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Pharmacodynamic study of the 7,8-dihydroxy-4-methylcoumarin-induced selective cytotoxicity toward U-937 leukemic cells *versus* mature monocytes: Cytoplasmic p21$^{Cip1/WAF1}$ as resistance factor

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ABSTRACT

The development of tumor-selective drugs with low systemic toxicity has always been a major challenge in cancer treatment. Our group previously identified the 7,8-dihydroxy-4-methylcoumarin (DHMC) as a potential chemotherapeutic agent due to its potent, selective anti-proliferative and apoptosis-inducing effects on several cancer cell lines over peripheral blood mononuclear cells. However, there are still no published reports that can explain such selectivity of action. Herein, we addressed this question by using the U-937 promonocytic leukemia cell line, which can be forced to differentiate into a monocyte-like phenotype *in vitro*. U-937 cells differentiation is dependent on the nuclear expression of p21$^{Cip1/WAF1}$, a protein that is absent in immature U-937 cells but present in both the nucleus and the cytoplasm of normal DHMC-resistant monocytes. Considering that induction of differentiation rendered U-937 cells resistant to DHMC, we evaluated the possible causal role of cytoplasmic p21$^{Cip1/WAF1}$ in the onset of such resistance by employing U-937 cells stably transfected with a $ZnCl_2$-inducible p21$^{Cip1/WAF1}$ variant lacking the nuclear localization signal (U-937/CB6-ΔNLS-p21 cells). Expression of cytoplasmic p21$^{Cip1/WAF1}$ did not induce differentiation of the cells but turned them resistant to DHMC through inhibition of JNK, a crucial mediator of DHMC-induced apoptosis in U-937 cells. Sub-acute toxicity evaluation of DHMC in Balb/c mice indicated that DHMC administered intraperitoneally at doses up to 100 mg/kg induced no systemic damage. Collectively, our results explain for the first time the selective cytotoxicity of DHMC for tumor cells over normal monocytes, and encourage further *in vivo* studies on this compound as potential anti-leukemic agent.

1. Introduction

Although successful in many cases, the clinical application of chemotherapeutic agents against malignant tumors still has two major limitations: the lack of selectivity and its consequent toxic side effects; and drug resistance, which reduces therapeutic efficacy. With focus on these two issues, over the last years our laboratory has devoted much effort to identifying new prototype compounds for the development of novel, more efficient anti-leukemic agents. Our research has led to the identification of both natural and synthetic coumarins with anti-proliferative, differentiating and/or cytotoxic activities in different leukemic cell lines [1–6]. In this regard, the synthetic 7,8-dihydroxy-4-methylcoumarin (denoted as DHMC from here onwards, Scheme 1) has

Abbreviations: ATRA, all-*trans*-retinoic acid; BSA, bovine serum albumin; db-cAMP, dibutyryl cyclic adenosine monophosphate; DHMC, 7,8-dihydroxy-4-methylcoumarin; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; IP, intraperitoneal; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NLS, nuclear localization signal; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PI, propidium iodide; rhC5a, recombinant human complement factor C5a; SDS, sodium dodecyl sulfate; SNK, Student–Newman–Keuls; ΔNLS, deleted nuclear localization signal.

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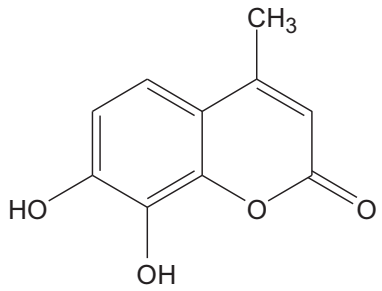


Scheme 1. 7,8-Dihydroxy-4-methylcoumarin (DHMC).

particularly gotten much interest due to its apoptosis-inducing activity in several cancer cell lines [2,3,6,7]. In previous publications we established the structural requirements for apoptosis induction by DHMC in the human U-937 leukemic cell line [2,6]. Also, we determined that the cited process is mediated by caspase-9 activation [6] and correlates with an increase in the phosphorylated (active) fraction of the c-Jun N-terminal kinase (JNK), a decrease in the phosphorylated fractions of the extracellular signal-regulated kinase (ERK) and the serine/threonine protein kinase Akt, and unchanged phosphorylated levels of the p38 mitogen-activated protein kinase (p38 MAPK) [3]. Most tellingly, our group and other have reported that the cytotoxic activity of DHMC is selective for malignant cells over normal peripheral blood mononuclear cells (PBMC) [3,6,7], drawing the attention to this coumarin as potential candidate for further design of selective anti-tumor drugs. However, so far there are no reports in the scientific literature that can provide an explanation for the aforementioned selective cytotoxicity.

The physiological transition from immature hematological cells to mature hematological cells entails changes in intracellular oxidation potential and protein expression patterns [8]. In this regard, Asada et al. demonstrated that nuclear expression of $p21^{Cip1/WAF1}$ plays a critical role in the differentiation of U-937 promonocytic cells into monocytes induced by *in vitro* treatment with vitamin D3 or ectopic expression of $p21^{Cip1/WAF1}$ [9], and described the translocation of $p21^{Cip1/WAF1}$ from the nucleus to the cytoplasm during such differentiation process [9].

The $p21^{Cip1/WAF1}$ protein is a cyclin-dependent kinase (CDK) inhibitor classically associated to the control of cell cycle and DNA replication. However, increasing evidence supports the involvement of $p21^{Cip1/WAF1}$ in different cellular processes such as stress response, apoptosis and tumorigenesis [10,11]. Of particular interest, several recent publications indicate that cytoplasmic $p21^{Cip1/WAF1}$ confers resistance to cell death induced by multiple chemotherapeutic drugs, and it has been proposed as a predictive and prognostic biomarker of chemotherapy resistance in different cancer types [12–17]. On the basis of this evidence, we hypothesized that the selective toxicity of DHMC toward leukemic U-937 cells might be predominantly determined by the differential occurrence and subcellular localization of $p21^{Cip1/WAF1}$ protein between these cells and mature monocytes.

We report herein for the first time that cytoplasmic $p21^{Cip1/WAF1}$ expression in U-937 cells forced to undergo differentiation by *in vitro* treatment with all-*trans*-retinoic acid (ATRA) or dibutyryl cyclic adenosine monophosphate (db-cAMP) leads to a substantial inhibition of the anti-proliferative and cytotoxic activities of DHMC. Our present study also reveals that such DHMC-resistant phenotype would depend on $p21^{Cip1/WAF1}$-mediated blockade of JNK activation, a crucial step in the mechanism of DHMC apoptotic action in U-937 cells as evidenced in this manuscript. Finally, we present the results of the first sub-acute toxicity assay of DHMC, and show that it does not induce toxicity in bone marrow hematological precursors or other tissues in Balb/c mice. Taken collectively, our current findings provide the first mechanistic insight into the selective cytotoxicity of DHMC for immature U-937 leukemic cells over normal monocytes and, in view of its low systemic toxicity, justify further research on this compound as potential anti-leukemic agent.

2. Materials and methods

2.1. Reagents and antibodies

RPMI 1640 medium, gentamicin antibiotic, bovine serum albumin (BSA), dibutyryl-cyclic adenosine monophosphate (db-cAMP), *all*-trans-retinoic acid (ATRA), phosphate-buffered saline (PBS), recombinant human C5a (rhC5a), $ZnCl_2$, fluorescein isothiocyanate (FITC), propidium iodide (PI), JNK inhibitor SP600125 were obtained from Sigma–Aldrich Co., LLC. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Baker (Deventer, The Netherlands). Fetal calf serum (FCS) was obtained from PAA Laboratories GmbH (Austria). Anti-JNK, -p-JNK and -$p21^{Cip1/WAF1}$ primary antibodies, and anti-rabbit and -mouse secondary antibodies were purchased from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA). All commercial chemicals and solvents were of reagent grade and used without further purification unless otherwise specified.

2.2. Synthesis

DHMC was synthesized according to the Pechmann–Duisberg reaction as described by Riveiro et al. [2]. The compound was then purified by recrystallization from ethanol and characterized by IR and NMR spectra as well as by melting point determination. Finally, it was dissolved in DMSO and stored at −20 °C.

2.3. Cell culture

The U-937 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). On the other hand, the U-937/CB6-ΔNLS-p21 cells, stably transfected with a Zn^{2+}-inducible $p21^{Cip1/WAF1}$ variant lacking the nuclear localization signal domain (ΔNLS-$p21^{Cip1/WAF1}$), were generously provided by Dr. Minoru Asada from the Nippon Medical School, Tokyo, Japan. Both cell types were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium, supplemented with 10% FCS and 50 μg/ml gentamicin. Cells used in each experiment were in *exponential growth phase*.

Peripheral blood monocytes were isolated from healthy volunteers by standard density gradient centrifugation on Ficoll-Hypaque. Blood cells were purified by centrifugation on a discontinuous Percoll gradient with modifications of a previously described method [18]. Briefly, PBMC were suspended in Ca^{2+}- and Mg^{2+}-free Tyrode solution supplemented with 0.2% EDTA and incubated for 30 min at 37 °C. During this incubation, the osmolarity of the medium was gradually increased from 290 to 360 Osmol/dm^3 by addition of 9% NaCl. Three different Percoll fractions were layered in polypropylene tubes: 50% at the bottom followed by 46% and 40%. PBMC (5×10^6 cells/ml) were layered at the top, and they were centrifuged at $400 \times g$ for 20 min at 4 °C. Monocytes were recovered from the 50 to 46% interface. The purity was checked by FACS analysis using anti-CD14 monoclonal antibody (BD Pharmingen, USA) and was found to be >85%.

The use of human samples in this study was approved by the local Ethics Committee and was conducted according to the Declaration of Helsinki.

Before seeding, the viability of monocytes, U-937 and U-937/CB6-DNLS-p21 cells was tested by trypan blue assay. Cells were used only if viability was higher than 90%.

2.4. Sub-acute toxicity assay

2.4.1. Animals

Experiments were carried out in a total of fifty healthy male Balb/c mice raised at the Instituto de Biología y Medicina Experimental (IBYME)-CONICET. All animal studies were conducted in accordance with the highest standards of animal care as outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH, USA) and were approved by the Instituto de Biología y Medicina Experimental (IBYME)-CONICET Animal Research Committee. Mice were given food and potable water *ad libitum*.

2.4.2. Experimental design and drug treatment

Firstly, forty 6- to 8-week-old male Balb/c mice (22 g of average weight) were randomly divided into two main groups considering the two drugs to be tested: DHMC- or coumarin-treated group. Subsequently, five mice from each main group were assigned to one of four different drug doses and housed in polycarbonate cages, as follows:

Group I: 12.5 mg/kg body weight dose.
Group II: 25 mg/kg body weight dose.
Group III: 50 mg/kg body weight dose.
Group IV: 100 mg/kg body weight dose.

Other ten mice were randomly assigned to control and vehicle groups (5 in each; groups V and VI, respectively).

DHMC and coumarin were dissolved in the vehicle (DMSO: 1.8% NaCl; 1:1) and daily administered *via* intraperitoneal (IP) injection (100 μl for each dose) for 15 days. During the course of treatment, both the weight and behavior of each animal were controlled. Mortality, if any, was also recorded. Forty-eight hours after the last IP injection, 4 animals of each group were sacrificed by cervical dislocation. The organs were quickly blotted and processed for histopathological studies. The remaining animals were sacrificed 15 days after the last dose. Their organs were also subjected to histological studies.

2.4.3. Histopathology

Liver, intestine, kidney and bone marrow tissues from all mice were fixed for 24 h in 10% formalin. Tissues were embedded in paraffin, sectioned (4 μm) and dyed with commonly used stains, hematoxylin and eosin (HE), Masson trichrome (MT) and periodic acid-Schiff (PAS), in order to determine treatment-induced injury.

2.5. Measurement of cell proliferation

U-937 or U-937/CB6-ΔNLS-p21 cells, pre-treated or not with 20 μM SP600125, 120 μM $ZnCl_2$, 1 μM ATRA, 400 μM db-cAMP or vehicle, were seeded at 3.5×10^5 cells/ml of RPMI 1640 and treated with 100 μM or 250 μM DHMC or 0.1% (v/v) DMSO (vehicle control group) for a maximum of 24 h in a 5% CO_2 atmosphere at 37 °C. Later, they were collected at the indicated times and counted in a Coulter Z-1 cell counter. These results were simultaneously confirmed by cell counting in a Neubauer hemocytometer chamber. Assays were performed in triplicate in at least three independent experiments.

2.6. Cytotoxicity determination

Cells treated with differentiating agents ATRA or db-cAMP, $ZnCl_2$- or JNK inhibitor- treated cells and non-treated control cells were seeded at 3.5×10^5 ml^{-1} of RPMI 1640 and then treated with 100 μM or 250 μM DHMC or 0.1% (v/v) DMSO (vehicle control group) during 24 h in a 5% CO_2 atmosphere. After treatment, an aliquot of the medium was mixed with an equal volume of 0.4% trypan blue and incubated for 5 min, after which the number of viable cells was estimated by using a hemocytometer chamber. In all cases, assays were carried out in duplicate in at least three independent experiments.

2.7. Caspase-3 activity

U-937 or U-937/CB6-ΔNLS-p21 cells, pre-treated or not with 20 μM SP600125, 120 μM $ZnCl_2$, 1 μM ATRA, 400 μM db-cAMP or vehicle, were seeded (3.5×10^5 cells/ml) and treated with 100 μM, 250 μM DHMC or 0.1% (v/v) DMSO (vehicle control group) during 24 h. After that, cells were harvested and processed according to CASP3C caspase-3 colorimetric assay kit provided by Sigma Chemical Co. (St. Louis, MO, USA).

2.8. 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, and probed with the indicated primary antibodies. Membranes were first incubated with anti-p-JNK primary antibody, then stripped and re-probed for total JNK antibody to verify equal loading of protein. All blots were incubated with horseradish peroxidase conjugated anti-rabbit or anti-mouse antibodies and developed by enhanced chemiluminescence (ECL) following the manufacturer's instructions (Amersham Life Science, England).

Densitometry analyses, carried out under conditions that yielded a linear response, were performed using ImageJ free software (http://rsbweb.nih.gov/ij/index.html).

2.9. Determination of U-937 cell differentiation markers

2.9.1. Surface myeloid CD11b and CD14 antigens assay

The expression of CD11b and CD14 was detected by direct immunofluorescence staining. U-937 cells (3.5×10^5 cells/ml) were treated with 0.1% (v/v) DMSO (vehicle control group), 1 μM ATRA or 400 μM db-cAMP (positive control group) for 48 h. Treated and control cells were washed twice in PBS and incubated with a saturated concentration of phycoerythrin (PE) anti-CD11b or anti-CD14 antibody (BD Pharmingen, USA) at 4 °C for 30 min. In all cases isotype-matched control monoclonal antibodies were used, and a gate (R1) was defined in the analysis to exclude all nonviable cells and debris, based on size and propidium iodine staining. Analysis was performed using a FACS flow cytometer and CellQuest software (BD Biosciences, USA). The results are expressed as mean fluorescence intensity respect to control (non-treated cells).

2.9.2. Chemotaxis assay

The "*in vitro*" chemotactic response of U-937 cells to C5a complement factor was assayed using the micropore filter technique (Transwell 3521, Costar Corp., Cambridge, MA, USA). Briefly, after 48 h of treatment, 1×10^5 control, vehicle or treated cells were seeded onto the top compartment of chemotactic chambers, in 0.1 ml of RPMI 1640, and placed in a 24-well tissue culture plate. A polyvinylpyrrolidone-free polycarbonate filter with a pore size of 5 μm separated the top and bottom compartments. The bottom compartment was filled with 0.6 ml of medium with or without 5×10^{-9} M rhC5a. Chambers were incubated for 5 h at 37 °C in a 5% CO_2 atmosphere. Migrated cells were collected and counted using a cellular meter Coulter Z-1.

2.10. Indirect immunofluorescence

U-937/CB6-ΔNLS-p21 cells (4×10^5 ml^{-1}) were firstly incubated with the indicated agents for 48 h in RPMI 1640 medium with 10% SFB and 50 μg/ml gentamicin in a 5% CO_2 atmosphere. On the other hand, monocytes were immediately processed after isolation. In all cases, cells (approximately 10×10^6/treatment) were fixed and permeabilized with 4% formalin for 20 min. After washing, cells were re-suspended in PBS buffer containing 10% (w/v) BSA for 40 min. Later, cells were incubated overnight with anti-p21$^{Cip1/WAF1}$ antibody at 4 °C. Then, samples were incubated with the secondary antibody conjugated with FITC (Jackson Immuno Research Laboratories, Inc., PA, USA). Nuclei were dyed with PI and cells were suspended in Vectashield H-1000® (Vector Laboratories, Inc., CA, USA) and stored at −20 °C until visualization. Images were obtained with a confocal microscope Nikon C1 (objectives 40× PlanApo AN 0.95 Oil/20× PlanApo AN 0.40 Oil/10× PlanApo AN 0.25 Oil) and analyzed with Adobe Photoshop® CS5 software (Adobe Systems, Inc., CA, USA).

2.11. Statistical analysis

Results are expressed as mean ± S.E.M. of at least three independent experiments. Statistical analysis was performed by one-way ANOVA followed by Student–Newman–Keuls (SNK) *a posteriori* test or by Student test for independent samples with equal or different variance, as appropiate, using InfoStat software (Grupo InfoStat, Córdoba, Argentina). A p-value of 0.05 or less was considered statistically significant. In order to satisfy requirements to carry out the one-way ANOVA test, logarithmic transformation of data was applied when necessary, to get homoscedasticity of the variable.

3. Results

3.1. Differentiated U-937 cells become resistant to the pro-apoptotic action of DHMC

During their differentiation process, U-937 cells express p21$^{Cip1/WAF1}$ in both the nucleus and cytoplasm [9]. In agreement, normal mature peripheral monocytes constitutively express p21$^{Cip1/WAF1}$ (Fig. 1, line A). Conversely, the expression levels of this protein in immature U-937 leukemic cells is substantially lower than in monocytes (Table 1, monocytes *vs.* U-937 control cells, $p < 0.001$), and can even be considered absent (Fig. 1, line B). As expected, a 48-h treatment of U-937 cells with the differentiating agents ATRA and db-cAMP, in concentrations of 1 μM and 400 μM, respectively, significantly increased the expression levels of p21$^{Cip1/WAF1}$ in these cells (Table 1, U-937 control cells *vs.* U-937 + ATRA and U-937 control cells *vs.* U-937 + db-cAMP, $p < 0.001$. Fig. 1, lines C and D, respectively). Moreover, as shown in Fig. 2A, the occurrence of p21$^{Cip1/WAF1}$ in 1 μM ATRA-treated U-937 cells correlated with the induction of CD11b differentiation marker protein, while db-cAMP (400 μM) induced both CD11b expression as well as chemotactic response to C5a complement factor, a physiological capacity of mature monocytes [19] (Fig. 2A). Neither ATRA nor db-cAMP promoted the expression of CD14 (Fig. 2A), indicating that both differentiating agents were capable of inducing the characteristic partial differentiation status of the cells.

In order to evaluate the effect of U-937 cell differentiation on the pharmacological activities of DHMC, the sensitivity of undifferentiated (control) and differentiated U-937 cells to this coumarin was compared. Fig. 2B shows that after 24 h of treatment there was a significant decrease ($p < 0.001$) in the anti-proliferative activity of 250 μM DHMC in ATRA- and db-cAMP-differentiated cells with respect to control ones. Likewise, under the same treatment conditions, differentiated U-937 cells were more resistant to the cytotoxic effect of 250 μM DHMC, as evaluated by trypan blue exclusion and caspase-3 activity assays (Fig. 2C and D, respectively).

3.2. Cytoplasmic p21$^{Cip1/WAF1}$ confers U-937 cells resistance upon DHMC anti-proliferative and pro-apoptotic effects

With the aim of evaluating the possible involvement of p21$^{Cip1/WAF1}$ in the resistance shown by differentiated U-937 cells to the pro-apoptotic effect of DHMC, a series of assays were performed employing U-937 cells stably transfected with a variant of p21$^{Cip1/WAF1}$ (U-937/CB6-ΔNLS-p21) lacking the nuclear localization signal or sequence (NLS). In these cells, the ΔNLS-p21$^{Cip1/WAF1}$ protein cannot translocate from the cytoplasm to the nucleus, and its expression is under the control of a sheep metallothioneine heavy metal-regulated promoter [9]. Thus, induction of cytoplasmic ΔNLS-p21$^{Cip1/WAF1}$ expression can only occur after treatment of the cells with salts containing metals such as Zn or Cd.

As shown in Fig. 3, wild type U-937 cells did not register increased levels of p21$^{Cip1/WAF1}$ after a 48-h treatment with 120 μM Cl_2Zn (Line A. Table 1, U-937 control cells *vs.* U-937 + Zn^{2+}, $p > 0.05$). Similarly, U-937/CB6-ΔNLS-p21 cells did not express ΔNLS-p21$^{Cip1/WAF1}$ spontaneously (Fig. 3, line B). However, exposure of these cells to the salt induced a significant expression of ΔNLS-p21$^{Cip1/WAF1}$ (Fig. 3, line C. Table 1, U-937/CB6-ΔNLS-p21 control cells *vs.* U-937/CB6-ΔNLS-p21 + Zn^{2+}, $p < 0.001$). In contrast to our previous observation for wild type U-937 cells and in agreement with Asada et al. [9], expression of ΔNLS-p21$^{Cip1/WAF1}$ did not correlate with occurrence of differentiation in U-937/CB6-ΔNLS-p21 clone (data not shown). U-937 cells treated with 120 μM Cl_2Zn for 48 h did not show any evidence of differentiation either (data not shown). In addition, pre-treatment with Cl_2Zn did not alter the anti-proliferative activity of DHMC on U-937 cells with respect to control untreated ones (Fig. 4A, left). Nevertheless, expression of cytoplasmic p21$^{Cip1/WAF1}$ (ΔNLS-p21$^{Cip1/WAF1}$) in U-937/CB6-ΔNLS-p21 previously exposed to 120 μM Cl_2Zn for 48 h substantially decreased ($p < 0.001$) the anti-proliferative effect of DMHC (Fig. 4A, right). Analogously, while wild type U-937 pre-treated with Zn^{2+} were as sensitive to DHMC as the non-pre-treated ones (Fig. 4B, left), Cl_2Zn pre-treatment in U-937/CB6-ΔNLS-p21 rendered these cells significantly more resistant ($p < 0.001$) to the effect of the coumarin than control non-pre-treated cells (Fig. 4B, right). In line with these observations, Cl_2Zn pre-treatment did not alter the pro-apoptotic effect of DHMC in wild type U-937 cells (Fig. 4C, left). Finally, it can be seen in Fig. 4C (right) that the induction of ΔNLS-p21$^{Cip1/WAF1}$ expression by Cl_2Zn pre-treatment in U-937/CB6-ΔNLS-p21 cells significantly reduced ($p < 0.01$) DHMC-mediated caspase-3 activation in these cells with respect to non-Cl_2Zn-treated control cells after 24 h.

All in all, these findings indicate that the resistance that differentiated U-937 cells show shows to the anti-proliferative and/or cytotoxic effects of DHMC would be due to the presence of p21$^{Cip1/WAF1}$ in their cytoplasm.

3.3. JNK has a key role in the anti-proliferative and cytotoxic effects of DHMC in U-937 cells

Cell death by apoptosis is governed by complex signaling networks, containing "switches" responsible for cross talks between pro-survival and pro-apoptotic intracellular pathways. Previous studies from our laboratory indicated that the pro-apoptotic activity of DHMC in U-937 cells correlated with JNK activation [3]. Thus, in an attempt to more directly evaluate the involvement of JNK in the mechanism of DHMC action, U-937 cells were co-treated with 100 μM DHMC and the JNK inhibitor SP600125 at 20 μM for 24 h, after which the anti-proliferative and

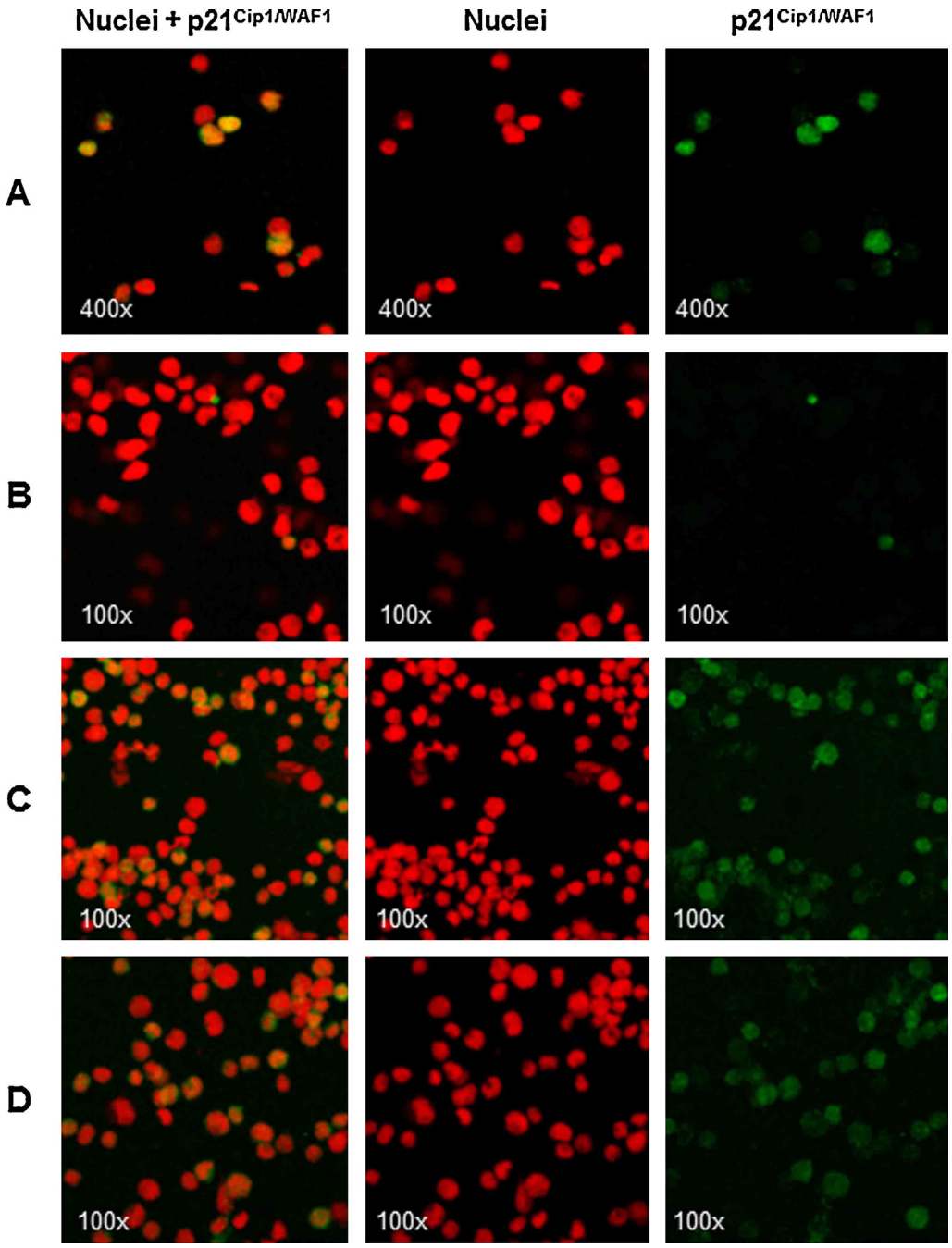


Fig. 1. Expression of $p21^{Cip1/WAF1}$ in normal monocytes, U-937 cells and U-937 cells differentiated with ATRA or db-cAMP. The above panels show the patterns of $p21^{Cip1/WAF1}$ expression in normal monocytes (A), U-937 cells (B), and U-937 cells subjected to a 48-h treatment with 1 μM ATRA (C) or 400 μM db-cAMP (D). In all cases, cells were processed for indirect immunofluorescence analysis. FITC (green) was used to stain the $p21^{Cip1/WAF1}$ protein, whereas PI (red) was used to stain cell nuclei. The panels are representative of at least three independent experiments in which similar patterns were obtained for the indicated cells/treatments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cytotoxic activities of DHMC were determined (Fig. 5A and B, respectively). As expected, JNK inhibition significantly reduced ($p < 0.001$) both the anti-proliferative and cytotoxic effects of the coumarin in U-937 cells. Moreover, it can be inferred from Fig. 5C that JNK would be a key mediator of the pro-apoptotic action of DHMC in U-937 cells, in this case evaluated by measuring caspase-3 activity.

3.4. Cytoplasmic $p21^{Cip1/WAF1}$ inhibits DHMC-mediated activation of JNK in U-937 cells

Results so far indicated that the anti-proliferative and cytotoxic effects of DHMC on U-937 promonocytic cells were substantially attenuated when these expressed $p21^{Cip1/WAF1}$ protein in their cytoplasm, providing a suitable explanation for the naturally occurring resistance of mature monocytes to the cytotoxic effect of the drug. Thus, based on our observations indicating a crucial role for JNK as mediator of DHMC-induced pharmacological effects in U-937 leukemic cells, and the well-established capacity of $p21^{Cip1/WAF1}$ to inhibit the JNK pathway [20,21], we evaluated the potential influence of $p21^{Cip1/WAF1}$ on JNK phosphorylation.

As shown in Fig. 6, DHMC induced a time-dependent increase in the phosphorylation of JNK in U-937/CB6-ΔNLS-p21 cells. However, no effect of the coumarin on JNK was found if these cells were pre-treated with Cl_2Zn to induce cytoplasmic ΔNLS-$p21^{Cip1/WAF1}$

Table 1
$p21^{Cip1/WAF1}$ expression in different cell types and/or treatment conditions.

Cell type/treatment	$p21^{Cip1/WAF1}$- or ΔNLS-$p21^{Cip1/WAF1}$-expressing cells (%)
Monocytes	82.86 ± 4.04
U-937 control cells	11.02 ± 9.30
U-937 + ATRA	53.33 ± 5.61
U-937 + db-cAMP	57.81 ± 7.02
U-937 + Zn^{2+}	4.34 ± 2.15
U-937/CB6-ΔNLS-p21 control cells	4.43 ± 1.44
U-937/CB6-ΔNLS-p21 + Zn^{2+}	90.10 ± 6.88

The percentage of cells that express $p21^{Cip1/WAF1}$ or ΔNLS-$p21^{Cip1/WAF1}$, depending on cell type and/or treatment, was determined by counting at least 500 cells in different visual fields of three comparable independent assays. Results are shown as the mean ± SEM.

expression prior to DHMC treatment (Fig. 6). These findings indicate that this protein would inhibit the phosphorylation of JNK in U-937 cells, conferring them resistance to the DHMC-induced anti-proliferative and cytotoxic activities.

3.5. Sub-acute toxicity study of DHMC in Balb/c mice

There are many research publications on the toxicity of coumarin (Scheme 2), both in animals as well as in humans [22]. On the other hand, no toxicological studies of DHMC have been carried out to date. Thus, with the aim of determining the effect of the drug on bone marrow hematological precursors as well as in other tissues, a sub-acute toxicological assay of DHMC

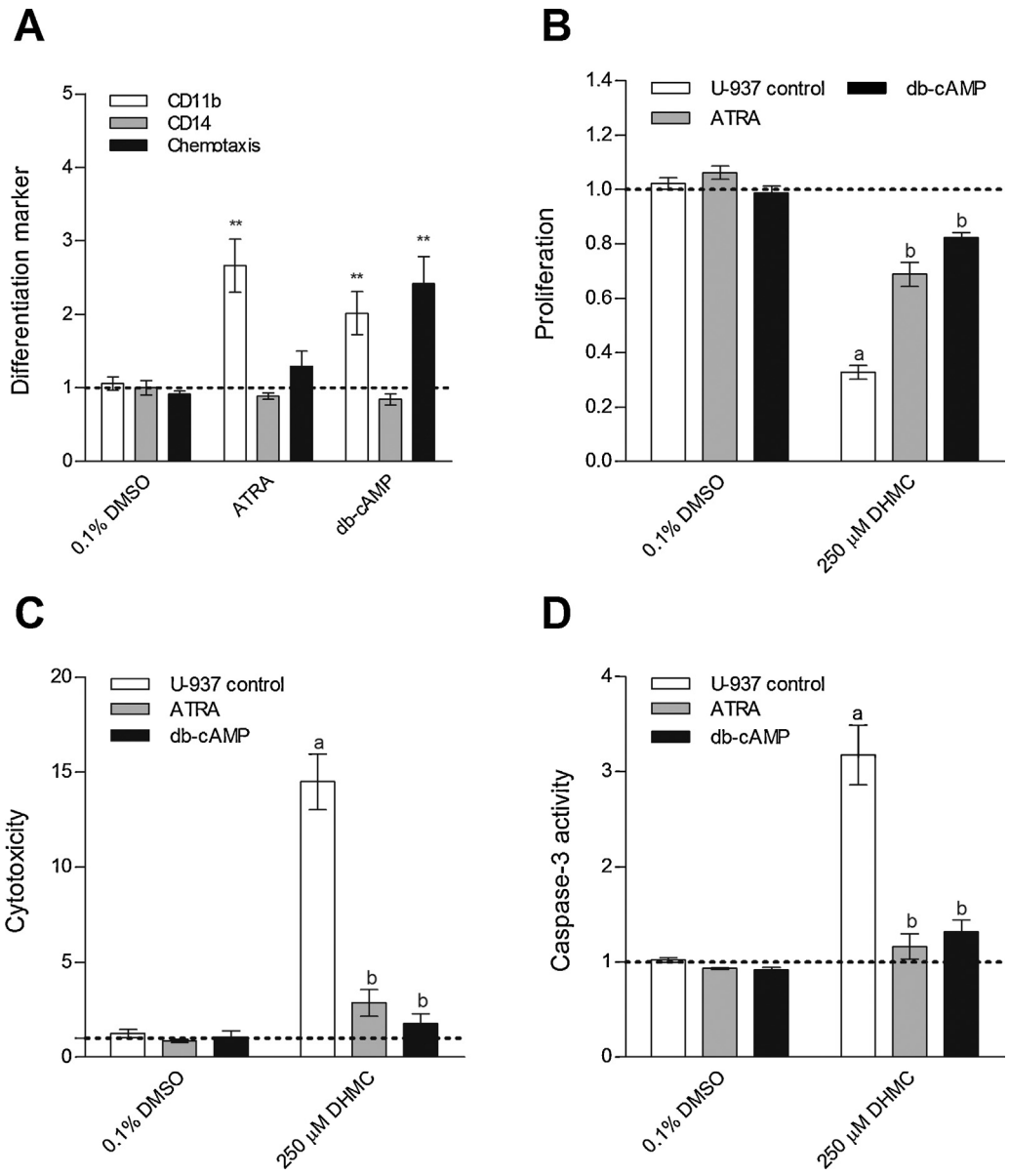


Fig. 2. Effect of U-937 cell differentiation on the pharmacological activities of DHMC. Cells were treated with 1 μM ATRA, 400 μM db-cAMP or 0.1% DMSO (v/v) (vehicle control group) for 48 h, after which the expression of CD11b and CD14 membrane proteins, and chemotaxis in response to rhC5a, were evaluated as indicators of U-937 differentiation (A). Cells were then washed and incubated with 250 μM DHMC or DMSO 0.1% (v/v) (vehicle control group). After 24 h of treatment, the effects of DHMC on cell proliferation (B), viability (C) and apoptosis (D) were evaluated by determination of cell number, trypan blue exclusion assay and measurement of caspase-3 activity, respectively. In all cases, the effects are presented relative to those measured for the corresponding untreated controls. Significant differences were determined by one-way ANOVA test ($p < 0.001$) followed by SNK *a posteriori* test. In graphic A, the two asterisks (**) indicate that the level of differentiation marker expression induced by a given differentiating agent is significantly different ($p < 0.01$) to that induced by the corresponding vehicle. Different letters above the bars indicate significant differences between pre-treatments (1 μM ATRA, 400 μM db-cAMP or 0.1% DMSO) on the activity of DHMC ($p < 0.001$). Logarithmic transformation of the variable was applied before the ANOVA test if required. Each bar and vertical line represents the mean ± SEM ($n > 3$).

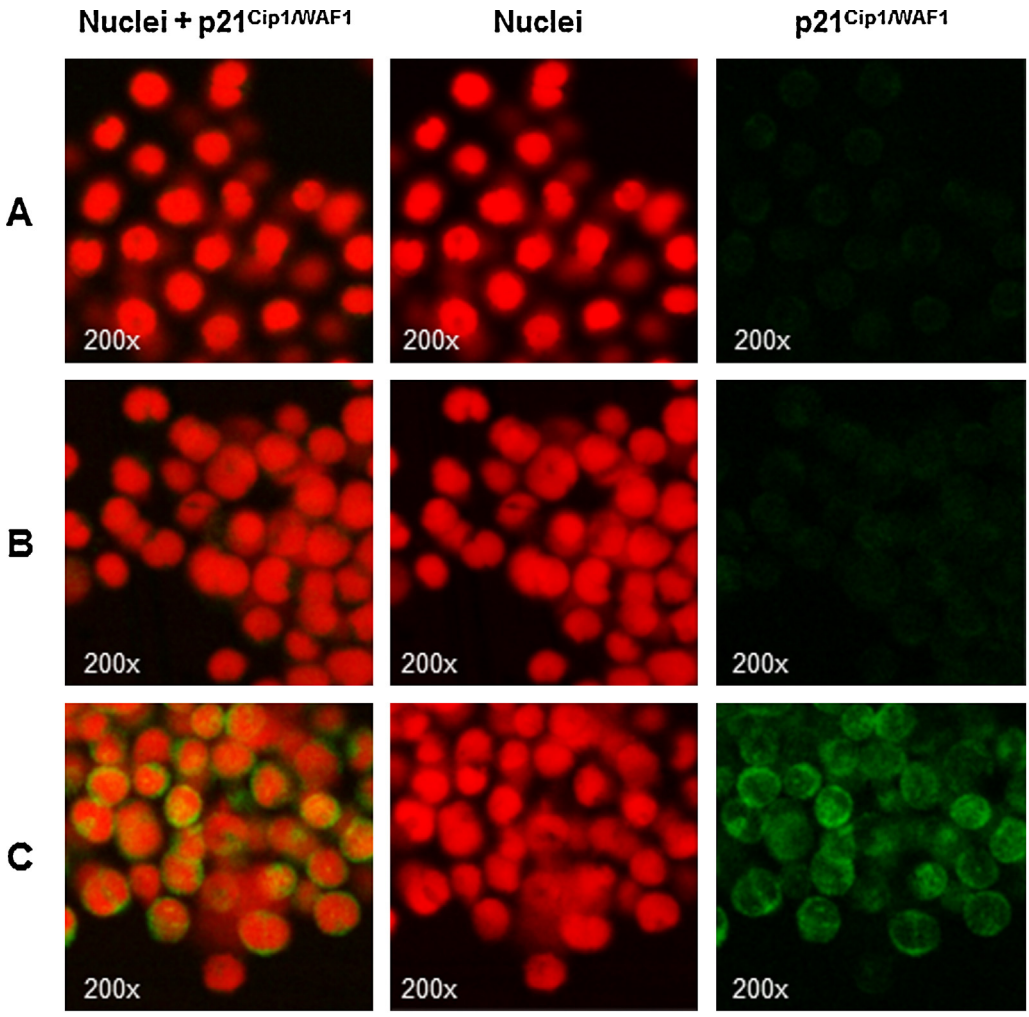


Fig. 3. Evaluation of cytoplasmic ΔNLS-$p21^{Cip1/WAF1}$ expression in U-937/CB6-ΔNLS-p21 cells treated with Zn^{2+}. After 48 h, 120 μM $ZnCl_2$-treated U-937 cells, untreated U-937 cells and 120 μM $ZnCl_2$-treated U-937/CB6-ΔNLS-p21 cells (lines A, B and C, respectively) were processed for indirect immunofluorescence analysis in order to determine the percentage of cells expressing $p21^{Cip1/WAF1}$ (U-937 cells) or ΔNLS-$p21^{Cip1/WAF1}$ (U-937/CB6-ΔNLS-p21). FITC (green) was used to stain the protein, whereas PI (red) was used to stain cell nuclei. Pictures are representative of at least three independent experiments in which similar patterns were obtained. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was carried out in Balb/c mice, employing coumarin as reference compound.

During the treatment period, both coumarin and DHMC were well tolerated, as judged from the fact that clinical appearance – consisting of evaluation of mobility, ruffled hair, and hunched back posture – and body weight of coumarin-, DHMC- and vehicle-treated mice were indistinguishable from those of untreated control animals (Fig. 7). No animal deaths were registered during the experiment.

After 15-day treatments, no significant lesions were observed in mice bone marrow, kidney and intestine tissues for all doses of DHMC and coumarin (Fig. 8, lines A, B and C, respectively). The same was observed in animals sacrificed 15 days after their last dose (data not shown). On the other hand, while mice treated with the vehicle, coumarin or DHMC showed small focal necrosis areas spread throughout the liver, regardless of the drug dose (Fig. 8, line D), untreated-control animals presented normal tissue histology. These findings were observed both in mice sacrificed 48 h and 15 days after their last dose, and they suggest that the cited liver damage was induced by the vehicle.

4. Discussion

The development of chemotherapeutic agents with ability to selectively target tumor cells and low rate of generation of drug-resistant variants has remained a constant challenge for over more than six decades. In recent years, coumarin compounds have attracted much attention due to the wide variety of pharmacological activities that they display [23–25]. In this regard, over the past four years more than forty inventions have been approved for patents worldwide, which relate to both novel and well-known coumarin derivatives acting as active ingredients in medicines, and/or being presented as potential therapeutic agents based on their anti-tumor effects [25]. Indeed, the use of these compounds as chemotherapeutic agents with cytotoxic, differentiating and/or enzyme-inhibiting activities seem to be their most promising clinical application [24,25].

DHMC has been well studied due to its pro-apoptotic activity in several cancer cell lines [2,3,6,7]. Recently, our group elucidated the structural requirements for DHMC-triggered apoptosis in U-937 cells [2,6], further establishing that activation of the intrinsic pathway and generation of reactive oxygen/nitrogen species (ROS/RNS) are key events in the mechanism of apoptosis induction by the coumarin [3,6], which also involves down-regulation of p-Akt and p-ERK 1/2 [3]. However, the current knowledge of the signaling pathways that mediate the pro-apoptotic activity of DHMC does not allow spelling out the cause of its selectivity for cancer over normal blood cells.

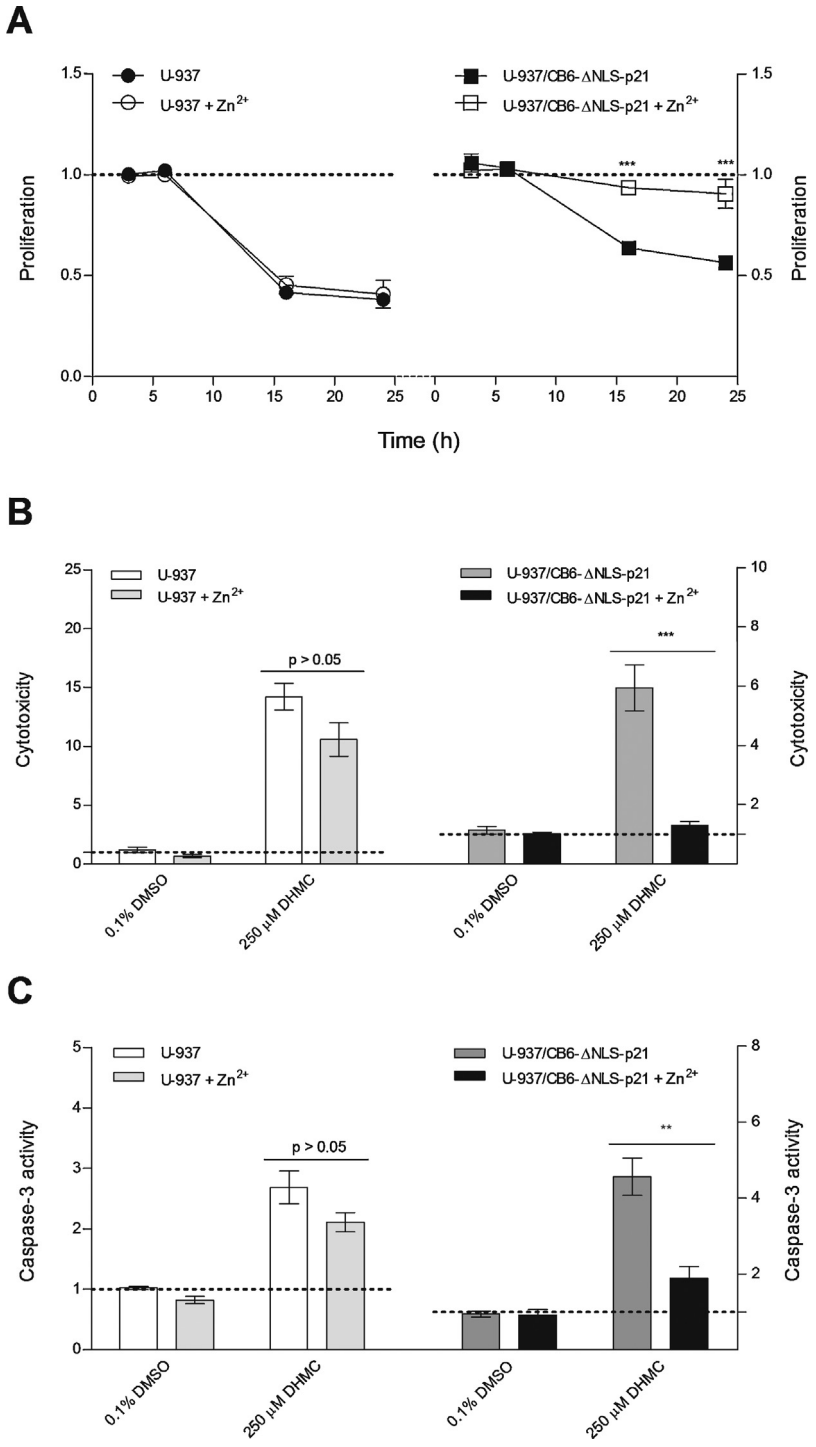


Fig. 4. The anti-proliferative and pro-apoptotic activities of DHMC are abrogated in ΔNLS-p21$^{Cip1/WAF1}$-expressing U-937/CB6-ΔNLS-p21 cells. The expression of ΔNLS-p21$^{Cip1/WAF1}$ in U-937/CB6-ΔNLS-p21 cells was induced by treatment of the cells with 120 μM Cl_2Zn for 48 h. Simultaneously, U-937 cells were also incubated with the salt in order to determine the possible influence of this treatment on the effect of DHMC. Both cell types, pre-treated or not with Cl_2Zn, were then washed and incubated with 250 μM DHMC or 0.1% (v/v) DMSO (vehicle control group) for 24 h. During this period, the anti-proliferative activity of DHMC was evaluated at different times (3, 6, 16 and 24 h) both in U-937 and U-937/CB6-ΔNLS-p21 cells (A; left and right graphics, respectively). The viability of control, DHMC- and 0.1% DMSO-treated cells was determined by trypan blue exclusion assay in U-937 and U-937/CB6-ΔNLS-p21 cells after 24 h (B; left and right graphics, respectively). In the same way, caspase-3 activity was determined in both cell types and their corresponding controls after 24 h of DHMC treatment (C; left and right graphics, respectively). In all cases, the effects are presented relative to those measured for untreated control cells. The statistical analysis of the influence of Zn^{2+} on the effects of DHMC in U-937 and U-937/CB6-ΔNLS-p21 cells at different times (A), and after 24 h (B and C), was performed using the Student test for unpaired samples with equal or unequal variances, as appropriate (***$p < 0.001$; **$p < 0.01$). Each bar and vertical line represents the mean ± SEM ($n > 3$).

Herein, we not only reveal the key role played by the JNK pathway in DHMC-induced apoptosis in U-937 promonocytic leukemic cells, but also provide the first evidence that activation of this pathway by DHMC, as well as its consequent pro-apoptotic effect, are inhibited in the presence of cytoplasmic p21$^{Cip1/WAF1}$. These findings point to cytoplasmic p21$^{Cip1/WAF1}$ as the crucial factor determining the previously described resistance of normal monocytes to the cytotoxic effect of DHMC [3,6,7].

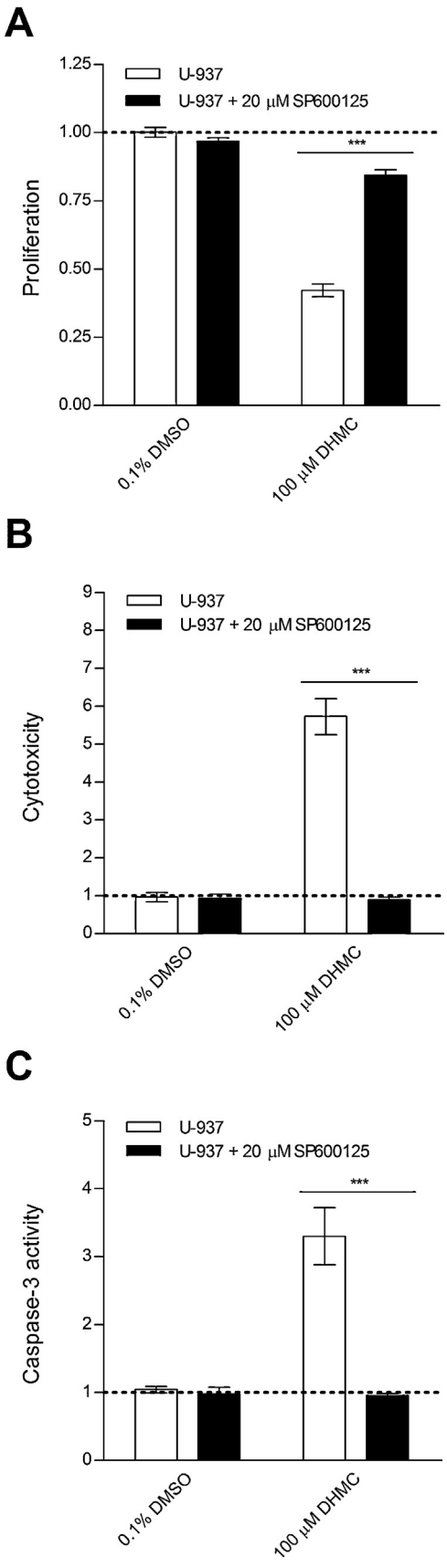


Fig. 5. Role of JNK in the anti-proliferative and cytotoxic activities of DHMC in U-937 cells. After a 1-h exposure to the JNK inhibitor SP600125 (20 μM), U-937 cells were co-treated with the inhibitor and 100 μM DHMC or DMSO 0.1% (v/v) (vehicle control group) for 24 h. Simultaneously, U-937 control cells were treated with 100 μM DHMC or vehicle. The statistical analysis of the effect of SP600125 on the anti-proliferative (A), cytotoxic (B) and pro-apoptotic (C) effects of 100 μM DHMC was performed using the Student test for paired samples with equal or unequal variances, as appropriate ($^{***}p < 0.001$). In all cases, the effects are presented relative to those measured for untreated control cells. Each bar and vertical line represents the mean ± SEM ($n > 3$).

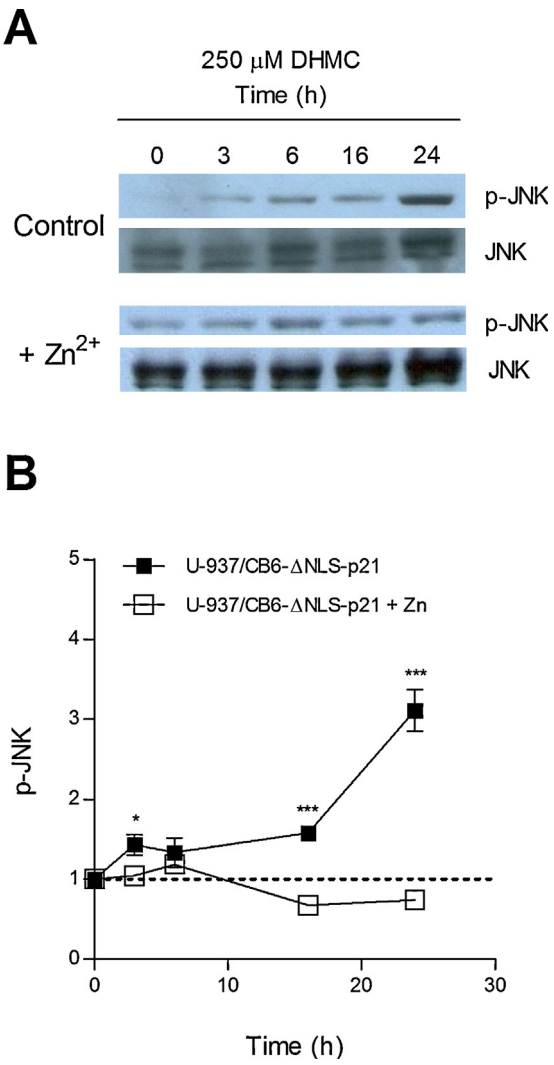
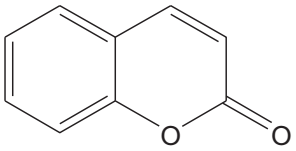


Fig. 6. ΔNLS-p21$^{Cip1/WAF1}$ interferes with DHMC-induced activation of JNK in U-937/CB6-ΔNLS-p21 cells. After 24 h, U-937/CB6-ΔNLS-p21 cells incubated (+Zn^{2+}) or not with 120 μM Cl_2Zn (control) were washed, treated with 250 μM DHMC and harvested at the indicated times (3, 6, 16 and 24 h). Equal amounts of proteins for each time condition were subjected to SDS–page and analyzed by Western blot with anti-p-JNK and -JNK antibodies (A). Graphic B shows the densitometry analyses of p-JNK and JNK Western blots employing ImageJ program. Data in *Y* axis represent densitometric values for p-JNK with respect to time 0, relative to total JNK. In A data are representative of at least three independent experiments in which the same pattern was obtained. In graphic B, dots indicate the mean ± SEM of one experiment, representative of other three independent ones. The statistical analysis of the influence of Zn^{2+}-induced ΔNLS-p21$^{Cip1/WAF1}$ expression on DHMC-dependent p-JNK activation was performed using Student test for unpaired samples with equal or unequal variances, as appropriate ($^{***}p < 0.001$; $^{*}p < 0.05$).

It has been well demonstrated that p21$^{Cip1/WAF1}$ exerts its anti-apoptotic effect against several stimuli in different cell models by N-terminal domain-mediated interactions with JNK, pro-caspase-3 and the apoptosis signal-regulating kinase 1 (ASK1) [20,21,26,27], or by induction of G_1 arrest in response to p21$^{Cip1/WAF1}$ binding to cyclin A and cyclin E/CDK2 [reviewed in [28]]. Of note, ΔNLS-p21$^{Cip1/WAF1}$ protein employed herein cannot translocate to the nucleus, hence ruling out the latter potential intracellular pathway for p21$^{Cip1/WAF1}$ action in U-937 cells. Therefore, it is most likely that the resistance to DHMC-induced



Scheme 2. Coumarin.

apoptosis conferred by cytoplasmic $p21^{Cip1/WAF1}$ may be due to inhibition of JNK, ASK1 and/or procaspase-3, which could occur simultaneously or sequentially. It is worth remarking that the MAPK kinase kinase (MAP3K) ASK1 lies up-stream of JNK, and can induce its activation in response to various stressors [29]. Thus, ASK1 could be the link between DHMC-generated pro-oxidant species and up-regulation of p-JNK in U-937 cells, since its kinase activity is inhibited by reduced thioredoxin [29], a ROS/RNS-oxidable protein that can sense changes in the intracellular redox balance [30]. Certainly, this hypothesis requires further investigation.

A question that emerges from the findings presented herein is whether the potential chemotherapeutic application of DHMC could be limited by tumor expression of $p21^{Cip1/WAF1}$. However, DHMC may not necessarily act through the same mechanism of action in all tumor cells in which it induces apoptosis. In this regard, according to Goel et al., generation of ROS/RNS in A549 human lung cancer cells subjected to DHMC treatment is not a key determinant for apoptosis induction by this coumarin [7]. Instead, our previous findings demonstrated that the pro-oxidant effect of DHMC and the subsequent increase in ROS/RNS intracellular levels are crucial for induction of apoptosis in U-937 promonocytic leukemia cells [6]. Also, the DHMC precursor 7,8-diacetoxy-4-methylcoumarin, which originates DHMC by intracellular deacetylation, induces mitochondria-mediated apoptosis in the A549 cell line. Nevertheless, contrary to what we described for U-937 cells [3], this effect would be mediated by Akt activation and down-regulation of p-JNK and p-38 MAPK [31]. Therefore, it is most likely that the resistance to DHMC that $p21^{Cip1/WAF1}$ might confer to a given cell lineage would crucially depend on the occurrence of $p21^{Cip1/WAF1}$ mediated-interference with key steps in the mechanism of apoptosis induction by the coumarin in such particular cell type. Certainly, the screening for pro-apoptotic activity of DHMC in cell lines and/or patient-derived primary cell cultures with different expression levels of cytoplasmic $p21^{Cip1/WAF1}$ will shed light on this issue.

Because the incidence of cancer increases with advancing age, during drug based anti-leukemic therapy, attention must be paid to the presence of age-related organ dysfunction, chemotherapy-induced side effects such as end organ-targeted toxicity, or induction of myelosuppression and the consequent neutropenia by cytotoxic agents [32]. With the aim of evaluating the potential induction of toxicity by DHMC in immature hematological cells as well as in renal and hepatic cells – usually susceptible to the toxic effects of several anti-tumor drugs -, and intestine – lined up with cells that have a high turn-over and are also drug-sensitive -, we performed a sub-acute toxicology assay in Balb/c mice. Interestingly, DHMC induced no significant toxicity in any of these tissues, even at a dose of 100 mg/kg. Assuming that this drug was fully absorbed and homogeneously distributed when administered intraperitoneally, and considering a mice average weight of approximately 22 g and a volemia plus extracellular fluids of about 3 ml [33], the aforementioned dose would be equivalent to an *in vitro* DHMC concentration of 4.34 mM, which is more than fifty times higher than the concentration needed to inhibit U-937 cell proliferation by 50% after 48 h of treatment [6]. What is more, DHMC showed the same toxicological profile as coumarin, a

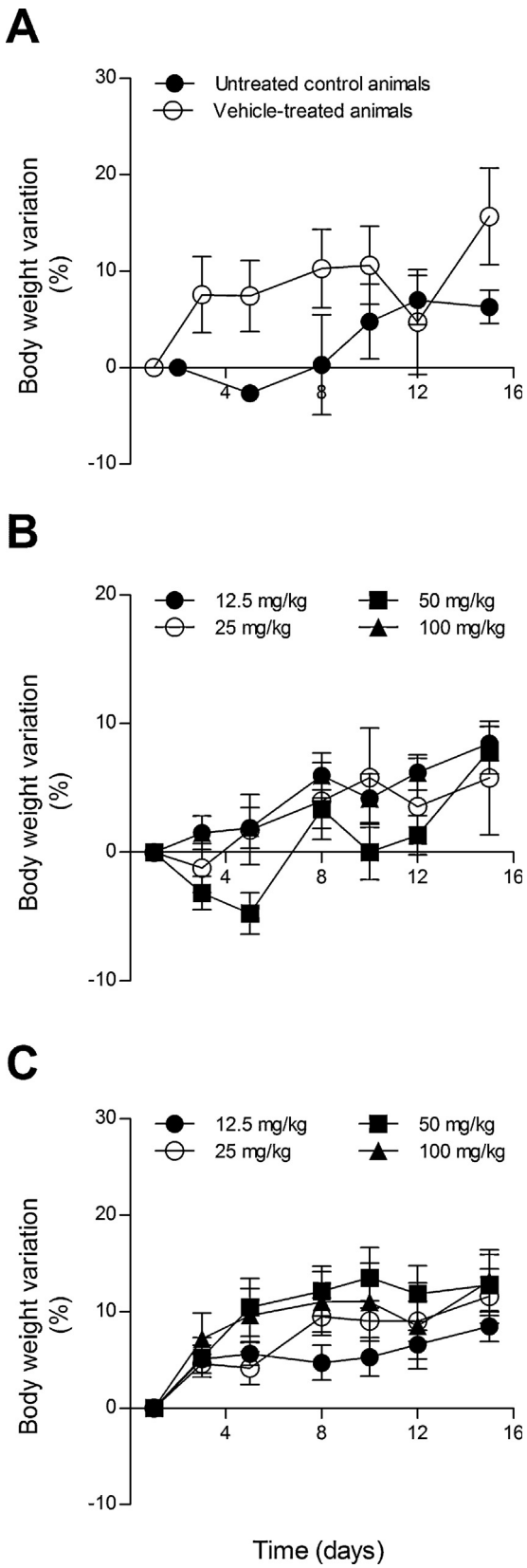


Fig. 7. Balb/c mice body weight variation during treatment with DHMC and coumarin. The graphs show the body weight percentage variation, with respect to the original weight (day 0), during a 15-day sub-acute toxicity assay for untreated and vehicle-treated control animals (A), mice treated with the indicated doses of coumarin (B) and animals treated with the indicated doses of DHMC (C). Each point and vertical line represents the mean ± SEM, respectively ($n = 5$).

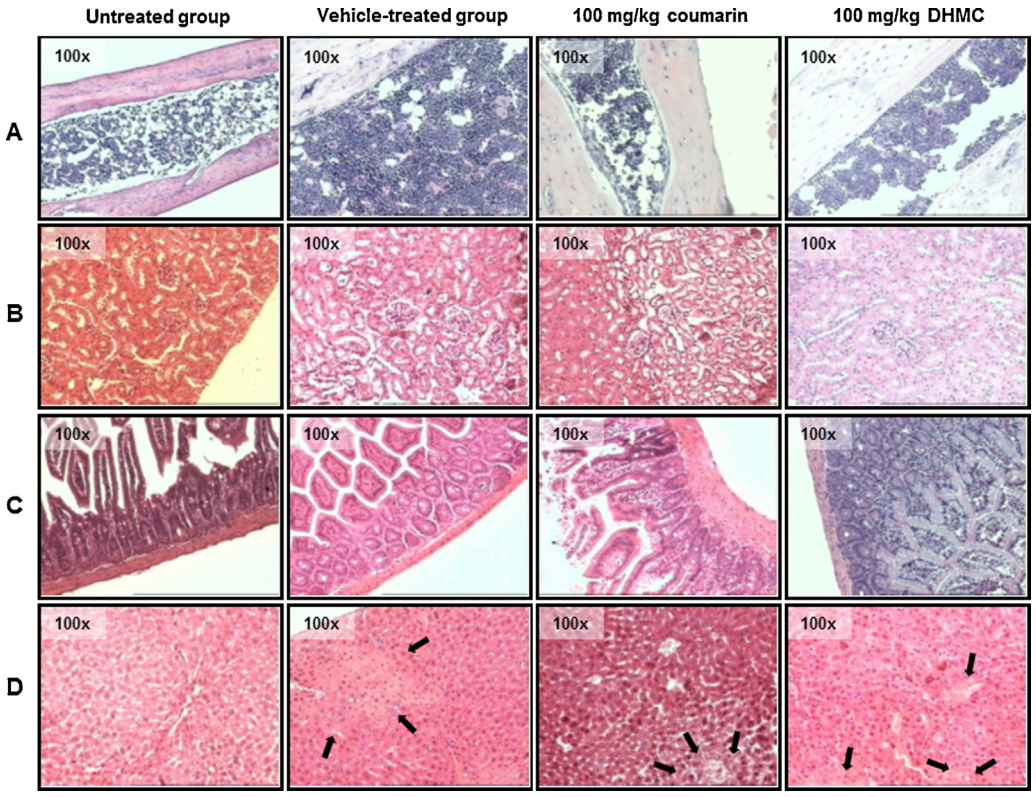


Fig. 8. Histopathological study of the effects of DHMC- and coumarin treatment in Balb/c mice. After 48 h of the last administered dose of DHMC and coumarin, three mice from each experimental group were sacrificed in order to evaluate the presence of histopathological lesions in selected organs. The above panels show photographs of bone marrow (A), kidney (B), intestine (C) and liver (D) from control (untreated) mice, vehicle-treated mice and mice injected with coumarin and DHMC at a dose of 100 mg/kg. Only the highest doses are shown as representative of the rest of the treatments with these compounds. Black arrows point to focal necrosis areas in hepatic tissue. Comparable results were obtained in all animals of each treatment group.

compound with current applications in multiple fields including food, cosmetics and pharmaceutical industries [22,34]. On the basis of these observations, induction of toxicity should not be a matter of concern when evaluating the *in vivo* anti-tumor activity of DHMC. Indeed, our data encourage further studies evaluating the pharmacological efficacy of this compound in human xenograft models, in which the use of the vehicle employed herein should be avoided to prevent hepatic damage.

All in all, our present results convincingly explain the selective cytotoxicity of DHMC for tumor cells over normal monocytes, and at the same time reveal new important clues into the mechanism of action of this coumarin in leukemic cells that will surely guide future investigations. In this regard, we are of the opinion that a deep understanding of the mode of action of cytotoxic drugs, as well as of the mechanisms underlying drug resistance, is certainly the key to optimizing treatment outcomes. Importantly, we also provide the first evidence that DHMC has no systemic toxicity under the conditions of this study, encouraging further *in vivo* research on this compound as potential anti-leukemic agent. Overall, we expect that our findings will contribute to the future development of DHMC-derived coumarins with clinical application in chemotherapy regimens.

Disclosure of potential conflicts of interest

The authors declare no conflicts of interest.

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