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Biochemical mechanisms underlying the pro-apoptotic activity of 7,8-dihydroxy-4-methylcoumarin in human leukemic cells

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ABSTRACT

The search for new drugs requires a deep understanding of the molecular basis of drug action, being necessary the elucidation of the mechanism of action with the understanding of the relationship between structure and activity. In the present study, we evaluated the pro-apoptotic activity of 7,8-dihydroxy-4-methylcoumarin (DHMC) and its underlying mechanisms in human leukemic cells. Here, we present evidence that DHMC induced selective and concentration-dependent apoptosis in human leukemic cells. The pro-apoptotic effect of DHMC was mediated by activation of the JNKs and inhibition of the ERK1/2 and PI3K/Akt pathways, with no participation of the p38 cascade after 24 h of treatment. Indeed, down-regulation of the proto-oncogene c-myc as well as induction of the cell cycle inhibitor $p21^{WAF1/CIP1}$ through a p53 independent mechanism were observed in U-937 cells. These findings suggest that DHCM may have a potential therapeutic role in the future treatment of hematological malignancies.

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1. Introduction

In leukemias early hematopoietic progenitors fail to respond to external stimuli within the bone marrow microenvironment. They do not undergo programmed cell death (apoptosis) and fail to differentiate into discrete types of blood cells, acquire immortality, self-perpetuate at the expense of other cells, and then spill into the peripheral blood [1]. Despite the numerous progresses achieved in the treatment of leukemias over the last years, many problems as cellular heterogeneity, development of multidrug-resistance, heterogeneous molecular abnormalities, and lack of selective action of antineoplastic agents still remain [2]. Nowadays, a variety of anticancer drugs including ara-C, cisplatin, etoposide, and taxol which interact with diverse intracellular molecular targets have been shown to engage the final common pathway

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Abbreviations: DHMC, 7,8-dihydroxy-4-methylcoumarin; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; TBE buffer, tris-borate–EDTA; NAC, N-acetyl-L-cysteine; IC_{50}, inhibitory proliferation concentration 50; CC_{50}, cytotoxic concentration 50; HO, Hoechst 33342; PI, propidium iodide.

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of programmed cell death, resulting in the apoptosis of human leukemic cells [3–5]. These conventional therapies cause serious side effects and, at best, merely extend the patient's lifespan by a few years, supporting the necessity to develop alternative therapeutic strategies. In our effort to discover and develop new potential and selective anti-leukemic compounds, we continue our studies using coumarins as leader molecules.

Coumarins are a group of heterocyclic compounds synthesized by numerous green plant species as well as by some bacteria and fungi [6], that have been widely used as a fragrance in food and cosmetic products [7]. Numerous biological activities have been attributed to simple coumarins and analogues as anti-coagulant [8], anti-inflammatory [9,10] anti-viral [11] and antimicrobial effects [12,13], enzyme inhibition [14,15], scavenging of reactive oxygen species [16,17] and central nervous system actions [18].

Different natural and synthetic coumarins are of great interest due to their described anti-tumoral properties in sensitive neoplasm. Several authors reported that numerous nitrated, hydroxylated or isopentenylated coumarins and derived pyrano- and furanocoumarins show strong anti-proliferative activity and induce apoptosis in various cancer cell lines [19–22]. The hydroxylated coumarin, esculetin, exhibits anti-tumoral effects by inhibiting cell cycle progression and inducing programmed cell death in HL-60 cells [23,24]. Lopez-Gonzalez et al. [25] showed that coumarin and its metabolite 7-hydroxycoumarin have cytostatic and pro-apoptotic actions in non-small cell lung carcinoma. On the other hand, several coumarins show synergistic effects with retinoic acid on the differentiation of human leukemic HL-60 cells [26]. Furthermore, daphnetin behaves as a potent anti-proliferative and differentiation agent in human renal cell carcinoma [27]. We have previously reported that a group of natural coumarins exhibit anti-proliferative and differentiation activity in the promonocytic U-937 cell line [28] and more recently we have observed that hydroxylated coumarins induce apoptosis in human leukemic cells (unpublished observations). These studies strongly support the potential therapeutic applications of coumarins, making these molecules attractive for their further evaluation as novel therapeutic agents for cancer treatment.

However, the anti-tumor mechanisms of coumarins are not well understood. Several researchers have investigated different intracellular events in an attempt to elucidate the mechanism of action of active coumarins in cancer cells. Cellular events as modulation of mitogen-activated protein kinases (MAPKs) [29–31], inhibition of protein kinases including EGF receptor tyrosine kinase, PKA and PKC [32] or topoisomerase II activity [30] have been evaluated. Chu et al. [23] reported that esculetin induces apoptosis by the mitochondrial pathway in the HL-60 cell line. Moreover, in the last years it has been proposed that the pro-oxidant action of plant derived phenolics rather than their antioxidant action may be an important mechanism for their apoptosis-inducing properties in cancer cells [33]. In scopoletin-induced apoptosis in HL-60 cells it has been suggested that the generation of reactive oxygen species may contribute to the induction of cell death [34].

In the present study, we focused on the effect of DHMC on the major stress signaling pathways: the mitogen-activated protein kinases, phosphoinositide-3-kinase/Akt signaling cascade (PI3K/Akt) and c-myc proto-oncogene, which are intimately involved in the regulation of cell growth, differentiation and apoptosis in hematopoietic cells.

In this work, we present evidence that DHMC induced selective apoptosis in human leukemic cells mediated by activation of the JNKs pathway and inhibition of the ERK1/2 pathway, with no participation of the p38 cascade after 24 h of treatment. Cell exposed to DHMC for 18 h showed inhibition of the PI3K/Akt pathway, an important survival pathway in leukemic cells. Furthermore, down-regulation of c-myc proto-oncogene and induction of the cell cycle inhibitor $p21^{WAF1/CIP1}$ in a p53 independent mechanism was also observed.

2. Materials and methods

2.1. Reagents and antibodies

Cell culture medium, antibiotics, bovine serum albumin (BSA), phosphate-buffered saline (PBS), dibutyryl cyclic adenosine monophosphate (dbcAMP), N-acetyl-L-cystein (NAC), dimethyl sulfoxide (DMSO), proteinase K and 4β-phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Company (St. Louis, MO). Fetal calf serum was purchased from Natocor (Argentina). LY294002 was from Tocris Cookson (Ellisville, MO). All other chemicals used were of analytical grade. Anti-c-Myc, -phospho-Akt1/2/3, -ERK1, -caspase 3, -PARP, -actin, rabbit antibodies; anti-p21, -phospho-p38, -phospho-ERK1/2, -phospho-JNK, -Akt1, -p38, -JNK1 mouse antibodies and anti-CD88 goat antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

7,8-Dihydroxy-4-methylcoumarin (DHMC) was synthesized through the Pechmann reaction between a properly substituted phenol and ethyl acetoacetate. DHMC was dissolved in 0.01% (v/v) DMSO and stored at −20 °C.

2.2. Cell culture

The human promonocytic U-937 cell line and the human promyelocytic HL-60 cells (American Type Culture Collection, Rockville, MD) were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium, supplemented with 10% fetal calf serum and 50 μg/ml gentamicin. The cell suspension was split at the third day and diluted 1 day before experimental procedures.

Peripheral blood mononuclear cells from heparinized samples of healthy donors isolated by centrifugation on Ficoll-Hypaque were provided by Dr. Mónica Vermeulen (IIHEMA; Academia Nacional de Medicina, Buenos Aires, Argentina). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium, supplemented with 10% fetal calf serum and 50 μg/ml gentamicin.

2.3. Assessment of cell viability

Cell viability was monitored by the trypan blue exclusion assay. Cells growing in exponential phase were seeded at 10^5 cells in 1 ml of RPMI 1640 in a 24 well culture plate and incubated in a 5% CO_2 atmosphere. Cells were exposed to

different concentrations of DHMC (0.15 μM–2.0 mM) or 0.0001% (v/v) DMSO (control group). After incubation for 48 h, an aliquot of each treatment was collected and mixed with an equal volume of 0.4% trypan blue and further incubated for 5 min after which the number of viable cells was estimated in a hemocytometer chamber. Cytotoxic concentration 50 (CC_{50}) values were calculated by the equation for sigmoidal dose response using Prism 4.00 for Windows (GraphPad Software, San Diego, CA). Assays were carried out by triplicate in at least three independent experiments.

2.4. Cell growth experiments

Cells growing in exponential phase were seeded at 10^4 cells in 150 μl of RPMI 1640 in a 96 well culture plate and incubated in a 5% CO_2 atmosphere. U-937 cells were exposed to different concentrations of DHMC ranging from 0.15 μM to 2.0 mM or to 0.0001% (v/v) DMSO (control group) for 48 h and followed by incubation in the presence of 0.5 μCi of [^{3}H] methyl-thymidine (Specific Activity 740.0 GBq/mmol) (Perkin-Elmer, USA), added 12 h before the end of the experiment. Cells were then harvested in an automatic cell harvester (Nunc, Maryland, USA). The incorporation of the radioactive nucleotide was measured in a Pharmacia Wallac 1410 liquid scintillation counter and expressed as incorporation percentage respect to the control group (cells treated with DMSO). Inhibitory proliferation concentration 50 (IC_{50}) values were calculated with the equation for sigmoidal dose response using Prism 4.00 for Windows (GraphPad Software, San Diego, CA). Assays were performed by quadruplicate in at least four independent experiments. On the other hand, the number of cells was determined using a cellular meter Coulter Z-1. Briefly, 10^5 cells/ml were seeded in 24 wells plates and treated with different concentrations of DHMC, 400 μM dbcAMP (positive control), or 0.0001% (v/v) DMSO (control group), for 3 days. Cells were then collected at different times according to each experiment and the number of cells was determined by a cellular meter. Cell density in culture did not exceed 1.5×10^6 cells/ml.

2.5. Detection of DNA fragmentation by gel electrophoresis

DNA from untreated or coumarin-treated cells was analyzed for endonucleolytic DNA damage, using horizontal agarose gel electrophoresis. Treated or untreated cells (3×10^6 cells) incubated for 24 h were collected, washed with PBS and lysed in a buffer containing 100 mM NaCl, 4 mM EDTA, 50 mM Tris–HCl, 0.5% SDS pH 8, and proteinase K (100 mg/ml) at 55 °C for 2 h. DNA was then extracted and purified as described by Parborell et al. [35]. Electrophoresis was performed for 2.30 h at 40 V in 1.8% agarose gels containing ethidium bromide at a final concentration of 0.1 mg/ml in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA at pH 8.0). DNA banding was visualized with an UV transilluminator.

2.6. Evaluation of morphological changes

Cells growing in exponential phase were seeded at 10^5 cells in 2 ml of RPMI 1640 in a 24 well culture plate and incubated in a 5% CO_2 atmosphere. Cells were exposed to different DHMC concentrations (0.15 μM–2.5 mM) or 0.0001% (v/v) DMSO (control group). The morphology of treated and untreated cells was examined following incubation for 24 or 48 h. Cells (10^5) were suspended in 100 μl PBS containing 2 μg/ml Hoechst 33342 nuclear dye (HO; Sigma, St. Louis, USA) and 12 μg/ml propidium iodide (PI; Sigma, St. Louis, USA). After incubation at 37 °C for 8 min, cells were observed in a fluorescence microscope (Zeiss, Germany) under UV light at 430 nm (HO) and 630 nm (PI). The number of apoptotic or necrotic cells was estimated by using a hemocytometer chamber. Ten randomly selected fields were acquired from each treatment and one hundred cells were minimally counted. Assays were carried out by triplicate in at least three independent experiments.

2.7. Determination of U-937 cell differentiation markers

2.7.1. Nitrobluetetrazolium differentiation assay

U-937 cells (2×10^5/ml) were exposed for 48 h to different concentrations of DHMC, 1 μM ATRA (positive control) or 0.0001% (v/v) DMSO (control group). Cells were then washed and resuspended in 200 μl RPMI 1640 media containing 1 mg/ml NBT and 1 μg/ml PMA. After incubation at 37 °C for 30 min, cells were pelleted and dissolved in 200 μl DMSO and absorbance determined at 570 nm.

2.7.2. Surface myeloid CD11b antigen assay

The expression of CD11b was detected by direct inmunofluorescence staining. U-937 cells (2×10^5 cells/ml) were treated with different concentrations of DHMC, 400 μM dbcAMP (positive control) or 0.0001% (v/v) DMSO (control group) for 48 h. Treated and control cells were washed twice in PBS and incubated with a saturated concentration of phycoerythrin anti-CD11b antibody (Coulter-Immunotech, France) or an equivalent concentration of isotype-matched control at 4 °C for 30 min. Cells were washed twice with PBS supplemented with 1% FCS and immediately analyzed in a FACScan flow cytometer (Becton-Dickinson, CA, USA). For each sample, a minimum of 5000 events were acquired. Percentages of positive cells between specific CD-immunolabelled cells and their negative controls were established for both untreated and treated cells.

2.7.3. Expression of the C5a receptor (CD88) assay

CD88 expression was evaluated by Western blot analysis using an anti-CD88 goat antibody as detailed below.

2.8. Western blots

For Western blot assays cells were lysed in 50 mM Tris–HCl pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol, 10% glycerol and 0.05% bromophenol blue and sonicated to shear DNA. Total cell lysates were resolved by SDS-PAGE, immunoblotted with the indicated primary antibodies, followed by horseradish peroxidase conjugated anti-rabbit, anti-mouse or anti-goat IgG (Santa Cruz Biotechnology, CA), and developed by enhanced chemiluminesence (ECL) following the manufacturer's instructions (Amersham Life Science, England). Band intensity was quantified by densitometry using Scion Image (Scion Corporation, Frederick, MD) software.

2.9. Statistical analysis

Results are expressed as the mean ± S.E.M. of at least three independent experiments. Statistical analysis was performed by one-way ANOVA followed by the Student–Newman–Keuls test. A p of 0.05 or less was considered statistically significant.

3. Results

3.1. 7,8-Dihydroxy-4-methylcoumarin caused growth inhibition and apoptotic cell death in leukemic cells but not in normal peripheral blood mononuclear cells

We have recently compared the pro-apoptotic activity of diverse structurally related coumarins derivatives by performing a structure–activity relationship study, where we describe the presence of two adjacent phenolic hydroxyl groups in the coumarin nucleus as the most important factor involved in the pro-apoptotic activity in leukemic cells (unpublished observations). In order to gain insight into the mechanism of action in the present work we studied the effect of DHMC on leukemic cells, as leader molecule of this type of coumarins.

U-937 cell growth, assessed by [^{3}H] thymidine incorporation, was inhibited by DHMC in a concentration-dependent manner. The inhibitory concentration 50 (IC_{50}) value was 48.65 ± 1.50 μM whereas the cytotoxic concentration 50 (CC_{50}) was 165.40 ± 1.80 μM, determined by the trypan blue dye exclusion assay.

To determine whether DHMC-evoked decreased viability in U-937 cells at cytotoxic micromolar concentrations resulted from the induction of apoptosis, different assays were performed. After 24 h of treatment, DHMC-treated U-937 cells exhibited internucleosomal DNA fragmentation in a concentration-dependent manner (Fig. 1A). Furthermore, DNA fragmentation was not observed at 6 h but it was evident at 24 h supporting that the induction of DNA ladders was time-dependent (Fig. 1B). Apoptotic and necrotic cells were evaluated by chromatin condensation with Hoechst 33342 (HO) and by nuclear staining with propidium iodide (PI). In cell incubated with concentrations of DHMC lower than 500 μM apoptosis was the prevailing form of cell death whereas necrosis was negligible (Fig. 2). U-937 cells showed intense staining, cell shrinkage, chromatin condensation (pyknosis) and nuclear segmentation, typical morphological changes associated with the apoptotic process (Fig. 2, inset). However, when cells were exposed to higher concentrations of DHMC the percentage of necrotic cells increased (Fig. 2). These findings suggest that DHMC induces apoptosis and/or secondary necrosis depending on the concentration.

Caspase 3 is a key enzyme involved in programmed cell death. Fig. 3 shows that cells incubated with DHMC exhibited at 24 h a decrease in inactive pro-caspase 3 and an increase in caspase 3. Proteolytic cleavage of the 116 kDa, poly (ADP-ribose)-polymerase protein (PARP) results in the separation of the N-terminal binding domain (24 kDa) from its C-terminal catalytic domain (89 kDa). Fig. 3 shows that cleaved PARP (89 kDa) increased at 3 h. These results clearly indicate that DHMC-induced apoptosis in U-937 cells through the activation of the caspase pathway.

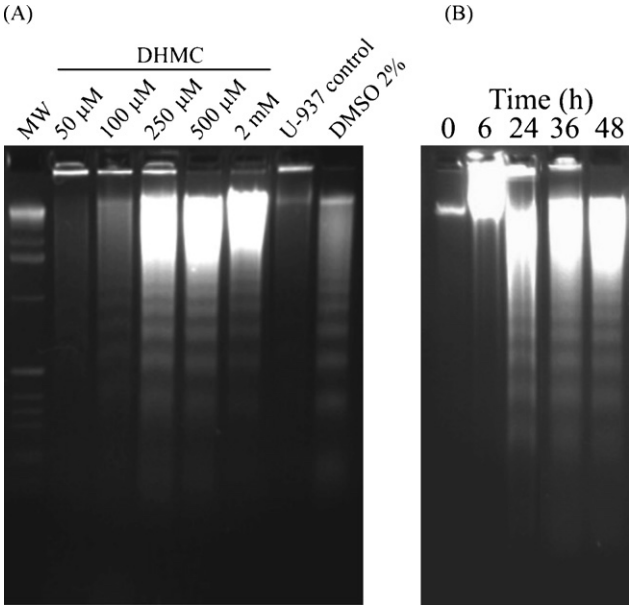

Fig. 1 – Effect of DHMC on the induction of internucleosomal fragmentation. (A) Cells were incubated with increasing concentrations of DHMC or 2% dimethylsulfoxide (positive control) for 24 h and the genomic DNA from treated and untreated cells was then subjected to agarose gel electrophoresis. Similar results were obtained in three separate experiments. (B) U-937 cells were treated with 250 μM DHMC for different time periods. After treatment, the cells were harvested and the isolated DNA was analyzed by agarose gel electrophoresis. Data are representative of at least three independent experiments.

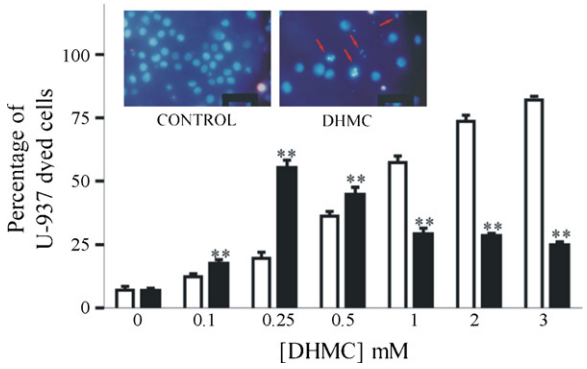

Fig. 2 – Effect of DHMC concentrations on the type of cell death in U-937 cells. Cells were incubated with increasing concentrations of DHMC for 24 h. Necrosis was assessed by the staining of nuclei with PI (□), whereas apoptosis was visualized by the appropriate changes of nuclei stained with Hoechst 33342 (■). Cells were observed in a fluorescence microscope under UV light at 430 nm (HO) and 630 nm (PI). Values are mean ± S.E.M. (n = 3); $^{**}p < 0.01$ vs. necrosis. Inset: morphological changes in U-937 cells associated with the apoptotic process induced by 250 μM DHMC for 24 h, visualized by the blue staining with Hoechst 33342. Arrows represent condensed chromatin, fragment nuclei and apoptotic bodies. Each picture is representative of three independent experiments.

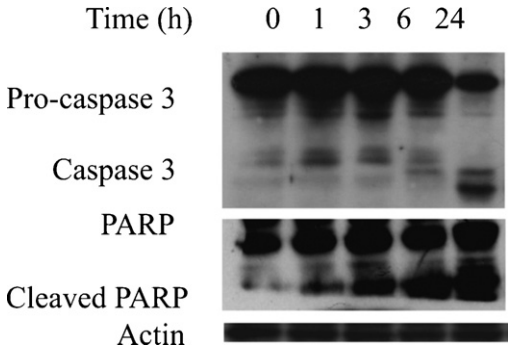

Fig. 3 – Effect of DHMC on the cleavage of pro-caspase 3 and PARP. Cells were treated with 250 μM DHMC and harvested at the indicated times. Equal amount of protein were subjected to SDS-PAGE and analyzed by Western blot with anti-caspase 3, PARP and actin antibodies. Data are representative of at least three independent experiments.

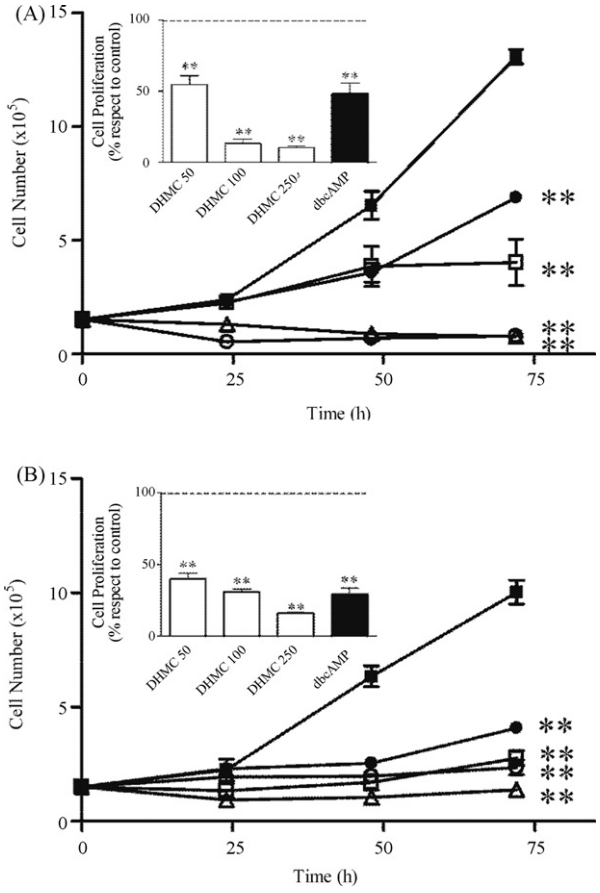

Fig. 4 – Effect of DHMC on cell proliferation in U-937 (A) and HL-60 (B) cell lines. Cells were treated with DHMC [50 μM (●), 100 μM (○), 250 μM (△) μM)], 400 μM dbcAMP (□) (positive control), or 0.0001% (v/v) DMSO (control group) (■) for 24, 48 and 72 h, collected and the cell number determined by a cellular meter Coulter Z-1. Results are the mean ± S.E.M. ($n = 3$); $^{**}p < 0.01$ vs. control group. Inset. U-937 (A) or HL-60 (B) cell proliferation following 48 h DHMC treatment. Data are expressed as proliferation percentage respect to control group. Control group proliferation considered 100% is showed as a dotted line. Results represent the mean ± S.E.M. ($n = 3$); $^{**}p < 0.01$ vs. control group.

In order to gain insight into the effect of DHMC on leukemic cells, DHMC-induced apoptosis was evaluated in the human promyelocytic cell line HL-60. In this cell line, the CC_{50} of DHMC was 145.70 ± 1.00 μM, whereas the IC_{50} was 27.46 ± 1.26 μM. The hydroxycoumarin-induced time-dependent growth inhibition in HL-60 cells as observed in U-937 cells (Fig. 4). To determine whether DHMC-induced apoptosis at micromolar concentrations in HL-60 cells as in U-937 cells, different assays were carried out. HL-60 cells were treated with different concentrations of DHMC for 24 h and the genomic DNA was then subjected to agarose gel electrophoresis. A ladder-like pattern of DNA fragments was clearly observed at 250 μM DHMC (Fig. 5A). As in U-937 cells, 250 μM DHMC increased the number of apoptotic cells and concentrations higher than 1 mM increased the percentage of necrotic cells (Fig. 5B). At 250 μM DHMC most HL-60 cells depicted morphological changes typically associated with the apoptotic process as condensed chromatin, nuclear fragmentation and typical apoptotic bodies (Fig. 5B, inset).

To assess the cell specificity of DHMC, we compared its cytotoxicity in U-937, HL-60 and normal peripheral blood mononuclear cells. Results showed that DHMC (250 μM) evoked a significant less cytotoxic effect in normal mononuclear cells after 24 h treatment than in leukemic cells (Fig. 6). Cytotoxic values of DHMC treatment in normal cells were 31.6 ± 5.9% whereas in U-937 and HL-60 were 86.3 ± 3.1 and 85.6 ± 5.9%, respectively. These data indicate that normal cells were more resistant to DHMC treatment than human leukemic cells.

The cellular morphological changes, DNA fragmentation, caspase activation and PARP cleavage observed in DHMC-treated U-937 or HL-60 are hallmarks of cell death through apoptosis. These findings support that DHMC, selectively reduces the viability of leukemic cells through the induction of apoptosis.

3.2. *7,8-Dihydroxy-4-methylcoumarin failed to induce cell differentiation in U-937 cells*

In addition to the conventional biological maturation inducers such as retinoic acid or vitamin D_3, various cytotoxic agents also trigger leukemic cell differentiation at low concentrations [36]. Finn et al. [27] reported that treatment with daphnetin (7,8-dihydroxycoumarin) at 10 and 50 μM, induces human renal carcinoma cell differentiation. Therefore, we evaluated whether low concentrations of DHMC (5–50 μM) triggered differentiation in leukemic cells. Differentiation–induction as a therapeutic strategy has the greatest impact on hematopoietic malignancies, most notably on leukemia. The U-937 cell line derived from a histiocytic lymphoma is an appropriated model to study the mechanism of cell differentiation [37,38]. In this cell line, dibutyryl cAMP (dbcAMP) as all trans retinoic acid (ATRA) induce monocyte maturation [37,38] and it can be monitored by changes of morphological, biochemical, and immunological properties.

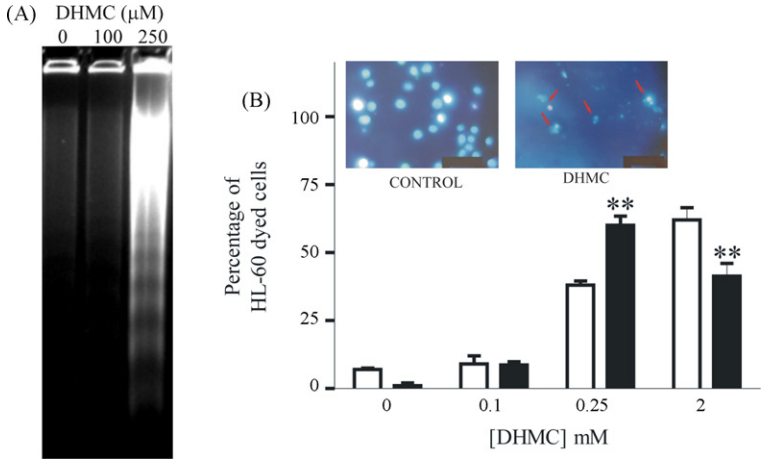

Fig. 5 – (A) Detection of internucleosomal DNA fragmentation in HL-60 cells. Cells were treated with different concentrations of DHMC for 24 h. Cells were then harvested and the isolated DNA analyzed by agarose gel electrophoresis. Data are representative of at least three independent experiments. (B) Effect of DHMC concentration on cell death in HL-60 cells. Cells were incubated with increasing concentrations of DHMC for 24 h. Necrosis was assessed by the staining of nuclei with PI (□), whereas apoptosis was visualized by the appropriate changes of nuclei stained with Hoechst 33342 (■). Cells were observed in a fluorescence microscope under UV light at 430 nm (HO) and 630 nm (PI). Values are mean ± S.E.M. ($n = 3$); $^{**}p < 0.01$ vs. necrosis. Inset: morphological changes in HL-60 cells associated with the apoptotic process induced by 250 μM DHMC for 24 h, visualized by the blue staining with Hoechst 33342. Arrows represent condensed chromatin, fragment nuclei and apoptotic bodies. Each picture is representative of three independent experiments.

As shown in Table 1, pretreatment of cells with non-toxic concentrations of DHMC (5–50 μM) for 48 h failed to induce U-937 cell differentiation to monocytes. In U-937-treated cells NBT reducing activity as well as CD11b and C5a receptor (CD88) expression were similar to control cells (DMSO-treated cells). These findings show that exposure of leukemic cells to low concentrations of DHMC failed to induce cell maturation.

3.3. *Regulation of c-myc proto-oncogene and cell cycle inhibitor p21$^{WAF1/CIP1}$ after 7,8-dihydroxy-4-methylcoumarin treatment in U-937 cells*

c-myc oncogene plays a central role in controlling the proliferation and differentiation of many normal and neoplastic tissues. Down-regulation of c-Myc expression results in programmed cell death or acceleration of an ongoing apoptotic response [39]. Our results showed that treatment of U-937 cells with 250 μM DHMC induced a time-dependent down-regulation of the c-Myc protein. The expression of c-Myc was decreased at 3 h after coumarin exposure (Fig. 7A and B), suggesting that this oncogene was involved in DHMC-induced apoptosis. Interestingly the co-treatment with the antioxidant *N*-acetyl-L-cysteine (NAC) delayed the down-regulation of c-Myc, as shown in Fig. 7C. A decrease in c-Myc expression was roughly detected after treatment for 24 h. These findings suggest that these events may represent downstream consequences of DHMC-induced oxidative stress.

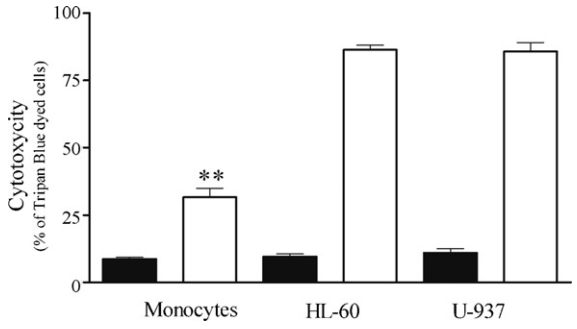

Fig. 6 – Effect of DHMC on cytotoxicity in U-937, HL-60 and normal peripheral blood mononuclear cells. Cells were treated with 250 μM DHMC (□) or 0.0001% (v/v) DMSO (control group) (■) during 24 h. Cellular toxicity was evaluated by the trypan blue assay as detailed in Section 2. Results represent the mean ± S.E.M. ($n = 3$); $^{**}p < 0.01$, compared with U-937 or HL-60 cell lines.

Cyclin-dependent kinase (CDK) inhibitors belonging to the Cip/Kip family have been implicated in programmed cell death and may be a link between the cell cycle and apoptosis [40]. Therefore, the time course of p21$^{WAF1/CIP1}$ expression was studied in U-937 cells. DHMC-treatment resulted in up-regulation of the endogenous cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$ after 6 h, when compared to U-937 control cells (Fig. 7A and B).

3.4. *Regulation of PI3K/Akt signaling cascade by the 7,8-dihydroxy-4-methylcoumarin in U-937 cells*

Phosphatidylinositol 3-kinase (PI3K) stimulation leads to the activation of a serine/threonine kinase termed Akt or PKB. Akt plays a central role in promoting survival of a wide range of cell types. The phosphorylation of Akt substrates, as the Bcl2 family member BAD and the protease Caspase 9, suppresses their pro-apoptotic function, thereby accounting at least in part for the potent survival effects of Akt [41]. To address the effect of DHMC on phosphorylated Akt (p-Akt), U-937 cells

Table 1 – Effect of DHMC on U-937 cell differentiation markers

	Cell differentiation markers		
	% of CD11b positive cells (mean ± S.E.M.) (a)	CD88 expression (b)	NBT reduction (absorbance 570 nm) (mean ± S.E.M.) (c)
U-937 control	53.00 ± 9.00	Negative	0.72 ± 0.02
5 μM DHMC	59.33 ± 5.13	Negative	ND
25 μM DHMC	55.00 ± 5.57	Negative	0.70 ± 0.02
50 μM DHMC	55.63 ± 6.66	Negative	0.69 ± 0.05
400 μM db-cAMP	96.33 ± 9.02[a]	Positive	ND
1 μM ATRA	ND	ND	1.26 ± 0.02[b]

U-937 cells were treated with DHMC (5–50 μM), 400 μM dbcAMP or 1 μM ATRA (positive controls), or 0.0001% (v/v) DMSO (control group) for 48 h. Monocytic markers evaluated: (a) percentage of CD11b positive cells was determined in a flow cytometer as described in Section 2. Results represent the mean ± S.E.M. ($n = 3$); [a]$p < 0.01$ compared with the control group. (b) C5a receptor (CD88) expression was analyzed by Western blot analysis with anti-CD88 antibody. Similar results were obtained in three independent experiments. (c) NBT reducing activity was evaluated by formazan production measured by absorbance at 570 nm. Values are mean ± S.E.M. ($n = 3$); [b]$p < 0.01$ compared with control group. ND: not determined.

were treated with 250 μM DHMC at various time periods. As shown in Fig. 8A, the exposure of U-937 cells to DHMC caused a marked decrease in p-Akt after 18 h. To further examine the effect of phosphorylation of Akt on DHMC-induced apoptosis, we studied whether inhibitors of this pathway could modulate DHMC response. To verify this hypothesis, LY294002, a specific inhibitor of PI3K was used. The co-treatment of DHMC and LY294002 caused substantial inhibition of p-Akt after 16 h of treatment (Fig. 8B). Fig. 8C illustrates that U-937 cells grown in the presence of 30 μM LY294002 were more sensitive to the apoptotic effect of 250 μM DHMC. PI3K/Akt inactivation by LY294002 increased the percentage of apoptotic cells induced by DHMC (73.5 ± 12.0%) as compared to that of DHMC or LY294002 alone (54.5 ± 0.7 and 21.0 ± 0.1%, respectively).

3.5. *Regulation of MAPKs signaling cascade by the 7,8-dihydroxy-4-methylcoumarin in U-937 cells*

Mitogen-activated protein kinases superfamily involves three main and distinct signaling pathways: the extracellular signal-regulated protein kinases (ERKs), the c-Jun N-terminal kinases or stress-activated protein kinases (JNKs/SAPK), and the p38 family [42]. ERK pathway is activated by mitogens and survival factors but ERKs are also activated by DNA damage caused by UV, cisplatin, etoposide, and ara-C in several cultured cells [43]. In vitro studies showed that JNKs and p38 activation are involved in diverse cellular responses such as cell differentiation, inflammation, cell cycle arrest and apoptosis, depending upon the cell types and the external stimuli [44,45].

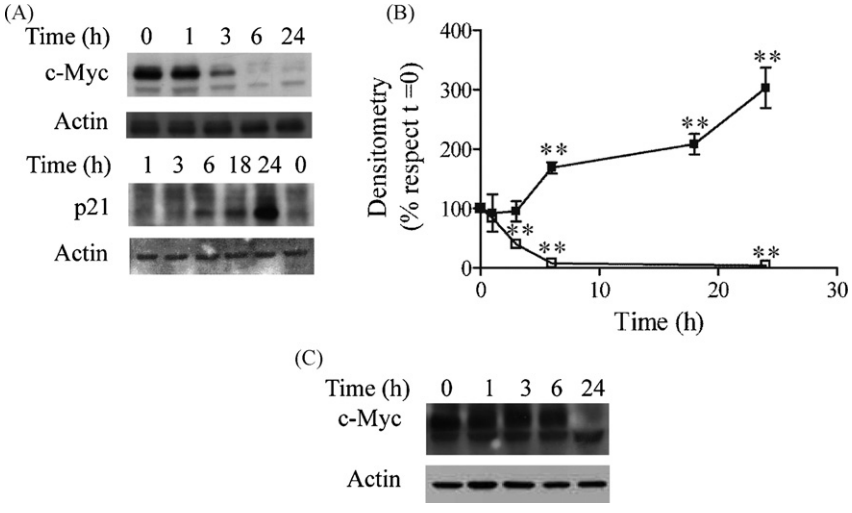

Fig. 7 – Effect of DHMC on c-Myc and p21$^{WAF1/CIP1}$ expression. (A) Cells were treated with 250 μM DHMC and harvested at the indicated times. Equal amount of protein was subjected to SDS-PAGE and analyzed by Western blot with anti-c-Myc, -p21$^{WAF1/CIP1}$ and -actin antibodies. Data are representative of at least three independent experiments. (B) Densitometric analysis relative to actin intensity obtained with the Scion Image program. Data are mean ± S.E.M. ($n = 3$); $^{**}p < 0.01$ respect to basal expression. (C) Cells were treated with 250 μM DHMC and 10 mM NAC and harvested at the indicated times. Equal amount of protein was subjected to SDS-PAGE and analyzed by Western blot with anti-c-Myc and actin antibodies. Data are representative of at least three independent experiments.

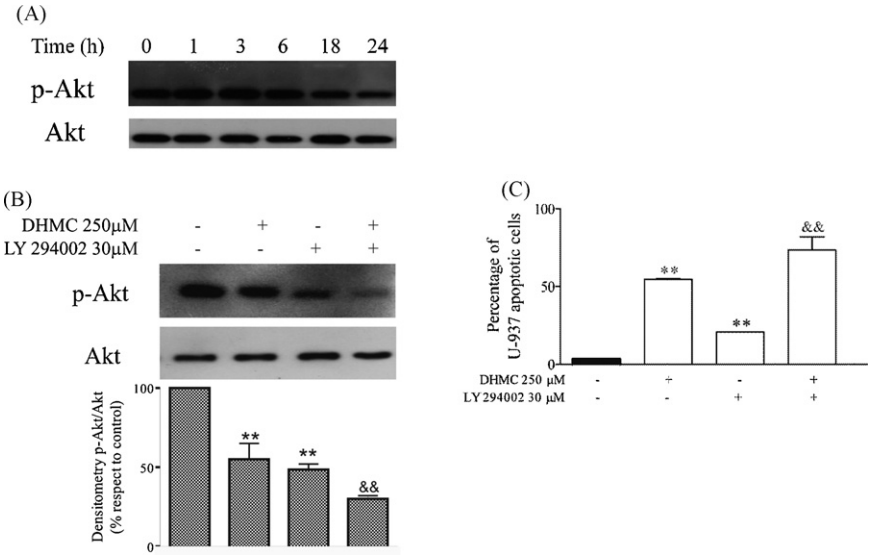

Fig. 8 – Effect of DHMC on p-Akt regulation. (A) Cells were treated with 250 μM DHMC and harvested at the indicated times. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with anti-p-Akt and Akt antibodies. Data are representative of at least three independent experiments. (B) Cells were treated with 250 μM DHMC and 30 μM LY294002, alone and in combination, for 16 h. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with anti-p-Akt and Akt antibodies. Densitometric analysis of p-Akt/Akt obtained with the Scion Image program relative to U-937 control conditions intensity. Data are mean ± S.E.M. ($n = 3$); $^{**}p < 0.01$ respect to control, $^{\&\&}p < 0.01$ respect to cells treated with 250 μM DHMC or 30 μM LY294002 alone. (C) Cells that displayed nuclear fragmentation by Hoechst 33342 staining were counted and expressed as the percentage of apoptotic cells, under control conditions (■) or after 250 μM DHMC and 30 μM LY294002, alone and in combination treatment (■) during 16 h. Data are mean ± S.E.M. ($n = 3$); $^{**}p < 0.01$ respect to control, $^{\&\&}p < 0.01$ respect to cells treated with 250 μM DHMC or 30 μM LY294002 alone.

In the present study, the effect of DHMC on MAPKs was studied in U-937 cells. DHMC caused specific inhibition of p-ERK1/2 after 6 h treatment (Fig. 9A). Those cells treated with 250 μM DHMC showed no phosphorylation of p38 at any studied times, suggesting that this kinase is not implicated in DHMC-induced apoptosis in U-937 cells (Fig. 9B). On the other hand, phosphorylated JNK, increased at 18 h (Fig. 9C). These findings support that ERKs and JNKs but not p38, are involved

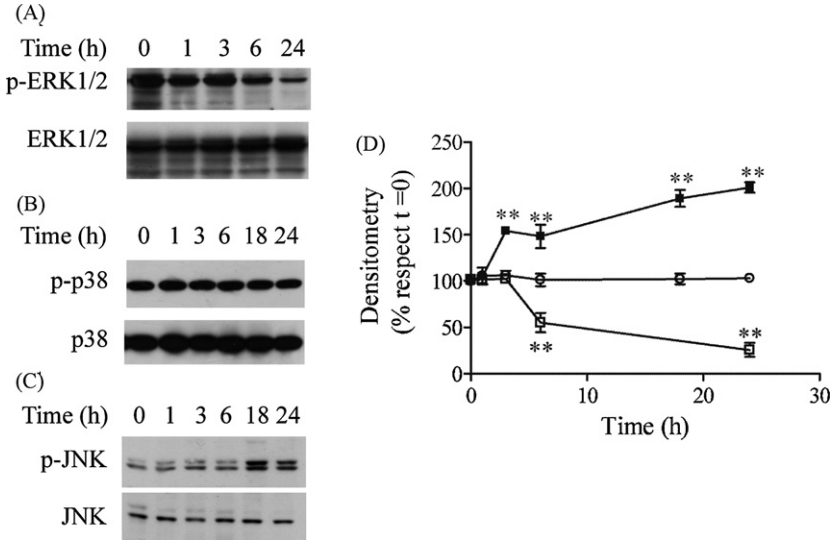

Fig. 9 – Effect of DHMC on the MAPK pathway. Cells were treated with 250 μM DHMC and harvested at the indicated times. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with (A) anti-p-ERK, -ERK, (B) -p-p38, -p38, (C) -p-JNK and -JNK antibodies. Data are representative of at least three independent experiments. (D) Densitometric analysis of p-ERK1/2/ERK (□), p-p38/p38 (○), -p-JNK/JNK (■) obtained with the Scion Image program relative to control conditions ($t = 0$) intensity. Data are mean ± S.E.M. ($n = 3$); $^{**}p < 0.01$ respect to basal expression.

in the transduction of cell death signals induced by DHMC in U-937 cells.

4. Discussion

Leukemias are hematological malignancies that occur as acute or chronic diseases. In leukemic states, transformed early progenitors fail to respond to differentiation stimuli and perpetuate presumably to an additional blockade of programmed cell death [2]. Therefore, the co-activation of differentiation and apoptosis is a potential approach for the treatment of leukemia.

Since the eighties, a number of in vivo studies have investigated the possible use of coumarins in the treatment of renal carcinoma [31], advanced malignant melanoma [46,47], non-small cell lung cancer [48] and breast cancer [49] showing in some of them a significant response rate. In our laboratory we have initiated a rational study of natural and synthetic coumarins as well as their analogues for the development of possible therapeutic agents for the treatment of human leukemias.

Leukemic cell differentiation can be stimulated by low concentrations of cytotoxic agents such ara-C or hybrid polar compounds as HMBA [50,51]. In addition, several coumarins derivatives induce monocytic differentiation of human leukemia cells [28,52]. Daphnetin (7,8-dihydroxycoumarin), a DHMC structural related coumarin exerts potent anti-proliferative and differentiation effects in a human renal cell carcinoma line [27]. In the present study, we evaluated whether DHMC at low concentrations induced the differentiation of U-937 cells. The exposure of cells to DHMC for 48 h failed to induce cell differentiation, evaluated through various differentiation markers such increased of nitrobluetetrazolium reduction and CD11b and CD88 expression (Table 1).

However, DHMC displayed anti-proliferative effects inducing programmed cell death in different human leukemia cell lines such U-937 and HL-60 (Fig. 4). This finding was subsequently confirmed using a selection of biochemical assays. DNA isolated from DHMC-treated U-937 or HL-60 cells showed the characteristic ladder pattern on agarose gels. The internucleosomal DNA degradation induced by DHMC was concentration- and time-dependent (Figs. 1 and 5). In addition morphological characteristics consistent with apoptotic cell death were also observed in cells treated with the hydroxylated coumarin (Figs. 2 and 5, inset). The response to DHMC in leukemic cells depended upon the concentration and duration of the stimulus. In both U-937 and HL-60 cell lines, concentrations of DHMC lower than 500 μM induced apoptosis but higher concentrations increased the percentage of necrotic cells (Figs. 2 and 5). Furthermore, an increased activity of pro-apoptotic caspase 3 and increased cleavage of PARP were observed in DHMC-treated U-937 cells (Fig. 3).

In accordance with present findings other studies show that different coumarin derivatives induce apoptosis in renal carcinoma cells, human leukemic cells and pancreatic cancer cells [31,53,54]. However, despite numerous studies, little is known about the cellular mechanism evoked by pro-apoptotic coumarin compounds in cancer cells [23,31,34]. Further studies will help to determine the exact mechanism of action and eventually highlight their potential application as possible therapeutics agents for the treatment in neoplasm.

Among the main stress signaling pathways in stress response to oxidative insults are the MAPK cascades, the PI3K/Akt, the nuclear factor kβ (NF-kβ) signaling system and p53 activation [55]. In a recent study, Kim et al. [34] suggested that oxygen radicals are likely implicated in scopoletin (6-metoxy-7-hydroxycoumarin)-induced apoptosis in leukemic cells and showed that NF-kβ and caspase 3 are activated.

MAP kinase signaling pathways transduce extracellular and intracellular stimuli into cellular responses. The relative balance between ERKs, JNK and p38 signaling may determine susceptibility to apoptosis induction [56]. In the present study, DHMC-treated U-937 cells showed activation of the JNKs pathway at 18 h but no detectable changes in phosphorylated-p38. Furthermore, down-regulation of p-ERK1/2 was observed at 6 h (Fig. 9). The underlying mechanism of DHMC-induced changes in the activation of MAPK cascades remains presently unknown although it is likely that coumarins act upstream of the MAPK cascades. In this sense, it has been reported that the anti-inflammatory and anticancer activity of certain coumarin derivatives may result from their allosteric MEK1 inhibitor activity [29]. Alternatively, it has been also suggested that oxidative stress can induce inhibition of the survival cascades, ERKs and PI3K/Akt, in different cellular models [55].

PI3K/Akt pathway is involved in cell survival signaling. This may be in part through Akt-mediated phosphorylation and functional inactivation of BAD, a pro-apoptotic Bcl-2 family protein and inhibition of caspase 9 and 3 activation, likely through direct phosphorylation of caspase 9. In addition, PI3K/Akt signaling, via inhibition of glycogen synthase kinase (GSK-3), increases the stability of the cyclin D1 and Myc proteins [41,57]. Therefore, we studied the Akt phosphorylated status in U-937 cells exposed to DHMC. Western blot analysis showed that Akt was highly expressed and phosphorylated in control cells, whereas in DHMC-treated cells Akt was inactivated at least within 18 h (Fig. 8A). In addition, as expected, the co-treatment with DHMC and LY294002 (a specific inhibitor of PI3K/Akt pathway) augmented DHMC-induced apoptosis (Fig. 8C) and Akt dephosphorylation (Fig. 8B). It has been reported that Akt protects cancer cells from apoptosis induced by anticancer therapies and that it is involved in drug-resistance [58]. Present findings show that DHMC treatment inactivates the Akt pathway, although further studies are necessary in order to elucidate the mechanism in leukemic cells. The proto-oncogene c-myc plays a pivotal role in cell cycle regulation, metabolism, apoptosis, differentiation, cell adhesion, and tumorigenesis, and further participates in the regulation of hematopoietic homeostasis. It is a transcription regulator that is part of an extensive network of interacting factors [39]. In the present study, c-Myc levels decreased after 3 h when U-937 cells underwent apoptosis induced by DHMC treatment (Fig. 7A and B). The decreased in c-Myc expression was observed before DNA fragmentation or typical apoptotic morphological changes, suggesting that variations in c-Myc levels might be associated with the apoptotic process induced by DHMC in human leukemia cells. On the other hand, the inactivation of Akt activates GSK-3, which desestabilizes Myc and targets it for ubiquitin-mediated degradation. Indeed, NAC treatment delayed c-Myc expression, suggesting that this

proto-oncogene pathway may be regulated as a result of a possible oxidative stress induced by DHMC (Fig. 7C).

The development of cell resistance to chemotherapy is one of the major causes of treatment failure in hematological malignancies. Often, resistance develops when the p53 pathway is impaired because several chemotherapeutic drugs mediate their pro-apoptotic effects through this via. Therefore, the development of new drugs that do not involve the p53 signaling pathway may constitute a novel therapeutic strategy for overcoming multidrug-resistance in leukemias. In the present work, we show that DHMC induced suicide by programmed cell death through an independent p53 pathway, because human leukemic HL-60 and U-937 cells are p53-null cell lines [59]. In accordance with this finding it has been previously reported that the cytostatic effect of coumarin compounds is likely p53-independent in human lung carcinoma cells [25].

The cell cycle progression is regulated by sequential activation and inactivation of a series of cyclin-dependent kinases (CDKs), which are positively regulated by cyclins and negatively by cyclin-dependent kinase inhibitors (CDKIs) [40]. Several studies have established that coumarin derivatives induce cell cycle disturbances in malignant cells [24,25,60,61]. Thus, we examined the expression of CDKI $p21^{WAF1/CIP1}$, in cells exposed to DHMC. An increase in $p21^{WAF1/CIP1}$ expression was observed following U-937 cells exposure to 250 μM DHMC for 18 h (Fig. 7). Up-regulation of $p21^{WAF1/CIP1}$ has been shown to correlate with an inhibition of cell growth and it is regarded as an indirect marker of apoptosis in cell systems where G1 phase blockade contributes to cell death. Moreover, the change in c-Myc levels observed after DHMC treatment, may also alter the operation of the cell cycle checkpoints, inducing arrest at G1 [40]. As it was previously discussed U-937 cell line is a p53-null leukemia cell line so the activation of $p21^{WAF1/CIP1}$ induced by DHMC occurred through an unknown p53-independent mechanism.

In conclusion, human leukemia cells exposed to DHMC showed activation of the JNK pathway with concomitant inhibition of the ERKs pathway and other transducer systems as PI3K/Akt as well as reduction of the early response gene c-myc with an up-regulation of $p21^{WAF1/CIP1}$ and caspase 3 in a p53-independent mechanism. So, the integration of the pro-survival and pro-apoptotic signaling pathways determines the final outcome in DHMC-treated U-937 cells. The therapeutic goal in cancer is to trigger tumor-selective cell death. DHMC exhibits low toxicity in normal cells but possess pro-apoptotic effect on malignant cells. Taken together, our findings support the possibility that DHMC might be an effective cytotoxic drug for the combined treatment of human leukemia. Nowadays, drug discovery requires knowledge of the structure and the understanding of the drug action and in time new drug-candidate molecules will emerge with this approach.

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REFERENCES

[1] Leszczyniecka M, Roberts T, Dent P, Grant S, Fisher PB. Differentiation therapy of human cancer: basic science and clinical applications. Pharmacol Ther 2001;90:105–56.
[2] Tsiftsoglou AS, Pappas IS, Vizirianakis IS. Mechanisms involved in the induced differentiation of leukemia cells. Pharmacol Ther 2003;100:257–90.
[3] Johnson SA. Clinical pharmacokinetics of nucleoside analogues: focus on haematological malignancies. Clin Pharmacokinet 2000;39:5–26.
[4] Roboz GJ. Treatment of acute myeloid leukemia in older patients. Expert Rev Anticancer Ther 2007;7:285–95.
[5] Thatte U, Bagadey S, Dahanukar S. Modulation of programmed cell death by medicinal plants. Cell Mol Biol (Noisy-le-grand) 2000;46:199–214.
[6] O'Kennedy R, Thornes RD. Coumarins: biology, applications and mode of action. Chichester, United Kingdom: John Wiley & Sons; 1997.
[7] Lake BG. Coumarin metabolism, toxicity and carcinogenicity: relevance for human risk assessment. Food Chem Toxicol 1999;37:423–53.
[8] Manolov I, Topashka-Ancheva M, Klouchek E. The synthesis, toxicological and comparative cytogenetic characteristics of the anticoagulant warfarin. Eksp Med Morfol 1993;31:49–60.
[9] Fylaktakidou KC, Hadjipavlou-Litina DJ, Litinas KE, Nicolaides DN. Natural and synthetic coumarin derivatives with anti-inflammatory/antioxidant activities. Curr Pharm Des 2004;10:3813–33.
[10] Hadjipavlou-Litina DJ. New diaminoether coumarinic derivatives with anti-inflammatory activity. Arzneimittelforschung 2000;50:631–5.
[11] Kostova I, Raleva S, Genova P, Argirova R. Structure–activity relationships of synthetic coumarins as HIV-1 inhibitors. Bioinorg Chem Appl 2006;68274.
[12] Periers AM, Laurin P, Ferroud D, Haesslein JL, Klich M, Dupuis-Hamelin C, et al. Coumarin inhibitors of gyrase B with N-propargyloxy-carbamate as an effective pyrrole bioisostere. Bioorg Med Chem Lett 2000;10:161–5.
[13] Schio L, Chatreaux F, Loyau V, Murer M, Ferreira A, Mauvais P, et al. Fine tuning of physico-chemical parameters to optimise a new series of novobiocin analogues. Bioorg Med Chem Lett 2001;11:1461–4.
[14] Ghani U, Ng KK, Atta ur R, Choudhary MI, Ullah N, James MN. Crystal structure of gamma-chymotrypsin in complex with 7-hydroxycoumarin. J Mol Biol 2001;314:519–25.
[15] Gnerre C, Catto M, Leonetti F, Weber P, Carrupt PA, Altomare C, et al. Inhibition of monoamine oxidases by functionalized coumarin derivatives: biological activities, QSARs, and 3D-QSARs. J Med Chem 2000;43:4747–58.
[16] Raj HG, Parmar VS, Jain SC, Goel S, Poonam, Himanshu. et al. Mechanism of biochemical action of substituted 4-methylbenzopyran-2-ones. Part I: dioxygenated 4-methyl coumarins as superb antioxidant and radical scavenging agents. Bioorg Med Chem 1998;6:833–9.
[17] Vajragupta O, Boonchoong P, Wongkrajang Y. Comparative quantitative structure–activity study of radical scavengers. Bioorg Med Chem 2000;8:2617–28.
[18] Molina-Jimenez MF, Sanchez-Reus MI, Benedi J. Effect of fraxetin and myricetin on rotenone-induced cytotoxicity in

SH-SY5Y cells: comparison with *N*-acetylcysteine. Eur J Pharmacol 2003;472:81–7.
[19] Appendino G, Bianchi F, Bader A, Campagnuolo C, Fattorusso E, Taglialatela-Scafati O, et al. Coumarins from Opopanax chironium. New dihydrofuranocoumarins and differential induction of apoptosis by imperatorin and heraclenin. J Nat Prod 2004;67:532–6.
[20] Finn GJ, Kenealy E, Creaven BS, Egan DA. In vitro cytotoxic potential and mechanism of action of selected coumarins, using human renal cell lines. Cancer Lett 2002;183:61–8.
[21] Kawaii S, Tomono Y, Ogawa K, Sugiura M, Yano M, Yoshizawa Y. The antiproliferative effect of coumarins on several cancer cell lines. Anticancer Res 2001;21:917–23.
[22] Kawaii S, Tomono Y, Ogawa K, Sugiura M, Yano M, Yoshizawa Y, et al. Antiproliferative effect of isopentenylated coumarins on several cancer cell lines. Anticancer Res 2001;21:1905–11.
[23] Chu CY, Tsai YY, Wang CJ, Lin WL, Tseng TH. Induction of apoptosis by esculetin in human leukemia cells. Eur J Pharmacol 2001;416:25–32.
[24] Wang CJ, Hsieh YJ, Chu CY, Lin YL, Tseng TH. Inhibition of cell cycle progression in human leukemia HL-60 cells by esculetin. Cancer Lett 2002;183:163–8.
[25] Lopez-Gonzalez JS, Prado-Garcia H, Aguilar-Cazares D, Molina-Guarneros JA, Morales-Fuentes J, Mandoki JJ. Apoptosis and cell cycle disturbances induced by coumarin and 7-hydroxycoumarin on human lung carcinoma cell lines. Lung Cancer 2004;43:275–83.
[26] Hofmanova J, Kozubik A, Dusek L, Pachernik J. Inhibitors of lipoxygenase metabolism exert synergistic effects with retinoic acid on differentiation of human leukemia HL-60 cells. Eur J Pharmacol 1998;350:273–84.
[27] Finn GJ, Creaven BS, Egan DA. Daphnetin induced differentiation of human renal carcinoma cells and its mediation by p38 mitogen-activated protein kinase. Biochem Pharmacol 2004;67:1779–88.
[28] Riveiro ME, Shayo C, Monczor F, Fernandez N, Baldi A, De Kimpe N, et al. Induction of cell differentiation in human leukemia U-937 cells by 5-oxygenated-6,7-methylenedioxycoumarins from Pterocaulon polystachyum. Cancer Lett 2004;210:179–88.
[29] Han S, Zhou V, Pan S, Liu Y, Hornsby M, McMullan D, et al. Identification of coumarin derivatives as a novel class of allosteric MEK1 inhibitors. Bioorg Med Chem Lett 2005;15:5467–73.
[30] Finn GJ, Creaven BS, Egan DA. Investigation of intracellular signalling events mediating the mechanism of action of 7-hydroxycoumarin and 6-nitro-7-hdroxycoumarin in human renal cells. Cancer Lett 2004;205:69–79.
[31] Finn G, Creaven B, Egan D. Modulation of mitogen-activated protein kinases by 6-nitro-7-hydroxycoumarin mediates apoptosis in renal carcinoma cells. Eur J Pharmacol 2003;481:159–67.
[32] Yang EB, Zhao YN, Zhang K, Mack P. Daphnetin, one of coumarin derivatives, is a protein kinase inhibitor. Biochem Biophys Res Commun 1999;260:682–5.
[33] Hadi SM, Asad SF, Singh S, Ahmad A. Putative mechanism for anticancer and apoptosis-inducing properties of plant-derived polyphenolic compounds. IUBMB Life 2000;50: 167–71.
[34] Kim EK, Kwon KB, Shin BC, Seo EA, Lee YR, Kim JS, et al. Scopoletin induces apoptosis in human promyeloleukemic cells, accompanied by activations of nuclear factor kappaB and caspase-3. Life Sci 2005;77:824–36.
[35] Parborell F, Irusta G, Vitale A, Gonzalez O, Pecci A, Tesone M. Gonadotropin-releasing hormone antagonist antide inhibits apoptosis of preovulatory follicle cells in rat ovary. Biol Reprod 2005;72:659–66.
[36] Martinsson P, Liminga G, Nygren P, Larsson R. Characteristics of etoposide-induced apoptotic cell death in the U-937 human lymphoma cell line. Anticancer Drugs 2001;12:699–705.
[37] Harris P, Ralph P. Human leukemic models of myelomonocytic development: a review of the HL-60 and U-937 cell lines. J Leukoc Biol 1985;37:407–22.
[38] Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer 1976;17:565–77.
[39] Hoffman B, Liebermann DA. The proto-oncogene c-myc and apoptosis. Oncogene 1998;17:3351–7.
[40] Kopnin BP. Targets of oncogenes and tumor suppressors: key for understanding basic mechanisms of carcinogenesis. Biochemistry (Mosc) 2000;65:2–27.
[41] Krasilnikov MA. Phosphatidylinositol-3 kinase dependent pathways: the role in control of cell growth, survival, and malignant transformation. Biochemistry (Mosc) 2000;65: 59–67.
[42] Cowan KJ, Storey KB. Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stress. J Exp Biol 2003;206:1107–15.
[43] Jarvis WD, Johnson CR, Fornari FA, Park JS, Dent P, Grant S. Evidence that the apoptotic actions of etoposide are independent of c-Jun/activating protein-1-mediated transregulation. J Pharmacol Exp Ther 1999;290: 1384–92.
[44] Chen YR, Meyer CF, Tan TH. Persistent activation of c-Jun N-terminal kinase 1 (JNK1) in gamma radiation-induced apoptosis. J Biol Chem 1996;271:631–4.
[45] Paul A, Wilson S, Belham CM, Robinson CJ, Scott PH, Gould GW, et al. Stress-activated protein kinases: activation, regulation and function. Cell Signal 1997;9:403–10.
[46] Marshall ME, Butler K, Cantrell J, Wiseman C, Mendelsohn L. Treatment of advanced malignant melanoma with coumarin and cimetidine: a pilot study. Cancer Chemother Pharmacol 1989;24:65–6.
[47] Nolte H, Pedersen L, Mouridsen HT. Combined treatment of advanced malignant melanoma with coumarin and cimetidine. Anticancer Res 1987;7:449–50.
[48] Marshall ME, Mendelsohn L, Butler K, Cantrell J, Harvey J, Macdonald J. Treatment of non-small cell lung cancer with coumarin and cimetidine. Cancer Treat Rep 1987;71: 91–2.
[49] Purohit A, Woo LW, Barrow D, Hejaz HA, Nicholson RI, Potter BV, et al. Non-steroidal and steroidal sulfamates: new drugs for cancer therapy. Mol Cell Endocrinol 2001;171:129–35.
[50] Richon VM, Webb Y, Merger R, Sheppard T, Jursic B, Ngo L, et al. Second generation hybrid polar compounds are potent inducers of transformed cell differentiation. Proc Natl Acad Sci USA 1996;93:5705–8.
[51] Wang Z, Wang S, Fisher PB, Dent P, Grant S. Evidence of a functional role for the cyclin-dependent kinase inhibitor p21CIP1 in leukemic cell (U-937) differentiation induced by low concentrations of 1-beta-D-arabinofuranosylcytosine. Differentiation 2000;66:1–13.
[52] Kawaii S, Tomono Y, Katase E, Ogawa K, Yano M. Effect of coumarins on HL-60 cell differentiation. Anticancer Res 2000;20:2505–12.
[53] Pae HO, Oh H, Yun YG, Oh GS, Jang SI, Hwang KM, et al. Imperatorin, a furanocoumarin from Angelica dahurica (Umbelliferae), induces cytochrome c-dependent apoptosis in human promyelocytic leukaemia, HL-60 cells. Pharmacol Toxicol 2002;91:40–8.
[54] Lewis A, Ough M, Li L, Hinkhouse MM, Ritchie JM, Spitz DR, et al. Treatment of pancreatic cancer cells with dicumarol induces cytotoxicity and oxidative stress. Clin Cancer Res 2004;10:4550–8.

[55] Yoon SO, Yun CH, Chung AS. Dose effect of oxidative stress on signal transduction in aging. Mech Ageing Dev 2002;123:1597–604.
[56] Tibbles LA, Woodgett JR. The stress-activated protein kinase pathways. Cell Mol Life Sci 1999;55:1230–54.
[57] Sears RC, Nevins JR. Signaling networks that link cell proliferation and cell fate. J Biol Chem 2002;277: 11617–20.
[58] Brognard J, Clark AS, Ni Y, Dennis PA. Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. Cancer Res 2001;61:3986–97.
[59] 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.
[60] Finn GJ, Creaven BS, Egan DA. A study of the role of cell cycle events mediating the action of coumarin derivatives in human malignant melanoma cells. Cancer Lett 2004;214:43–54.
[61] Jimenez-Orozco FA, Lopez-Gonzalez JS, Nieto-Rodriguez A, Velasco-Velazquez MA, Molina-Guarneros JA, Mendoza-Patino N, et al. Decrease of cyclin D1 in the human lung adenocarcinoma cell line A-427 by 7-hydroxycoumarin. Lung Cancer 2001;34:185–94.