DMSO and ATRA. On the contrary, DMSO and ATRA induced the highest number of differentiated cells compared with that induced by 3,4-DHPEA. It should be noted, however, that the 3,4-DHPEA affect on cell differentiation was statistically significant, particularly because in the control sample, no differentiated cells were found (Fig. 3B). The degree of differentiation was also quantified by extracting the intracellular insoluble formazan deposits derived from the reduction of NBT and measuring the optical density at 715 nm. The expression of the results as OD per  $1 \times 10^9$  of viable cells showed that 3,4-DHPEA at 100  $\mu\text{mol/L}$  induced a differentiation grade similar to that caused by DMSO and ATRA (Fig. 3C).

To investigate the molecular mechanisms by which 3,4-DHPEA may alter the cell cycle progression and the differentiation state of HL60 cells, the expression of cyclins, CDK6 and CDKi, was quantified by Western blot analysis. The cell extracts were obtained after 24 h of treatment with increasing concentrations of 3,4-DHPEA, DMSO, and ATRA; in addition, another positive control represented by phorbol 12-myristate 13-acetate (PMA) was included in these experiments, because PMA is well known to induce marked effects on the expression of the above reported proteins. Treatment of cells with 3,4-DHPEA did not significantly alter the expression of cyclins E, B1, and A, although cyclin D3 was increased (180  $\pm$  26% of control,  $P < 0.05$ ) at 75  $\mu\text{mol/L}$  (Fig. 4). A similar effect was also induced by DMSO and ATRA (185  $\pm$  18% and 170  $\pm$  22%

of control, respectively,  $P < 0.05$ ), but not by PMA (Fig. 4). Instead, PMA induced an increment of cyclin B1 (272  $\pm$  36% of control,  $P < 0.05$ ) and the reduction of cyclin A (23  $\pm$  16% of control,  $P < 0.05$ ). Similarly to DMSO and PMA, 3,4-DHPEA



**FIGURE 3** Effect of increasing concentrations of 3,4-DHPEA, DMSO (1.3%), and ATRA (1  $\mu\text{mol/L}$ ) on proliferation (A), apoptosis and differentiation (B), and differentiation (C) of HL60 cells after exposure for 72 h. Differentiation was quantified either by counting the cells after the NBT reduction assay (B) or by dissolving the black-blue formazan deposits and measuring the optical density at 715 nm (C). Values are means  $\pm$  SD,  $n = 5$ . Means without a common letter differ,  $P < 0.05$ ; in B, the capital letters refer to apoptosis and the small letters refer to differentiation.



**FIGURE 4** Effect of increasing concentrations of 3,4-DHPEA, DMSO (1.3%), ATRA (1  $\mu\text{mol/L}$ ), and PMA (0.2  $\mu\text{mol/L}$ ) on the expression of the cell cycle-related proteins of HL60 cells after exposure for 24 h, then analyzed by Western blotting. Figure shows the results of 1 experiment, which is representative of the 3 that were performed that produced similar results.

reduced the amount of CDK6 expression ( $45 \pm 6\%$  of control,  $P < 0.05$ ) at 100  $\mu\text{mol/L}$ . Regarding CDKi, p15 was not significantly modified by all treatments, whereas p21 and p27 were induced by 3,4-DHPEA with a maximum effect at 75  $\mu\text{mol/L}$  ( $190 \pm 32\%$  and  $233 \pm 38$  of control, respectively,  $P < 0.05$ ). PMA had a similar enhancing activity (Fig. 4). In agreement with the protein level, the p21 and p27 mRNA levels also increased after treatment with increasing doses of 3,4-DHPEA, reaching a maximum effect at 75  $\mu\text{mol/L}$  and 50  $\mu\text{mol/L}$ ,

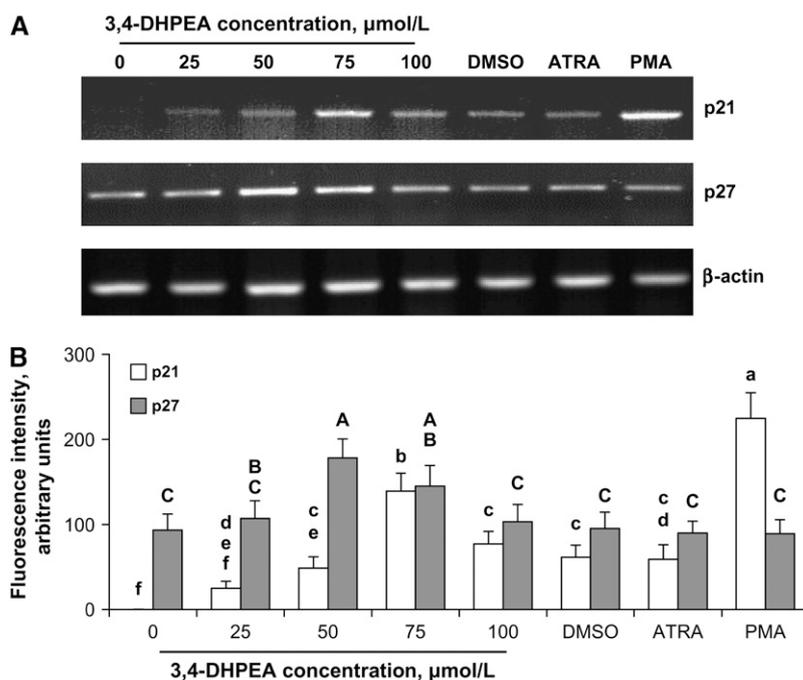
respectively (Fig. 5A and 5B). It is interesting to note that, under these experimental conditions, the mRNA for p21 in control cells was not detected (Fig. 5A). In addition, the mRNA for p27 was not altered by all the other inducers used (Fig. 5B).

## Discussion

In this study, our main finding was that 3,4-DHPEA is able to induce differentiation on a human tumor cell line. Similar to 3,4-DHPEA, other phenolic compounds isolated from tea and wine have been found to be able to induce differentiation on both promyelocytic cell lines (30,31) and other cellular systems (32). A recent study also showed that a complex mixture of phenol compounds isolated from olive oil can induce differentiation on HL60 cells (20). Although the NBT reduction assay used in this study could not discriminate granulocyte from monocyte, the observation that the cells did not form aggregates and did not adhere to the flask suggests that 3,4-DHPEA treatment induced differentiation toward the granulocytic lineage. This property is common to 2 well-known differentiating agents, DMSO and ATRA (used as positive controls), whereas PMA induces differentiation toward the macrophage lineage (27).

The results obtained on the synchronized HL60 cell cycle are of particular interest because they indicated that the cells in the S-phase are more susceptible to undergoing 3,4-DHPEA induced apoptosis than those in  $G_2/M$  and  $G_0/G_1$ . This evidence suggests that cells that do not actively proliferate and that remain in the  $G_0$  resting state should be more resistant to the 3,4-DHPEA induced apoptosis. This hypothesis is supported by previous findings, which showed that, in nonproliferating lymphocytes, 3,4-DHPEA is not able to induce apoptosis (22). It has recently been found that 3,4-DHPEA causes growth arrest and apoptosis in human colon carcinoma HT-29 cells (33). In this case, the effects were evident at higher concentrations of 3,4-DHPEA (200–400  $\mu\text{mol/L}$ ), and the growth arrest was associated with the accumulation of cells in S and  $G_2/M$  phases of the cell cycle (33). These differences may be attributed to the different cell

**FIGURE 5** Effect of increasing concentrations of 3,4-DHPEA, DMSO (1.3%), ATRA (1  $\mu\text{mol/L}$ ), and PMA (0.2  $\mu\text{mol/L}$ ) on p21<sup>WAF1/Cip1</sup> and p27<sup>Kip1</sup> mRNA expression of HL60 cells after exposure for 24 h, then analyzed by RT-PCR. The figure shows the agarose gel electrophoresis of 1 experiment (A) and the densitometric analysis of 3 gels (B) expressed as means  $\pm$  SD. Means without a common letter differ,  $P < 0.05$ ; in B, the capital letters refer to p27 and the small letters refer to p21.



types and consequently to different mechanisms that may be involved. In HT-29 cells, it was shown that 3,4-DHPEA interferes with several signaling pathways that control proliferation and apoptosis, and that these effects were mediated by activation of a specific serine/threonine protein phosphatase PP2A (33). The hypothesis that a similar activation of PP2A can be responsible for the effects induced by 3,4-DHPEA on HL60 cells remains to be determined.

The molecular mechanisms by which 3,4-DHPEA interferes with the cell cycle and induces apoptosis and differentiation on HL60 cells are not known; however, we show here that 3,4-DHPEA is able to modify the expression of important proteins involved in the regulation of these processes. The cell cycle is regulated by the sequential expression of cyclins, which activate various CDK, and by the selective induction of different CDKi (23,24). Although in this study, we did not show proof of relevant alterations in the expression of some cyclins (A, B1, and E) and CDKi p15, we showed that 3,4-DHPEA induced an increment of cyclin D3, CDKi p21<sup>WAF1/Cip1</sup> and p27Kip1, and a decrease of CDK6. All these effects could be involved in the inhibition of the G<sub>1</sub>-S transition. By binding to CDK6, cyclin D3 activates the kinase activity, which through phosphorylation of pRb induces the release of the transcription factor E2F that controls the expression of genes implicated in the DNA synthesis and the G<sub>1</sub>-S transition. This process could be partially compromised by the 3,4-DHPEA treatment by means of the reduction of CDK6 expression and the upregulation of the CDKi p21<sup>WAF1/Cip1</sup> and p27Kip1. Both these proteins are able to bind to cyclin D/CDK6 complex and inhibit the pRb phosphorylation (23,24).

In addition, p21<sup>WAF1/Cip1</sup> and p27Kip1 were implicated in the control of differentiation and apoptosis of HL60 cells. It was shown that overexpression of p21<sup>WAF1/Cip1</sup> and p27Kip1 accelerated both the monocytic and granulocytic differentiation of HL60 cells triggered by PMA and DMSO, respectively (34). Interestingly, it was also found that overexpression of p21<sup>WAF1/Cip1</sup> and p27Kip1 induces an increase of the level of cyclin D3 (34). Further evidence supporting the involvement of these CDKi in differentiation were recently obtained by antisense RNA experiments that showed that a reduction of p21<sup>WAF1/Cip1</sup> and p27Kip1 expression impaired the terminal differentiation of HL60 cells (35). From these findings, it can be hypothesized that the differentiating activity of 3,4-DHPEA on HL60 cells may be mediated by the upregulation of these CDKi. The effect of 3,4-DHPEA on the transcription of the gene coding for p21<sup>WAF1/Cip1</sup> was particularly striking considering that in the control cells the mRNA for p21<sup>WAF1/Cip1</sup> was not detected, probably because it was below the detection limit of our method; although in the 3,4-DHPEA treated cells, it was clearly evident. In many cases, the induction of p21<sup>WAF1/Cip1</sup> required the functional p53 tumor suppressor protein that is activated by DNA damage (36). However, in HL60 cells, the gene encoding for p53 brings various deletions that cause the lack of its expression (37). Therefore, the observed increase of p21<sup>WAF1/Cip1</sup> mRNA indicates that 3,4-DHPEA is able to activate a p53-independent pathway for p21<sup>WAF1/Cip1</sup> expression in HL60 cells, as reported for other agents (38).

Regardless of the mechanisms involved, the results of this study are of biological significance in a nutritional context because the dose of 3,4-DHPEA that was used in this study could be reached in vivo, as demonstrated by a recent intervention study that showed that a single ingestion of 40 mL of olive oil containing a modest amount of phenols (366 mg/kg) resulted in a plasma concentration of 3,4-DHPEA of close to 20  $\mu$ mol/L (39).

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