Bioorganic & Medicinal Chemistry 20 (2012) 5537–5549

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Structure-anti-leukemic activity relationship study of *ortho*-dihydroxycoumarins in U-937 cells: Key role of the δ-lactone ring in determining differentiation-inducing potency and selective pro-apoptotic action

Ramiro Vázquez [a,b,*], María E. Riveiro [a], Mónica Vermeulen [c], Eliana Alonso [b], Carolina Mondillo [d], Graciela Facorro [e], Lidia Piehl [e], Natalia Gómez [b], Albertina Moglioni [b], Natalia Fernández [b], Alberto Baldi [a], Carina Shayo [a], Carlos Davio [b,*]

[a] *Laboratorio de Patología y Farmacología Molecular, Instituto de Biología y Medicina Experimental (IBYME)-CONICET, Vuelta de Obligado 2490 (C1428ADN), Buenos Aires, Argentina*
[b] *Cátedra de Química Medicinal, Facultad de Farmacia y Bioquímica-UBA, Junín 954 (C1113AAD), Buenos Aires, Argentina*
[c] *Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Av. Las Heras 3092 (C1425ASU), Buenos Aires, Argentina*
[d] *Laboratorio de Endocrinología Molecular y Transducción de Señales, Instituto de Biología y Medicina Experimental (IBYME)-CONICET, Vuelta de Obligado 2490 (C1428ADN), Buenos Aires, Argentina*
[e] *Cátedra de Física, Facultad de Farmacia y Bioquímica-UBA, Junín 954 (C1113AAD), Buenos Aires, Argentina*

ARTICLE INFO

Article history:
Received 11 May 2012
Revised 8 July 2012
Accepted 16 July 2012
Available online 2 August 2012

Keywords:
Coumarins
Catechol
Apoptosis
Differentiating activity
Selective cytotoxicity
Leukemia

ABSTRACT

Previous studies indicated the need of at least one phenolic hydroxyl group in the coumarin core for induction of cytotoxicity in different cell lines. Herein, we present an exhaustive structure-activity relationship study including *ortho*-dihydroxycoumarins (*o*-DHC) derivatives, cinnamic acid derivatives (as open-chain coumarin analogues) and 1,2-pyrones (representative of the δ-lactone ring of the coumarin core), carried out to further identify the structural features of *o*-DHC required to induce leukemic cell differentiation and apoptosis in U-937 cells. Our results show for the first time that the δ-lactone ring positively influences the aforementioned biological effects, by conferring greater potency to compounds with an intact coumarin nucleus. Most tellingly, we reveal herein the crucial role of this molecular portion in determining the selective toxicity that *o*-DHC show for leukemic cells over normal blood cells. From a pharmacological perspective, our findings point out that *o*-DHC may be useful prototypes for the development of novel chemotherapeutic agents.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Chemotherapeutic agents constitute the most effective strategy in the fight against leukemic malignancies. However, chemotherapeutics currently used in cancer therapies are often significantly cytotoxic and non-specific. Over the years, the identification of novel effective drugs for the treatment of leukemia has remained a focus of intense interest. In this regard, a major challenge is to develop anti-cancer agents that are highly effective and exhibit selective toxicity for cancer cells.

Coumarins (known as 1,2-benzopyrones or *ortho*-hydroxycinnamic acid-8-lactones) comprise a very large class of naturally occurring phenolic derivatives found in plants, fungi, bacteria and animals.[1,2] Coumarin could be considered the resulting fusion of benzene and an 1,2-pyrone ring (δ-lactone). Thus, to study the coumarin nucleus, this could be separated into a benzenoid and an 1,2-pyrone constituent, each with different chemical properties. Coumarins have attracted considerable interest for decades because of their large variety of pharmacological activities, namely anti-bacterial,[3] oral anti-coagulant,[4,5] anti-mutagenic[6] and anti-inflammatory properties,[7] as well as capacity to inhibit human platelet aggregation,[8] reactive oxygen species (ROS) scavenging capacity[9] and anti-HIV activity.[10] As for their anti-tumor effects, the apoptosis and differentiation-inducing activities of coumarins

Abbreviations: ATP, adenosine triphosphate; CC_{50}, cytotoxic concentration 50; CI95, confidence interval 95%; db-cAMP, dibutyryl cyclic adenosine monophosphate; DCF-DA, 2′,7′-dichlorofluorescein diacetate; DHCA, dihydrocaffeic acid; DHMC, 7,8-dihydroxy-4-methylcoumarin; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ESR, electron spin resonance; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; IC_{50}, inhibitory proliferation concentration 50; *o*-DHC, *ortho*-dihydroxycoumarins; PARP, poly-(ADP-ribose)-polymerase; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PI, propidium iodide; rhC5a, recombinant human complement factor C5a; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate.

* Corresponding authors. Tel.: +54 11 4783 2869; fax: +54 11 4786 2564 (R.V.); tel.: +54 11 4964 8233 (C.D.).
E-mail addresses: ramirobioq@hotmail.com (R. Vázquez), carlosdavio@hotmail.com (C. Davio).

0968-0896/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2012.07.043

extend to several different cell line models in vitro, and they appear to be the most promising in terms of cancer treatment.[11]

For the last several years, the central focus of our laboratory has been the screening of novel compounds with potential application as differentiating and/or cytotoxic agents acting selectively on hematopoietic cancer cells. In this regard, in previous publications we described the differentiation-inducing activity of natural 6,7-methylenedioxy-5-oxycoumarins isolated from *Pterocaulon polystachyum* in U-937 cells, as well as the structural requirements for such activity.[12,13] Later, we reported the pro-apoptotic effect of 7,8-dihydroxycoumarin (DHMC, **1**, Table 1) on U-937 and HL-60 myeloid leukemia cells, activity that would be dependent on oxidative stress generation.[14,15]

Generally speaking, the pharmacological and biochemical properties of simple coumarins depend upon their structures. In this respect, previous reports by our group have documented the prominent role of phenolic residues, particularly when constituting a catechol moiety, in the pro-apoptotic activity of hydroxycoumarins in U-937 cells.[14,15] With the aim of further identifying the structural features of *ortho*-dihydroxycoumarins (*o*-DHC) required for apoptosis induction, in this work we performed an exhaustive structure-anti-leukemic activity relationship (SAR) study of a series of **1** derivatives, cinnamic acid derivatives (open-chain coumarin analogues) and 1,2-pyrones (representative of the δ-lactone ring of the coumarin system), as a means of dissecting the coumarin ring of **1** to analyze the relative contribution of its constituent parts, particularly the δ-lactone ring. Moreover, based on the aforementioned capacity of *P. polystachyum*-derived cumarins to induce U-937 cell differentiation,[12,13] we also evaluated the differentiating potential of *o*-DHC in U-937 cells, as well as the structural determinants of such activity.

To the best of our knowledge, our results demonstrate for the first time the close link between the capacity of *o*-DHC to generate free radical species and the induction of caspase-9 mediated apoptosis in U-937 cells. We also provide the first compelling evidence that the δ-lactone ring of the coumarinic system has a fundamental role in both the generation and stabilization of such species as well as in the pro-apoptotic action of *o*-DHC. Most tellingly, we demonstrate herein that the δ-lactone ring not only determines the selective toxicity that *o*-DHC show for leukemic cells over normal peripheral blood cells but also confers greater differentiating potency, highlighting the importance of the integrity of the coumarin ring for the biological actions of *o*-DHC in U-937 cells.

Collectively, our results point to *o*-DHC as potential candidates for the design of novel anti-leukemic drugs.

2. Results and discussion

2.1. Effects of coumarins, 1,2-pyrones and cinnamic acid derivatives on U-937 cells viability and proliferation

Firstly, in order to shortlist potential pro-apoptotic and/or differentiating compounds from the coumarin-like derivatives listed in Table 1 (**1**–**14**), we evaluated the effects of these compounds on U-937 cells viability and proliferation after 48 h of treatment. Table 2 shows that coumarins bearing an *ortho*-dihydroxy substitution in their structure (DHMC, **1**; fraxetin, **2**; esculetin, **3** and **4**) exerted a higher cytotoxicity and growth inhibitory activity compared to **5**, which has a *meta*-dihydroxy susbtitution (Table 2, 1–**4** vs **5**, $p < 0.05$), or monohydroxy derivatives (Table 2, 1–**4** vs **10**, **13** and **14**, $p < 0.05$). Likewise, substitution of one of the adjacent hydroxyl groups by a methoxyl residue (**8**), a chloro atom (**9**) or an acetyl group (**11**) led to a significant decrease in coumarin activity on cellular viability compared with that of *o*-DHC (Table 2, **8**, **9** and **11** vs **1**–**4**, $p < 0.05$). Similar results were obtained when both hydroxyl groups were replaced with methoxyl residues (Table 2, **6** and **7** vs **1**–**4**, $p < 0.05$). Finally, the monoamine derivative **12** was found inactive. Taken collectively, these results indicate the need of a catechol moiety in the coumarin ring for induction of toxicity in the U-937 leukemic cell line.

Of note, although neither coumarin **10** nor quinolone **13** induced cytotoxicity in U-937 cells, the latter showed a lesser CI_{50} value than its coumarin analogue **10** ($p < 0.05$; Table 2), suggesting that the higher stability that the δ-lactam confers to the quinolone system would correlate with an increased potency of growth inhibitory activity with respect to the coumarin bearing a δ-lactone ring.

In line with data presented herein for coumarin-like compounds, only catechol-containing cinnamic acid derivatives (**19** and **20**) showed inhibition of cell proliferation, whereas 3,4-dimethoxy-substituted derivatives **21** and **22**, the monohydroxy derivative **24** or the non-hydroxy derivative **23** were totally inactive. Interestingly, CC_{50} values for **19** and **20** were higher than 2000 μM (Table 2).

As shown in Table 2, the 1,2-pyrones did not display any biological activity at any time or concentration evaluated.

In good agreement with the results described above, further FACS studies showed that only *o*-DHC were able to significantly increase ($p < 0.001$) the percentage of U-937 cells in the sub G0/G1 phase of the cell cycle (Fig. 1A). What is more, clonogenic survival assays indicated that U-937 cells subjected to a 24-h exposure to *o*-DHC at concentrations of 100 μM or higher showed significantly decreased ($p < 0.001$) capacity to form colonies in soft agar, pointing out that catechol-bearing coumarins induce irreversible loss of viability in these cells (Fig. 1B). Conversely, compounds **6**, **10**, **19** and **20** did not alter the clonogenic capacity of U-937 in soft agar (data not shown).

In sum, our findings point out that, although the δ-lactone portion does not exert biological activity by itself, it increases the potency of the growth inhibitory activity of the catechol moiety on U-937 cells, and plays a crucial role in determining the cytotoxic action of *o*-DHC.

2.2. Pro-apoptotic activity of coumarins and hydroxylated cinnamic acid derivatives on U-937 cells

As marker of the apoptotic process, we evaluated the exposure of phosphatidylserine on the outer leaflet of the plasma membrane by using annexin V binding assay. U-937 cells were treated with all the compounds listed in Table 1, but only results corresponding to some of them are shown. Figure 2A shows the distribution of U-937 cells pre-treated with **1**, **6** (for 24 h), **20** and 2% DMSO (for 48 h) over quadrants, after double staining with annexin V/propidium iodide (PI). Quantitative evaluation of the percentages of normal, early apoptotic, late apoptotic and necrotic U-937 cells after treatment with the cited compounds for 24 h indicated that only *o*-DHC **1**–**4** induced a reduction in the fraction of normal cells with respect to the control group, with an increase in early apoptotic and late apoptotic subpopulations ($p < 0.01$) (Fig. 2B). In contrast, as seen in Figure 2B, neither the catechol-bearing-compound **19** nor **20** provoked a significant reduction in the subpopulation of normal cells compared to the untreated group ($p > 0.05$). The remaining compounds listed in Table 1 did not decrease significantly the percentage of normal cells with respect to the control group after 24 h at a concentration of 500 μM (data not shown).

To confirm the above results, we also studied the activity of caspase-3 enzyme. As expected, *o*-DHC **1**–**4** (250 μM) induced caspase-3 activation in U-937 cells after 24 h of treatment (Fig. 2C), whereas the rest of the coumarin-like compounds in Table 1 (**5**–**14**) were ineffective even at twice the concentration of **1**–**4**. The lack of effect of some of these compounds (**5**–**9**) is shown in

Table 1
Compounds used in the SAR study

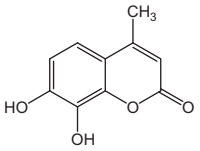
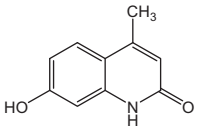
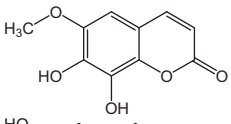
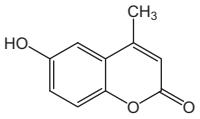
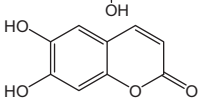
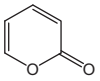
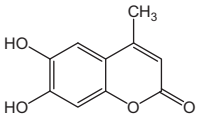
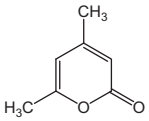
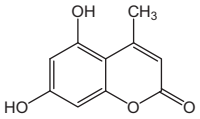
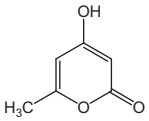
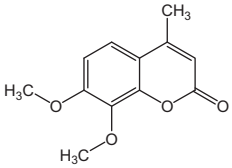
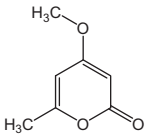
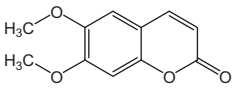
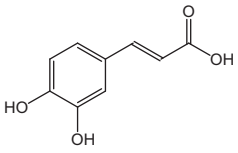
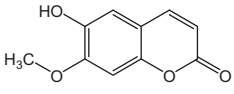
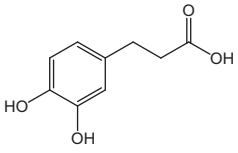
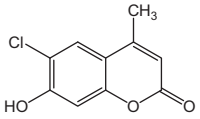
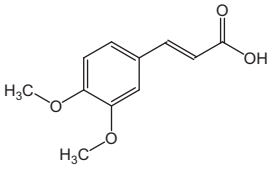
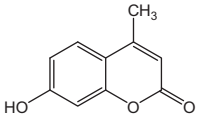
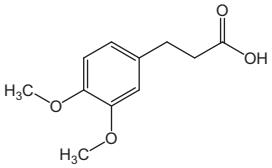
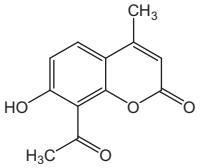
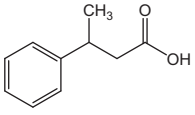
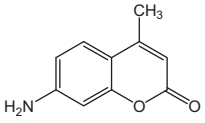
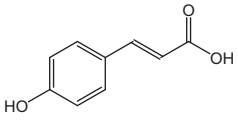
Number	IUPAC name	Structure	Number	IUPAC name	Structure
1	7,8-dihydroxy-4-methylcoumarin (DHMC)		**13**	7-hydroxy-4-methylquinolone	
2	6-methoxy-7,8-dihydroxy-4-methylcoumarin (Fraxetin)		**14**	6-hydroxy-4-methylcoumarin	
3	6,7-dihydroxycoumarin (Esculetin)		**15**	1,2-pyrone	
4	6,7-dihydroxy-4-methylcoumarin		**16**	4,6-dimethyl-1,2-pyrone	
5	5,7-dihydroxy-4-methylcoumarin		**17**	4-hydroxy-6-methyl-1,2-pyrone	
6	7,8-dimethoxy-4-methylcoumarin		**18**	4-methoxy-6-methyl-1,2-pyrone	
7	6,7-dimethoxycoumarin		**19**	Caffeic acid (CA)	
8	6-hydroxy-7-methoxycoumarin		**20**	3,4-dihydroxy-hydro-cinnamic acid (DHCA)	
9	6-chloro-7-hydroxy-4-methylcoumarin		**21**	3,4-dimethoxycinnamic acid	
10	7-hydroxy-4-methylcoumarin		**22**	3,4-dimethoxy-hydrocinnamic acid	
11	7-hydroxy-8-acetyl-4-methylcoumarin		**23**	3-phenylbutiric acid	
12	7-amine-4-methylcoumarin		**24**	4-hydroxycinnamic acid (*p*-coumaric acid)	

Table 2
Anti-proliferative and cytotoxic activity of coumarins, 1,2-pyrones and cinnamic acid derivatives on U-937 cells

Compound	IC_{50} (μM) CI95	CC_{50} (μM) CI95	Compound	IC_{50} (μM) CI95	CC_{50} (μM) CI95
Coumarins			**14**	627.7 (507.2–748.2)	>2000
1	83.2 (65.7–105.4)	120.2 (109.0–132.6)	***1,2-Pyrones***		
2	142.9 (69.8–292.3)	70.2 (54.2–91.1)	**15**	>2000	>2000
3	143.1 (120.9–169.4)	107.5 (93.6–123.4)	**16**	>2000	>2000
4	97.4 (77.5–122.4)	76.0 (66.2–87.4)	**17**	>2000	>2000
5	>2000	>2000	**18**	>2000	>2000
6	736.1 (540.7–1002)	>2000			
7	589.9 (463.8–750.3)	1289 (788.9–2109)	*Cinnamic acid derivatives*		
8	975.9 (898.8–1060)	>2000	**19**	316.5 (205.3–690.2)	>2000
9	771.3 (517.2–1150)	734.3 (616.6–875.0)	**20**	212.7 (142.4–420.7)	>2000
10	710.9 (674.5–747.3)	>2000	**21**	>2000	>2000
11	453.7 (340.2–605.0)	>1000	**22**	>2000	>2000
12	>2000	>2000	**23**	>2000	>2000
13	258.7–(202.0–331.4)	>1000	**24**	>2000	>2000

Results are expressed as the concentration that induces a 50% of cell proliferation (IC_{50}) and the concentration that decreases cell viability in 50% (CC_{50}). Values indicate means with 95% confidence intervals (CI95) ($n \geqslant 3$).

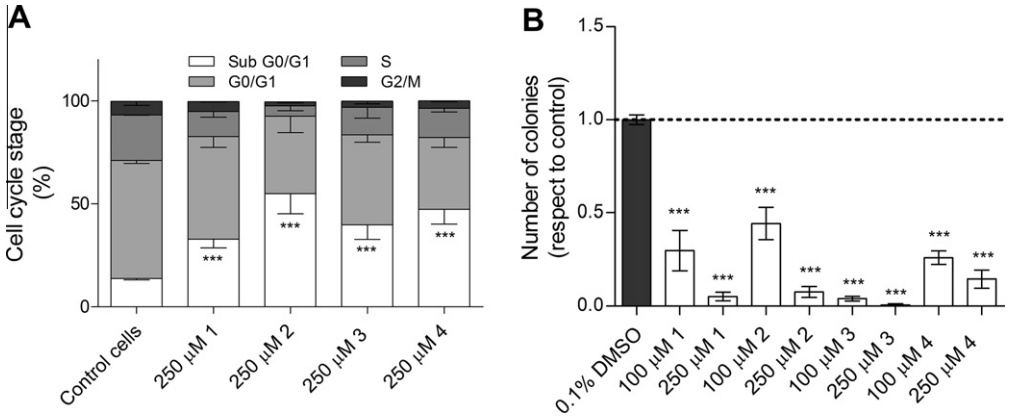


Figure 1. Characterization of the cytotoxic effect of *o*-DHC in U-937 cells. Exponentially-growing cells were exposed to compounds **1**–**4** at the indicated concentrations or to 0.1% (v/v) DMSO (vehicle control group). After 24 h of treatment, U-937 cell cycle alterations (A) and clonogenic capacity in soft agar (B) were evaluated as described in the Experimental section. In the case of Graphic B, cells from each treatment were washed and seeded in soft agar and the number of colonies generated in these conditions was determined 2–3 weeks later. Data in graphic A were log-transformed before performing a one-way ANOVA test ($p < 0.001$) followed by SNK a posteriori test in order to evaluate significant variations in the percentage of cells in the sub G0/G1 phase of the cell cycle in each treatment group with respect to control cells ($^{**}p < 0.01$ and $^{***}p < 0.001$). In Graphic B, one-way ANOVA test ($p < 0.01$) was performed followed by SNK a posteriori test ($^{***}p < 0.001$ vs vehicle control group). In all cases, each bar and vertical line represents the mean ± SEM ($n > 3$).

Figure 2C. Accordingly, we detected the appearance of the active 11 kDa fragment of caspase-3 and its cleaved substrate poly-(ADP-ribose)-polymerase (PARP) after a 24-h treatment with 250 μM **1**–**4** coumarins (Fig. 2D). Catechol-containing cinnamic acid derivatives **19** and **20**, on the other hand, were unable to induce caspase-3 activation in U-937 cells even after longer treatment and at higher concentrations than those used for *o*-DHC (Fig. 2B). These results were confirmed by Western blot assays, in which neither caspase-3 nor PARP cleavage were detected (data not shown).

In all, the above findings show that *o*-DHC proved to be more effective apoptosis inducers than monohydroxycoumarins or open-chain analogues, confirming that the presence of the catechol moiety is essential for coumarins to induce apoptosis in U-937 cells, but not sufficient if the coumarin structure is disrupted.

Goel et al. have reported the pro-apoptotic activity of **1** in a human non-small cell lung carcinoma cell line.[16] Also, anti-tumor effects induced by **3** have been observed in different human cancer cells, especially in leukemic ones.[17–21] This evidence indicates that *o*-DHC act as apoptosis inducers in various types of cancer cells, which broadens their potential chemotherapeutic applications.

2.3. o-DHC-induced apoptosis in U-937 cells would be triggered through activation of the intrinsic, caspase-9-mediated pathway

Caspase-mediated apoptosis is commonly accepted as the paradigm of programmed cell death. There are two major pathways of caspase activation. The 'intrinsic' pathway, mediated by caspase-9, and the 'extrinsic' one, executed mainly by the binding of specific ligands (such as TNF-α) to death receptors, which leads to caspase-8 activation. Both the intrinsic and the extrinsic pathways converge into the same final executor, caspase-3.

In order to determine which caspase-dependent pathway is responsible for *o*-DHC pro-apoptotic activity in U-937 cells, we evaluated the levels of cleaved caspases 8 and 9 in response to **1** treatment. As shown in Figure 3A and B, 250 μM **1** induced an increase in the cleavage of caspase-9 from 12 h onwards ($p < 0.01$), resulting in an increment of its active form in a time dependent manner. However, no changes were detected in the cleavage of caspase-8 (Fig. 3A and B). Moreover, while caspase-9 specific inhibitor Z-LEHD-FMK (100 μM) was able to inhibit **1**-triggered caspase-3 activation in U-937 cells, caspase-8 specific inhibitor

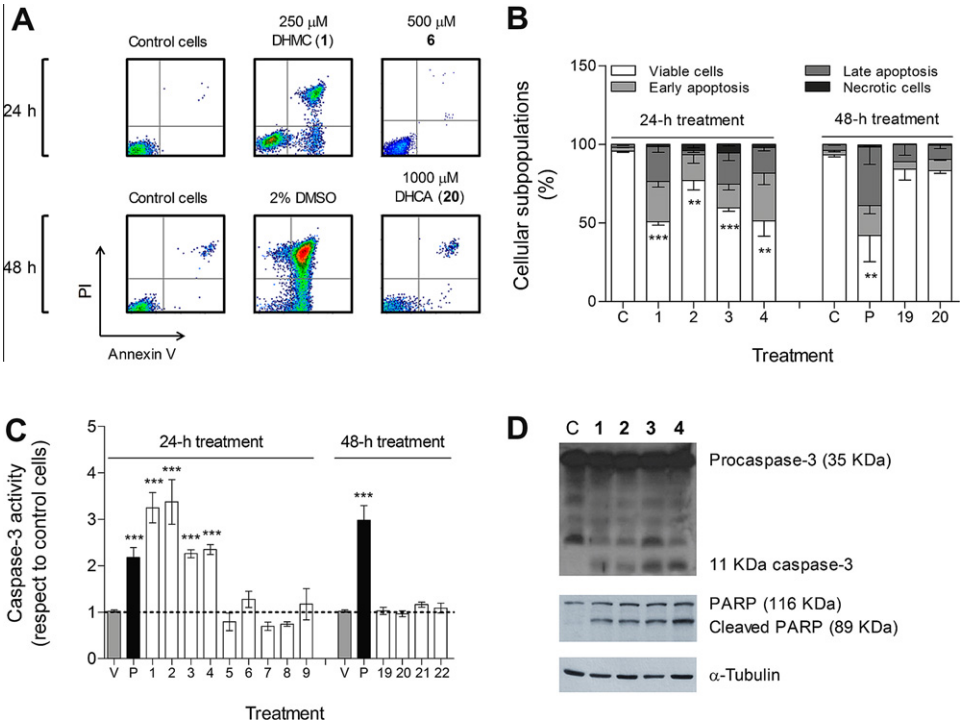


Figure 2. Pro-apoptotic activity of *o*-DHC in treated U-937 cells. After 24 h or 48 h of treatment, control cells (C), 0.1% (v/v) DMSO (vehicle control group, V), 2% (v/v) DMSO (positive control group, P) or U-937 cells treated with different compounds were analyzed in order to detect phosphatidylserine expression by annexin V binding assay (Graphic A and B) and caspase-3 activation (Graphics C and D). In every case, compounds **1**–**4** were tested at 250 μM, compounds **5**–**14** up to 500 μM (here are shown only **5**–**9**) and compounds **19**–**24** up to 1000 μM (here are shown only **19**–**22**). Graphic A shows the different cell subpopulations according to the Annexin V/PI staining pattern: cells labeled with annexin V only were considered to be at an early apoptotic stage, cells labeled with annexin V and PI were considered to be at a late apoptotic stage, and cells labeled with PI only were considered necrotic. Data in graphics B and C were log-transformed before performing a one-way ANOVA test ($p < 0.001$) followed by SNK a posteriori test in order to evaluate significant variations in the percentage of normal cells (B) and in caspase-3 activity (C) in each treatment group with respect to control cells ($^{**}p < 0.01$ and $^{***}p < 0.001$). Each bar and vertical line represents the mean ± SEM ($n > 3$). Graphic D shows determination of cleaved caspase-3 and PARP by Wetern blot. U-937 cells were treated with 250 μM *o*-DHC and harvested after 24 h. Equal amounts of protein were subjected to SDS–PAGE and analyzed by Western blot with anti-caspase-3, PARP and α-tubulin antibodies. Data are representative of at least three independent experiments.

Z-IETD-FMK (100 μM) was ineffective (Fig. 3C and D, respectively). These results demonstrate for the first time that apoptosis induced by **1** (as representative of *o*-DHC) in U-937 cells results from the activation of caspase-9 and the intrinsic pathway.

Similarly, Park et al. have reported mitochondria-mediated pro-apoptotic activity of **3** in U-937 cells.[18] It has also been described that the intrinsic pathway would mediate the pro-apoptotic effects of **1** and **3** in A549 human non-small cell lung carcinoma and in human cervical cancer cells, respectively.[16,21] With regard to the activation of the cited pathway, it is worth remarking that pro-oxidant species such as reactive oxygen/nitrogen species (ROS/RNS) have been reported to be involved in the pro-apoptotic activity of **3** in human cervical cancer cells.[21] Additionally, in a previous study we observed a significant attenuation of the pro-apoptotic effect of **1** on U-937 cells in the presence of the anti-oxidant agent N-acetylcysteine (NAC), which indirectly suggested the dependence of such effect on pro-oxidant species generation.[14] In all, the above evidence indicates that the redox properties of *o*-DHC would play a key role in their pro-apoptotic effect (see further).

2.4. Evaluation of differentiation-inducing activity of hydroxylated coumarins and cinnamic acid derivatives on U-937 cells

Because of their morphology, biochemical and staining properties, the U-937 human promonocitic leukemia cells have been classed as a cell line frozen in a window of the monocyte differentiation lineage corresponding to monoblasts and/or immature monocytes.[22,23] This non-APL M5 cell line can be forced to continue its differentiation program under the appropriate treatment.[24,25]

Previously, our group described the differentiating activity of derivatives of 5,6-methylenedioxy-5-oxy natural coumarins in U-937 cells.[12,13] On the basis of this evidence and considering that several pyranocoumarins and hydroxycoumarins have been reported to be differentiation inducers of HL-60 cells,[26–28] we studied the possibility that *o*-DHC might trigger U-937-cell differentiation at low concentrations, and apoptosis at higher ones. To perform this study, we considered **1** and **20** as representative of *o*-DHC and *ortho*-dihydroxycinnamic acid derivatives, respectively, evaluating the chemotactic response to rhC5a and the expression of CD11b, CD14 and C5a receptor (CD88) proteins as monocyte differentiation makers.[24,25,29,30]

Treatment of U-937 cells with 50 μM **1** for 72 h induced a significant increase in CD11b and CD14 protein expression (Fig. 4A). However, replacement of the two adjacent hydroxyl groups by methoxyl residues (**6**) rendered the compound completely ineffective as differentiation inducer, even when tested at a higher concentration than **1** (Fig. 4A). Regarding *ortho*-dihydroxycinnamic acid derivatives, **20** retained the ability to induce the expression of CD11b and CD14 proteins (Fig. 4A) in U-937 cells after 72 h, but only when used at higher concentrations than **1**. As in the case of coumarin compounds, when the hydroxyl groups in *ortho*-dihydroxycinnamic acid derivatives were substituted for methoxyl ones (**22**) the differentiation-inducing activity was abrogated (Fig. 4A).

CD88 is a monocyte marker commonly used in differentiation protocols. It is a G-protein coupled receptor (GPCR) associated with

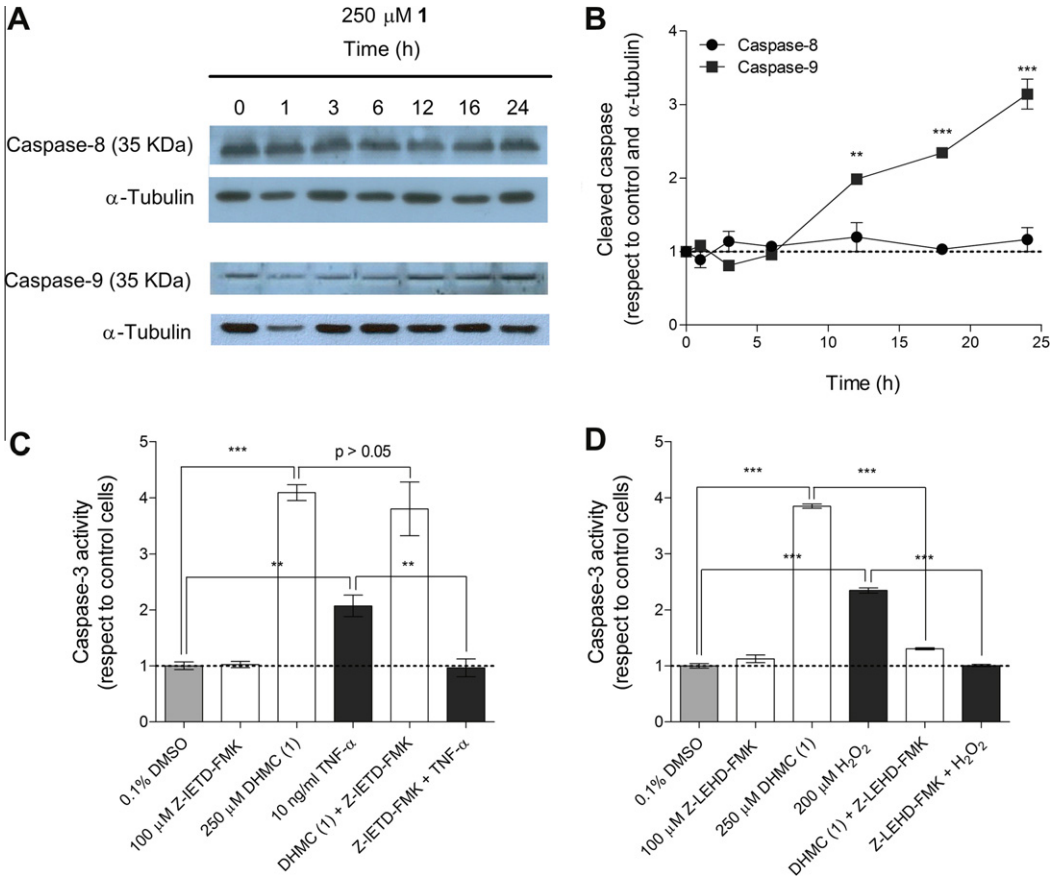


Figure 3. Effect of DHMC (**1**) on the activity of caspases 8 and 9 in the U-937 cell line. Cells were treated during 24 h with 250 μM DHMC (**1**) and harvested at the indicated times (1, 3, 6, 12, 18, 24 h). Untreated control cells were harvested after 24 h. Equal amounts of proteins were subjected to SDS–PAGE and analyzed by Western blot with anti-caspase-8 and -9, and anti-α-tubulin antibodies (A). Densitometry analyses of caspase-8 and -9 Western blots were obtained by employing ImageJ program (**B**). Data are representative of at least three independent experiments in which the same pattern was obtained. In other assays, cells were treated for 24 h with 0.1% (v/v) DMSO (vehicle control group), 100 μM Z-IETD-FMK (caspase-8 inhibitor), 250 μM DHMC (**1**), 10 ng/ml TNF-α (extrinsic pathway inducer, positive control), 250 μM DHMC (1) + 100 μM Z-IETD-FMK or 10 ng/ml TNF-α + 100 μM Z-IETD-FMK to evaluate the capacity of **1** to activate the extrinsic apoptosis pathway (C). In turn, with the aim of testing the activation of the caspase-9-dependent pathway, U-937 cells were treated for 24 h with 0.1% (v/v) DMSO (vehicle control group), 100 μM Z-LEHD-FMK (caspase-9 inhibitor), 250 μM DHMC (**1**), 200 μM H_2O_2 (intrinsic pathway inducer, positive control), 250 μM DHMC (**1**) + 100 μM Z-LEHD-FMK or 200 μM H_2O_2 + 100 μM Z-LEHD-FMK (D). The activity of caspase-3, final executor of both pathways, was determined in every case. In graphics B, C and D, one-way ANOVA test ($p < 0.001$) was performed followed by SNK a posteriori test ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$). Each bar and vertical line represents the mean ± SEM ($n > 3$).

Ca^{2+} release from intracellular stores which is involved in the chemotactic process in response to complement factor C5a factor.[29,30] Thus, we evaluated CD88 expression in U-937 cells by measuring both Ca^{2+} intracellular release and chemotaxis induced by rhC5a. Figure 4C shows that 50 μM **1** promoted CD88 expression, whereas **6**, bearing two adjacent methoxy groups, proved to be ineffective. In the same way, **20** was able to induce CD88 expression and this capacity was dependent on the presence of two phenolic hydroxyl groups, since **22** was found inactive (Fig. 4C). With regard to the chemotactic response to rhC5a, 50 μM **1** induced a significant increase in the level of the response with respect to vehicle-treated control cells (Fig. 4B). However, replacement of the two hydroxyl groups by methoxyl residues (**6**) abolished this activity (Fig. 4B). The same pattern was observed for compounds **20** and **22** (Fig. 4B).

Of note, expression of CD11b protein was also induced by coumarin derivatives bearing an H-bond donor in position 6 or 7 of the aromatic ring, such as an amine group or a hydroxyl residue (Fig. 4D, compounds **5** and **10**–**14**). However, this differentiation-inducing activity was abolished in the case of compounds with partial disruption of the catechol moiety, such as **8**, or compounds with a Cl atom adjacent to a hydroxyl group (**9**). Further studies are required to establish the influence of these residues on the adjacent hydroxyl group. This partial differentiating activity was not observed when treating the cells with the derivative of cinnamic acid bearing a hydroxyl group (**24**). The rest of the differentiation markers evaluated herein were not detected in response to these compounds (data not shown).

The 1,2-pyrone analogues (**15**–**18**) were incapable of inducing expression of any of the differentiation markers evaluated (data not shown), indicating that the aromatic portion of the coumarin nucleus is of major importance for the differentiation-inducing capacity of coumarins.

Taken collectively, our findings demonstrate the fundamental role of the catechol moiety in determining coumarin-induced expression of CD11b, CD14 and CD88 differentiation markers and chemotactic response in U-937 cells. Additionally, the loss of the coumarin ring integrity (**20**) entails loss of potency, pointing out that the δ-lactone constituent of the ring has influence on the differentiation-inducing activity of the aromatic portion.

2.5. The pro-oxidatnt properties of o-DHC correlate with their pro-apoptotic action in U-937 cells

Considering that oxidative stress mediates the pro-apoptotic activity of *o*-DHC in different cell models,[14,21] in this section we aimed to evaluate the potential influence of the pro-oxidant properties of these compounds on their previously described pharmacological activities.

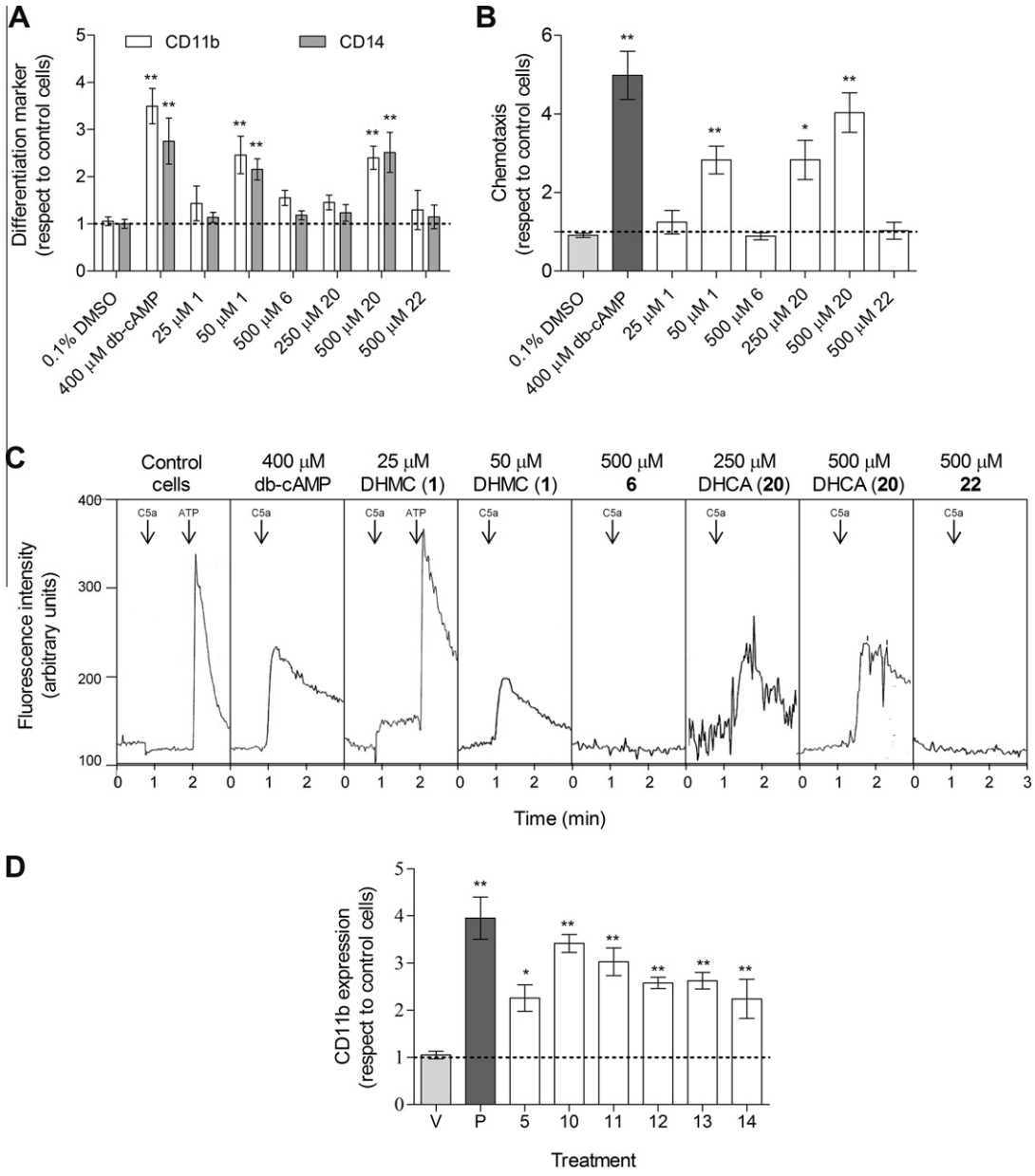


Figure 4. Differentiation markers in U-937 cells after 72 h of treatment. The effect of coumarinic and cinnamic acid derivatives on the expression of CD11b and CD14 (A), chemotactic ability (B) and intracellular Ca^{2+} mobilization induced by rhC5a due to C5a receptor (CD88) expression (C) was evaluated after a 72-h treatment of U-937 cells with DHMC (**1**) as representative of *ortho*-dihydroxycoumarins, compound **6**, DHCA (**20**), compound **22** and 0.1% (v/v) DMSO (vehicle control). Graphic D shows the expression of CD11b in response to a 72-h treatment with compounds **5**, **10**, **11**, **12**, **13** and **14** up to 500 μM. These compounds had no inducing effect on the other differentiation markers (CD14, CD88 and chemotaxis capacity. Data not shown). In graphics A, B and D, the differentiation markers are expressed relative to control cells and logarithmic transformation of the variable was required before a one-way ANOVA test ($p < 0.01$) followed by SNK a posteriori test ($^{**}p < 0.01$; $^{*}p < 0.05$). Each bar and vertical line represents the mean ± SEM ($n \geqslant 3$). In graphic C, arrows indicate the addition of rhC5a (C5a) or ATP as positive control of intracellular Ca^{2+} mobilization. Cells treated with 400 μM dibutyryl cyclic adenosine monophosphate (db-cAMP) served as positive control of the differentiation process. Similar results were obtained in at least three independent experiments.

Figure 5A shows detectable oxidation potential values for all the compounds listed in Table 1. Compounds **6**, **7**, **15**, **16**, **17**, **18** and **23** did not display oxidation signals under the evaluated conditions (Section 4.9.1). Based on these values, we classified the compounds into three groups. In the first group we included compounds with oxidation potential values lower than 350 mV, which comprised coumarins and cinnamic acid derivatives bearing the catechol moiety. Of these, only coumarins were able to induce apoptosis in U-937 cells, whereas both **1** and **20** showed differentiation-inducing activity. In accordance, Zhang et al. have reported that the δ-lactone portion of the coumarinic ring has an electron-withdrawing effect on the benzenoid part, although rather weak.[31] The second group included compounds with oxidation potential values between 350 and 550 mV. These were incapable of inducing apoptosis in U-937 cells, although some of them showed partial differentiation-inducing activity (CD11b expression). Compounds **21** and **22** constituted the third group, having oxidation potentials higher than 1100 mV. Collectively, these results indicate that the pro-apoptotic activity of *o*-DHC in U-937 cells, previously associated with oxidative stress induction, does not correlate with their oxidation potential values.

Electron spin resonance spectroscopy studies were also carried out in order to address the objective posed above. All the compounds in Table 1 were evaluated in RPMI 1640 medium pH

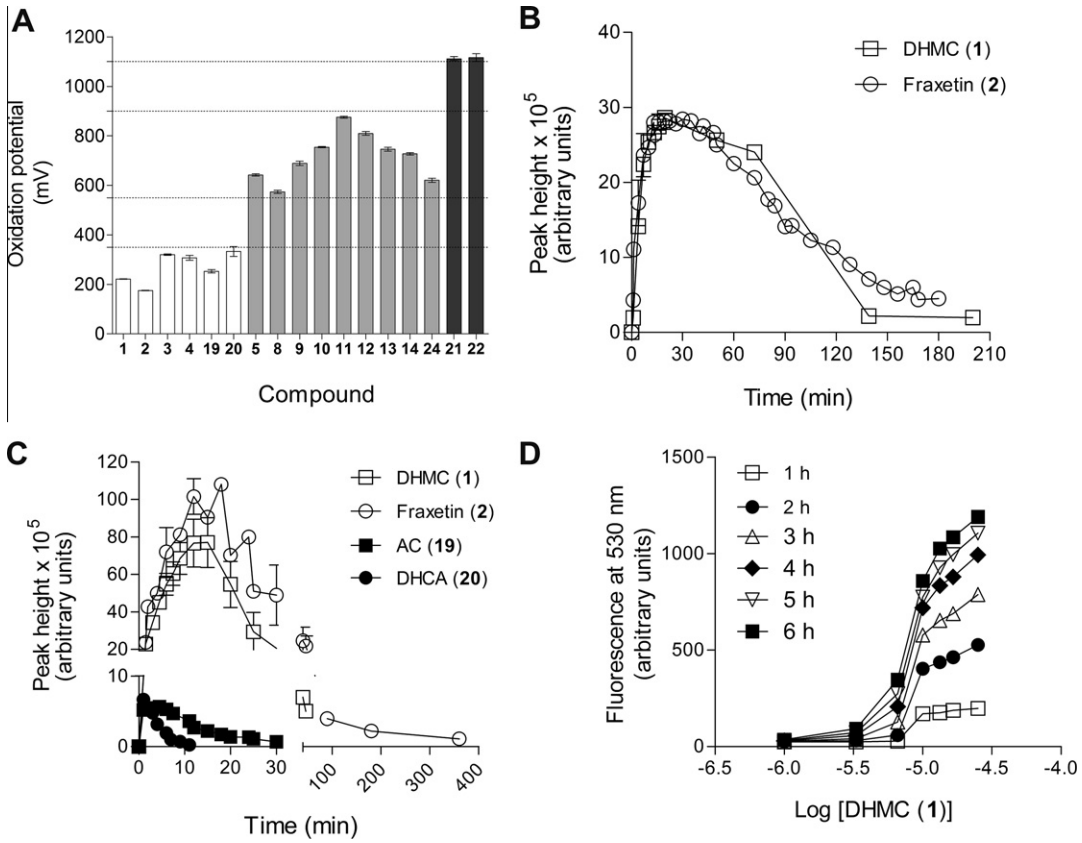


Figure 5. Influence of pro-oxidant properties on the biological activities of coumarins and cinnamic acid compounds. Oxidation potentials were determined by cyclic voltammetry in potassium phosphate buffer 0.1 M pH 7.4 (A). This graphic only shows those compounds listed in Table 1 with oxidation potential values detectable in the voltage-range evaluated. The ability of all the compounds listed in Table 1 to generate free radicals was evaluated by electron spin resonance. Only *o*-DHC were able to generate stable free radicals in RPMI 1640 medium pH 7.4 (B). Compounds **19** and **20** were only able to generate radical species in sodium carbonate buffer pH 9.0, but these were less stable compared to those generated by *o*-DHC (C). The pro-oxidant activity of DHMC (**1**) in U-937 cells was assayed by determination of DCF-DA oxidation (D). In graphic A, B and C, data are means ± SEM ($n = 3$). Graphic D is representative of at least three independent experiments in which the same pattern was obtained.

7.4 and Na_2CO_3 buffer pH 9. Under the first condition, only *o*-DHC (**1**–**4**) were able to generate detectable signals (data not shown). In order to increase the sensitivity of detection, we performed the same assay in Na_2CO_3 buffer pH 9, taking into account that this non physiological condition would lead to formation of phenoxide species. This time, *o*-DHC (**1**–**4**) as well as compounds **19** and **20** produced free radical signal (data not shown). With the aim of detecting differences in the formation kinetics and stability of the free radicals generated by *o*-DHC (**1**–**4**) and compounds **19** and **20**, we evaluated these two properties in two representative *o*-DHC selected at random (**1** and **2**) and in the cinnamic acid derivatives **19** and **20**. The production kinetics of free radicals derived from **1** and **2** in RPMI 1640 medium is shown in Figure 5B. Figure 5C shows the formation kinetics of free radicals produced by **1**, **2**, **19** and **20** in Na_2CO_3 buffer pH 9. As can be seen in this figure, the signal was more intense and sustained for coumarins with respect to compounds lacking the δ-lactone portion of the coumarin ring.

Considering the aforementioned pro-oxidant properties of *o*-DHC, we further evaluated the capacity of **1** to generate oxidizing species in U-937 cells using the method based on the ROS/RNS-dependent oxidation of DCF-DA. As shown in Figure 5D, **1**, considered as representative of *o*-DHC**,** induced DCF-DA oxidation in a time- and concentration-dependent manner. However, U-937 cells treated with 500 μM **6**, **10**, **20**, and **22** did not show DCF-DA oxidation even after 48-h treatments (data not shown). In agreement with our former observations regarding cytotoxic activity, these results point out that the catechol moiety is not sufficient to induce oxidative stress in U-937 when the coumarin ring is disrupted. Moreover, as shown in Table 3, this indicates a strong correlation between the ability of *o*-DHC to generate stable free radicals and the pro-apoptotic activity of these compounds in U-937 cells.

All in all, findings presented herein indicate for the first time that the fundamental role of the δ-lactone ring in the pro-apoptotic activity of *o*-DHC in U-937 cells depends upon its capacity to stabilize free radical species by unpaired electron delocalization. In contrast, the relative influence of the δ-lactone portion on the differentiation-inducing activity of coumarins in U-937 cells is a question that demands further investigation.

2.6. Evaluation of cytotoxic selectivity of o-DHC and cinnamic acid derivates against cancer cells with respect to normal blood cells

In this work we describe for the first time the dual pharmacological effects of **1** (as representative of *o*-DHC) on apoptosis and differentiation in U-937 cells, depending upon concentration and treatment duration. In accordance with other authors, we consider that this dual behavior can serve as a criterion for identification and prioritization of candidate compounds likely to be useful in the development of novel anti-leukemic agents.[32] Results shown herein indicate that it would be more advisable to use compounds bearing an intact coumarin nucleus in anti-leukemic drug design than cinnamic acid derivatives. In this regard, although **20** displayed differentiation-inducing activity, its potency was lower than that of **1**.

Table 3
Relationship among patterns of substitution, pro-oxidant properties and biological activities of the compounds used in the SAR study

Compound	Structural features	Apoptotic activity	Differentiating activity	Oxidation potential (mV)	Free radicals in culture conditions
Coumarins					
1[a]	*ortho*-Dihydroxyl moiety	Positive	CD11b/CD14/CD88 Chemotactic ability with rhC5a	<350	Positive
5	*meta*-Dihydroxyl moiety	Negative	CD11b	550–1100	Negative
6	*ortho*-Dimethoxyl groups	Negative	Negative	>1200	Negative
7	*ortho*-Dimethoxyl groups	Negative	Negative	>1200	Negative
8	6-Hydroxy-7-methoxy	Negative	Negative	550–1100	Negative
9	6-Chloro-7-hydroxy	Negative	Negative	550–1100	Negative
10	7-Hydroxy	Negative	CD11b	550–1100	Negative
11	7-Hydroxy-8-acetyl	Negative	CD11b	550–1100	Negative
12	7-Amine	Negative	CD11b	550–1100	Negative
13	7-Hydroxy (quinolone)	Negative	CD11b	550–1100	Negative
14	6-Hydroxy	Negative	CD11b	550–1100	Negative
1,2-Pyrones					
Compounds **15**–**18**	—	Negative	Negative	>1200	Negative
Cinnamic acid derivatives					
20[a]	*ortho*-Dihydroxy moiety	Negative	CD11b/CD14/CD88 Chemotactic ability with rhC5a	<350	Negative
21 and **22**	*ortho*-Dimethoxy groups	Negative	Negative	1100–1200	Negative
23	—	Negative	Negative	>1200	Negative
24	*para*-Hydroxyl group	Negative	Negative	550–1100	Negative

[a] DHMC (**1**) is considered representative of *ortho*-dyhydroxycoumarins (**1**–**4**), while DHCA (**20**) is considered representative of *ortho*-dyhydroxycinnamic acid derivatives (**19**–**20**).

Besides potency, another important criterion to consider in anti-cancer drug development is specificity of action. In the literature, most reports evaluating the anti-leukemic activity of coumarins are mainly based on the myeloid cell lines U-937 and HL-60. In an attempt to extend our results to the lymphoid linage, we evaluated the pro-apoptotic activity of **1**, **20** and derivatives in Jurkat cells (a human T-cell acute lymphoblastic leukemia-derived cell line). After 24 h, 250 μM **1** and 500 μM **20** were able to induce caspase-3 cleavage-mediated activation, resulting in the cleavage of PARP by the former (Fig. 6A). Results of the evaluation of the enzymatic activity of caspase-3 showed in Figure 6B confirm the formerly described **1** and **20**-mediated activation of caspase-3 in Jurkat cells ($p < 0.01$). On the other hand, dimethoxy-substituted derivatives of **1** and **20** did not induce apoptosis in Jurkat cells, indicating the necessity of the catechol moiety for the induction of such activity in these cells as well. Of note, while 500 μM **20** induced caspase-3 activation in Jurkat cells after 24 h, this compound was not able to induce apoptosis in U-937 cells even after 48 h at 1000 μM (Fig. 2). These findings suggest that **1** and **20** may have two different and independent mechanisms of action, being the U-937 cells naturally resistant to the mechanism of **20** and sensitive to **1**.

As pointed out above, potential anti-cancer drugs should be more active against tumorigenic cells than normal cells. Thus, it was pertinent to evaluate the selective toxicity of **1** and **20** (as representative of *o*-DHC **1**–**4** and *ortho*-dihydroxycinnamic acid derivatives, respectively) in normal human peripheral blood cells versus leukemic cell lines. Figure 6C shows that, while **1** exerted a more potent cytotoxic effect on U-937 cells than on normal peripheral monocytes ($p < 0.001$), **20** proved to be more active against the latter ($p < 0.001$). In both cell types, **1**- and **20**-mediated cytotoxic effects disappeared when the hydroxyl groups were replaced by methoxyl residues (compounds **6** and **22**), indicating their essential role in the induction of cytotoxicity. Interestingly, Figure 6D shows that **1** displayed the same selective toxicity pattern for lymphoid cells, preferentially affecting leukemic Jurkat cells over normal lymphocytes ($p < 0.001$). On the other hand, even though 500 μM **20** exerted the same level of cytotoxicity than **1** in Jurkat cells, it still proved more toxic to normal lymphocytes than Jurkat malignant cells ($p < 0.001$). Thus, it is likely that the δ-lactone portion of the coumarin ring plays a role in determining not only the potency of apoptosis- and differentiation-induction, but also the selective toxicity that *o*-DHC show for malignant cells over normal ones.

The chatechol moiety proved to be determinant for both **1** and **20**-induced cytotoxic effects. However, it is likely that these compounds act via completely different mechanisms. In this regard, several authors have reported that **19** and other related compounds have clastogenic effects in normal lymphocytic cells.[33,34] Specifically, Bhat et al. detected clastogenic activity of **19** in peripheral normal lymphocytes in the same range of concentrations herein evaluated for **20**.[34] In the same report, these authors stated that such cytotoxic activity would be mediated through a non-enzymatic mechanism implying direct action of the compound on mobilization and reduction of endogenous copper ions, with the consequent oxidative DNA breakage in the presence of these ions. Of interest, normal lymphocytes and monocytes as well as Jurkat malignant cells express p53 protein, whereas U-937 cells are p53-deficient. Given the well-established role of p53 in the induction of apoptosis following DNA damage, such differential p53 expression pattern would provide a suitable explanation for the resistance that U-937 cells show to **20** if its mode of action were mediated by DNA damage. Moreover, results from our group indicate that **1** does not show direct effects on DNA (unpublished data).

With regard to **1,** knowing its toxic effect in U-937 cells is mediated by free radicals and pro-oxidant species, differences in protein expression patterns among U-937 cells and mature monocytes and lymphocytes could help explain the apparent selective toxicity it shows for the leukemic cell line. In this respect, during maturation hematopoietic cells undergo changes in their internal redox balance due to a sequential increase in O_2 exposure, and in turn express several proteins to protect themselves from products of aerobic metabolism such as $O_2^{\cdot-}$ and other pro-oxidant species.[35]

3. Conclusion

The present work not only reports the dual effects of *o*-DHC on apoptosis and differentiation in U-937 cells, but also provides conclusive evidence that the δ-lactone ring constituent of the

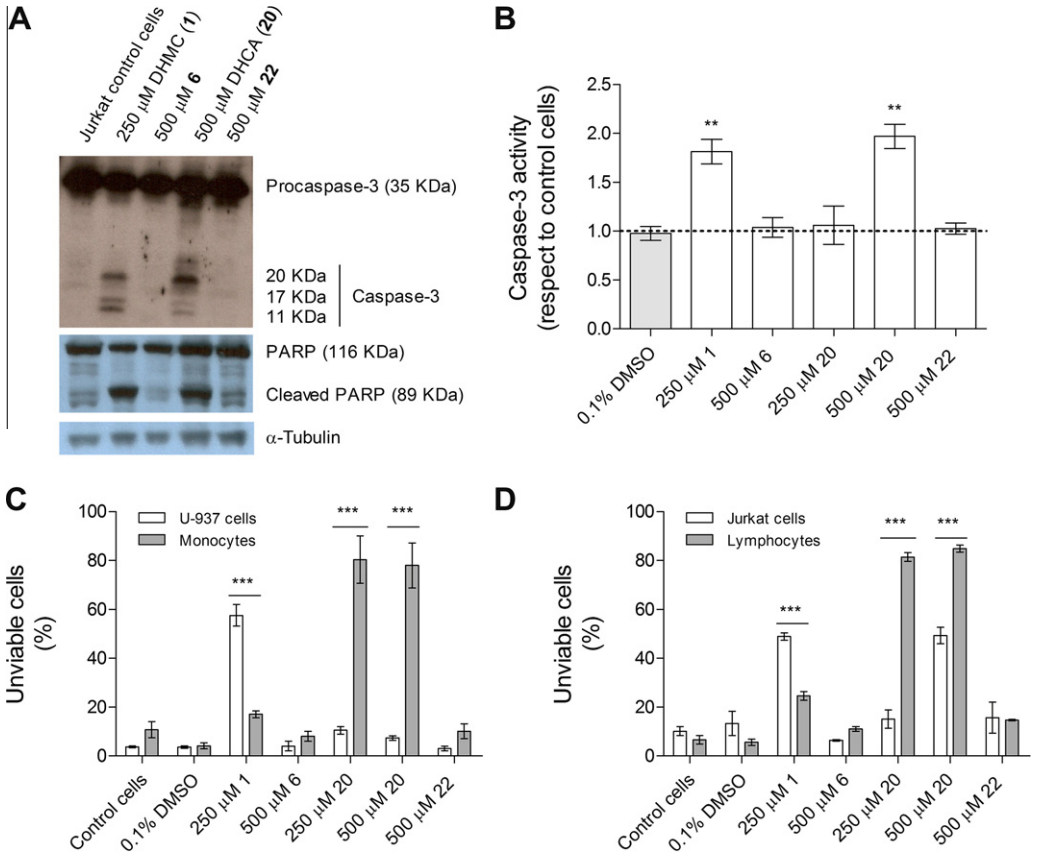


Figure 6. Selective cytotoxicity of DHMC (**1**) towards leukemic cells. After 24 h, Jurkat cells treated with 0.1% (v/v) DMSO (vehicle control group) or with the indicated compounds were analyzed by Western blot in order to detect caspase-3 and PARP cleavage (A) and enzymatic activity of caspase-3 (B). The cytotoxic effects of DHMC (**1**) (250 μM, pro-apoptotic concentration in U-937 cells), DHCA (**20**) (250 μM and 500 μM, differentiating concentration in U-937 cells), compounds **6** and **22** (500 μM) and 0.1% (v/v) DMSO (control vehicle group) were evaluated on U-937 and normal peripheral blood monocytes (C) and on Jurkat cells and normal peripheral blood lymphocytes (D) by Trypan Blue exclusion assay after 24 h of treatment. In Graphic A, equal amounts of protein were subjected to SDS–PAGE and analyzed by Western blot with anti-caspase-3, anti-PARP and anti-α-tubulin antibodies. Data are representative of at least three independent experiments. In graphics B–D, logarithmic transformation of the variable was required before a one-way ANOVA test ($p < 0.001$) followed by SNK a posteriori test ($^{**}p < 0.01$, $^{***}p < 0.001$). Data are means ± SEM ($n > 3$).

coumarinic system substantially contributes to both activities, conferring greater potency to compounds containing an intact coumarin nucleus. We also demonstrate the prominent role of free radical species in the pro-oxidant species-mediated pro-apoptotic activity of *o*-DHC in U-937 cells, and describe for the first time the caspase-3-mediated cytotoxic effect of **1** and **20** in Jurkat cells as well as the caspase-9-mediated pro-apoptotic effect of **1** in U-937 cells. Most tellingly, our results strongly indicate that the δ-lactone ring is crucial in determining the selective toxicity that *o*-DHC show for leukemic cells over normal peripheral blood cells. In conclusion, we have established herein the structural requirements for hydroxycoumarins to induce apoptosis and differentiation in the U-937 cell line, contributing to the literature on the subject with novel findings which we expect can provide useful insights for further investigation on *o*-DHC and their potential use as chemotherapeutic agents for the treatment of hematological malignancies.

4. Experimental section

4.1. Reagents and antibodies

RPMI 1640 medium, gentamicin antibiotic, bovine serum albumin (BSA), dibutyryl-cyclic adenosine monophosphate (db-cAMP), phosphate-buffered saline (PBS), Fura 2-AM, recombinant human C5a (rhC5a), RNase A and adenosine triphosphate (ATP) were obtained from Sigma Chemical Co. (St. Louis, USA). Dimethyl sulfoxide (DMSO) was obtained from Baker (Deventer, The Netherlands). Fetal calf serum (FCS) was purchased from PAA Laboratories GmbH (Austria). Anti-caspase-3, -8 and -9, and anti-poly (ADP-ribose) polymerase (PARP) rabbit antibodies, and anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Anti-α-tubulin antibody was purchased by Cell Signaling Technology, Inc (Beverly, USA). All commercial chemicals and solvents were of reagent grade and used without further purification unless otherwise specified.

4.2. Compounds

Compounds 7,8-dihydroxy-4-methylcoumarin (DHMC, **1**) and 7-hydroxy-4-methycoumarin (**10**) were synthesized as previously described by our group.[14,15] On the other hand, 7,8-dihydroxy-6-methoxycoumarin (fraxetin, **2**), 6,7-dihydroxycoumarin (esculetin, **3**), 6,7-dihydroxy-4-methylcoumarin (**4**), 5,7-dihydroxy-4-methylcoumarin (**5**), 7,8-dimethoxy-4-methylcoumarin (**6**), 6,7-dimethoxycoumarin (**7**), 6-hydroxy-7-methoxycoumarin (**8**), 6-chloro-7-hydroxy-4-methylcoumarin (**9**), and 7-hydroxy-8-acetyl-4-methylcoumarin (**11**) were obtained from INDOFINE Chemical Company, Inc. (Boston, USA). Compounds 7-amine-4-methylcoumarin (**12**), 7-hydroxy-4-methylquinolone (**13**), 1,2-pyrone (**15**), 4,6-dimethyl-1,2-pyrone (**16**), 4-hydroxy-6-methyl-1,2-pyrone (**17**), 4-methoxy-6-methyl-1,2-pyrone (**18**), caffeic acid (CA, **19**), 3,4-dihydroxy-hydrocinnamic acid (DHCA, **20**), 3,4-dimethoxycinnamic acid (**21**), 3,4-dimethoxy-hydrocinnamic acid

(**22**), 3-phenylbutiric acid (**23**) and *para*-coumaric acid (**24**) were obtained from Sigma Chemical Co. (St. Louis, USA).

All synthesized and commercial compounds listed on Table 1 were dissolved in DMSO and stored at −20 °C.

4.3. Cell culture

The U-937 and Jurkat cell lines (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% FCS and 50 μg/ml gentamicin. Cells used in each experiment were in the period of exponential replication.

Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers by standard density gradient centrifugation on Ficoll-Hypaque. Monocytes were purified by centrifugation on a discontinuous Percoll gradient with modifications of a previously described method.[36] Briefly, PBMC were suspended in Ca^{2+} and Mg^{2+}-free Tyrode solution supplemented with 0.2% EDTA and incubated during 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 *g* for 20 min at 4 °C. Monocytes were recovered at the 50–46% interface. The purity was checked by FACS analysis using anti-CD14 monoclonal antibody (BD Pharmingen) and was found to be >85%. Lymphocytes were recovered in the 46–40% interface. These cells were then incubated at 37 °C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium, supplemented with 10% FCS and 50 μg/ml gentamicin.

The use of human samples in this study was approved by the local Ethics Committee and was conducted according to the Declaration of Helsinki. Signed informed consent was obtained from the patients.

Before seeding, viability of U-937 and Jurkat cells and PBMC was tested by Trypan Blue assay. Cells were used only if viability was higher than 90%.

4.4. Measurement of the cytotoxicity concentration 50 (CC_{50})

Cells growing in exponential phase were seeded at 3.5×10^5 cells in 1 ml of RPMI 1640 in a 48 well culture plate and incubated in a 5% CO_2 atmosphere. Cells were exposed at different coumarin concentrations (0.15 μM to 2.0 mM) or 0.6% (v/v) DMSO (vehicle control group). After incubation for 48 h, 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 a hemocytometer chamber. CC_{50} values were calculated with the equation for sigmoidal dose response using Prism 5.00 for Windows (GraphPad Software, San Diego, CA). Assays were carried out in duplicate in at least three independent experiments.

4.5. Cell growth inhibition assays (IC_{50})

Cells growing in exponential phase were seeded at 3.5×10^5 cells in 200 μl of RPMI 1640 in a 96-well culture plate and incubated under a 5% CO_2 atmosphere at 37 °C. U-937 cells were exposed to different concentrations of compounds ranging from 0.15 μM to 2.0 mM or to 0.6% (v/v) DMSO (vehicle control group) followed by incubation with 0.5 μCi of [^{3}H]-methyl-thymidine (PerkinElmer, USA) added 12 h before the end of the experiment and 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. IC_{50} values were calculated using the equation for sigmoidal dose response using Prism 5.00 for Windows (GraphPad Software, San Diego, CA). Assays were performed in triplicate in at least three independent experiments and validated by parallelly determining cell numbers with a Coulter Z-1.

4.6. Clonogenic assay

U-937 cells were treated with different concentrations of compounds from Table 1 or 0.1% (v/v) DMSO (vehicle control group). After 24 h of incubation, 2.5×10^3 treated and control cells were washed extensively and resuspended in 1.5 ml of mixture containing 0.4% (w/v) agar select and 15% FCS-RPMI 1640 medium. Each cell suspension was seeded over a bottom layer containing 0.7% (w/v) agar select and 15% FCS-RPMI 1640 medium in a 6 well culture plate. Plates were incubated in a 5% CO_2 atmosphere for 4 weeks. The colonies were stained with crystal violet (0.5% w/v), and those containing at least 50 cells were scored using a stereomicroscope.

4.7. Determination of apoptosis markers

4.7.1. Cell cycle analysis

U-937 cells growing in exponential phase were treated with different concentrations of compounds from Table 1 or 0.1% (v/v) DMSO (vehicle control group) for 24 h. Then, cells were harvested and centrifuged at 1000 rpm for 5 min. Cell suspensions were fixed and permeabilized by vigorous addition of nine volumes of ice-cold 70% (v/v) ethanol and stored at -20 °C for a minimum of 24 h, prior to analysis. Cells at a density of approximately 10^6 were re-suspended in 800 μl of propidium iodide (PI) staining solution (20 μg/ml PI and 200 μg/ml RNase A in PBS, pH 7.4) and incubated in the dark at room temperature for 30 min. The percentages of cells in the sub-G0/G1, G0/G1, S and G2/M cell cycle phases were determined by a FACS Scan Flow Cytometer (Beckton-Dickinson CA, USA). Data from at least three independent experiments were analyzed using Cyflogic free software (http://www.cyflogic.com).

4.7.2. Determination of phosphatidylserine expression by annexin V binding assay

Cells growing in exponential phase were seeded at 3.5×10^5 cells/ml and treated with different concentrations of compounds or 2% (v/v) DMSO (positive control) for either 24 h or 48 h. After washing with cold PBS, 2.0×10^5 cells were incubated with FITC-labeled Annexin V and PI according to the protocol included in the Annexin V-FITC assay kit (Invitrogen) and analyzed using a FACS Scan Flow Cytometer (Beckton-Dickinson CA, USA). The different cell subpopulations were identified according to the Annexin V/PI staining pattern, as follows: cells labeled with annexin V only were considered to be at an early apoptotic stage, cells labeled with annexin V and PI were considered to be at a late apoptotic stage, and cells labeled with PI only were considered necrotic.

4.7.3. Caspase-3 activity

U-937 cells growing in exponential phase (3.5×10^5 cells/ml) were treated with different concentrations of compounds, 2% (v/v) DMSO (positive control) or 0.1% (v/v) DMSO (vehicle control group) during 24 h or 48 h. After that, cells were harvested and processed according to CASP3C caspase-3 colorimetric assay kit provided by Sigma Chemical Co. (St. Louis, USA). To determine whether the intrinsic or the extrinsic pathway was involved in the pro-apoptotic activity of *ortho*-dihydroxycoumarins, U-937 cells were pre-incubated 2 h with 100 μM Z-LEHD-FMK caspase-9 inhibitor (Sigma Chemical Co.) or 100 μM Z-IETD-FMK caspase-8 inhibitor (Sigma Chemical Co.) before the addition of **1**. Tumor

necrosis factor-α (TNF-α, Sigma Chemical Co.) and H_2O_2 (Sigma Chemical Co.) were employed as inducers of caspase-8 and 9 pathways activation, respectively.

4.7.4. Determination of cleaved caspase-3, -8 and -9, and PARP by Western blot

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, blotted and incubated with the indicated primary antibodies (Santa Cruz Biotechnology, CA), followed by horseradish peroxidase conjugated anti-rabbit (Santa Cruz Biotechnology, CA) and developed by enhanced chemiluminesence (ECL) following the manufacturer's instructions (Amersham Life Science, England). Densitometry analyses were performed using ImageJ free software (http://rsbweb.nih.gov/ij/index.html).

4.8. Determination of U-937 cell differentiation markers

4.8.1. Surface myeloid CD11b and CD14 antigens assay

The expression of CD11b was detected by direct inmunofluorescence staining. U-937 cells (3.5×10^5 cells/ml) were treated with different concentrations of compounds, 0.1% (v/v) DMSO (vehicle control group) or 400 μM db-cAMP (positive control group) for 72 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) 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 IP staining. Analysis was performed using a FACS flow cytometer and CellQuest software (BD Biosciences). The results are expressed as mean fluorescence intensity respect to control (non-treated cells).

4.8.2. Expression of the C5a receptor (CD88) assay

The C5a receptor (CD88) is a G-protein coupled receptor associated with Ca^{2+} release from intracellular stores.[29,30] Thus, we evaluated its expression by measuring Ca^{2+} intracellular release after rhC5a stimulus, using Fura 2-AM as a fluorescent indicator. U-937 cells (3.5×10^5 cells/ml) were treated with different concentrations of compounds, 400 μM db-cAMP (positive control group), or 0.1% (v/v) DMSO (vehicle control group) for 72 h. Cells of each experimental group were washed, resuspended and incubated in a buffered saline solution (BSS; 140 mM NaCl, 3.9 mM KCl, 0.7 mM KH_2PO_4, 0.5 mM $Na_2HPO_4{\cdot}12H_2O$, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 20 mM HEPES, 10 mM glucose, and 0.1% BSA, pH 7.5) in the presence of 2 μM Fura 2-AM. Stock solution of 2 mM de FURA 2-AM was prepared in DMSO. Cells were incubated for 30 min at 37 °C in an atmosphere of 5% CO_2, time by which Fura 2-AM was trapped intracellularly by esterase cleavage. Cells were then washed twice in BSS, and brought to a density of 2.0×10^6 cells/ml in BSS. Fluorescence was measured in a spectrofluorometer (Jasco, Tokyo, Japan) provided with the CA-61 accessory to measure Ca^{2+} with continuous stirring, with the thermostat adjusted to 37 °C and an injection chamber. During 8 min intracellular Ca^{2+} mobilization was registered every second by exposure to alternating 340 and 380 nm light beams, and the intensity of light emission at 505 nm was measured. In this way, light intensities and their ratio (F340/F380) were tracked. Different agents (rhC5a or ATP) were injected (5 μl) into the chamber as a 100-fold concentrated solution without interrupting recording. The preparation was calibrated by determining maximal fluorescence induced by 0.1% Triton X-100, and minimal fluorescence in the presence of 6 mM EGTA (pH 8.3).

4.8.3. Chemotaxis assay

The 'in vitro' locomotion of U-937 cells was assayed using the micropore filter technique (Transwell 3521, Costar Corp., Cambridge, MA). Briefly, 1×10^5 control, vehicle control or treated cells were seeded after 72 h of treatment onto the top compartment of the 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.

4.9. Evaluation of pro-oxidant properties

4.9.1. Determination of oxidation potential by cyclic voltammetry

As Wu and Dewald described,[37] all the compounds were diluted at 50 μM in potassium phosphate buffer 0.1 M ph 7.4 at 50 μM. The potential was cycled from 0.2 to 1.2 V and back to 0.2 V (vs Ag/AgCl) at a scan rate of 20 mV/s using a BAS 100B/W Electrochemical Workstation, Bioanalytical Systems, Inc. (West Lafayette, USA).

4.9.2. Electron spin resonance (ESR) experiments

The presence of free radicals intermediates of compounds listed on Table 1 diluted in RPMI 1640 medium or in carbonate buffer pH 9.0 was detected by ESR at 20 °C in an X-band ESR Spectrometer Bruker EMX Plus (Bruker Biospin GmbH, Germany).

In order to obtain a sensitive detection for compounds with very low levels of free radical intermediates, the spectrometer settings were: center field 3516 G, microwave power 10.0 mW, conversion time 5.12 ms, time constant 5.12 ms, modulation frequency 50 kHz, gain 2×10^4, resolution 1024 points, modulation amplitude of 1 G. At least 300 scans were accumulated.

With compounds with high levels of free radicals intermediates, kinetics studies were performed. For these studies, each compound was diluted to 2 mM in the respective medium ESR spectra were recorded at given acquisition times between 1 min and the time in which the signal disappear. Spectrometer settings were similar to those indicated above, except that modulation amplitude was 0.1 G and the number of scans varied from 10 to 25, depending on the signal intensity.

4.9.3. Determination of pro-oxidant species

U-937 cells were pre-incubated with 10 μM 2′,7′-dichlorofluorescein diacetate (DCF-DA) (Sigma Chemical Co.) for 30 min at 37 °C. After washing in PBS and re-suspension in RPMI 1640 medium, 5×10^4 cells were seeded and treated with different concentrations of 1 (10, 33, 66, 100, 133, 166 and 250 μM). Fluorescence, due to DCF-DA oxidation, was monitored during 6 hours (excitation λ 490 nm-emission λ 530 nm-cut off λ 515 nm) using the equipment *Flex Station 3* microplate reader (Molecular Devices Inc., USA). A sigmoidal concentration-response curve was plotted using Prism 5.00 for Windows (GraphPad Software, San Diego, CA). Results are from one representative experiment of three independent assays performed that showed the same pattern.

4.10. Statistical analysis

Results in Table 2 are expressed as the mean with a 95% confidence interval (IC95). The rest of the results are expressed as mean ± SEM of at least three independent experiments. Statistical analysis was performed by one-way ANOVA followed by the Student–Newman–Keuls (SNK) a posteriori test using InfoStat software (2009). 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 it was necessary to get homoscedasticity of the variable.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Grant support

This study was supported by grants PICT 07-01725 from Agencia Nacional de Promoción Científica y Tecnología, and UBACyT B042 from Universidad de Buenos Aires, Argentina.

References and notes

1. O'Kennedy, R.; Thornes, R. D. *Coumarins: Biology, Applications and Mode of Action*; John Wiley & Sons: New York, 1997.
2. Borges, F.; Roleira, F.; Milhazes, N.; Santana, L.; Uriarte, E. *Curr. Med. Chem.* **2005**, *12*, 887.
3. Laurin, P.; Klich, M.; Dupis-Hamelin, C.; Mauvais, P.; Lassaigne, P.; Bonnefoy, A.; Musicki, B. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2079.
4. Au, N.; Rettie, A. E. *Drug. Metab. Rev.* **2008**, *40*, 355.
5. Oldenburg, J.; Watzka, M.; Rost, S.; Müller, C. R. *J. Thromb. Haemost.* **2007**, *5*, 1.
6. Pillai, S.; Menon, S.; Mitscher, L.; Pillai, C.; Shankel, D. *J. Nat. Prod.* **1999**, *62*, 1358.
7. Fylaktakidou, K. C.; Hadjipavlou-Litina, D. J.; Litinas, K. E.; Nicolaides, D. N. *Curr. Pharm. Des.* **2004**, *10*, 3813.
8. Roma, G.; Braccio, M. D.; Carrieri, A.; Grossi, G.; Leoncini, G.; Grazia Signorello, M.; Carotti, A. *Bioorg. Med. Chem.* **2003**, *11*, 123.
9. Raj, H. G.; Parmar, V. S.; Jain, S. C.; Goel, S.; Poonam; Himanshu; Malhorta, S.; Singh, A.; Olsen, C. E.; Wengel, J. *Bioorg. Med. Chem.* **1998**, *6*, 833.
10. Kirkiacharian, S.; Thuy, D. T.; Sicsic, S.; Bakhchinian, R.; Kurkjian, R.; Tonnaire, T. *Farmaco* **2002**, *57*, 703.
11. Riveiro, M. E.; De Kimpe, N.; Moglioni, A.; Vázquez, R.; Monczor, F.; Shayo, C.; Davio, C. *Curr. Med. Chem.* **2010**, *17*, 1325.
12. Riveiro, M. E.; Shayo, C.; Monczor, F.; Fernández, N.; Baldi, A.; De Kimpe, N.; Rossi, J.; Debenedetti, S.; Davio, C. *Cancer Lett.* **2004**, *210*, 179.
13. Riveiro, M. E.; Maes, D.; Vázquez, R.; Vermeulen, M.; Mangelinckx, S.; Jacobs, J.; Debenedetti, S.; Shayo, C.; De Kimpe, N.; Davio, C. *Bioorg. Med. Chem.* **2009**, *17*, 6547.
14. Riveiro, M. E.; Moglioni, A.; Vazquez, R.; Gomez, N.; Facorro, G.; Piehl, L.; Rubin de Celis, E.; Shayo, C.; Davio, C. *Bioorg. Med. Chem.* **2008**, *16*, 2665.
15. Riveiro, M. E.; Vazquez, R.; Moglioni, A.; Gomez, N.; Baldi, A.; Davio, C.; Shayo, C. *Biochem. Pharmacol.* **2008**, *75*, 725.
16. Goel, A.; Prasad, A. K.; Parmar, V. S.; Ghosh, B.; Saini, N. *FEBS Lett.* **2007**, *581*, 2447.
17. Chu, C. Y.; Tsai, Y. Y.; Wang, C. J.; Lin, W. L.; Tseng, T. H. *Eur. J. Pharmacol.* **2001**, *416*, 25.
18. Park, C.; Jin, C. Y.; Kim, G. Y.; Choi, I. W.; Kwon, T. K.; Choi, B. T.; Lee, S. J.; Lee, W. H.; Choi, Y. H. *Toxicol. Appl. Pharmacol.* **2008**, *227*, 219.
19. Lin, T. H.; Lu, H. J.; Yin, Y. F.; Tseng, T. H. *Chem. Biol. Interact.* **2009**, *180*, 61.
20. Park, C.; Jin, C. Y.; Kwon, H. J.; Hwang, H. J.; Kim, G. Y.; Choi, I. W.; Kwon, T. K.; Kim, B. W.; Kim, W. J.; Choi, Y. H. *Toxicol. In Vitro* **2010**, *24*, 486.
21. Yang, J.; Xiao, Y. L.; He, X. R.; Qiu, G. F.; Hu, X. M. *J. Asian Nat. Prod. Res.* **2010**, *12*, 185.
22. Abrink, M.; Gobl, A. E.; Huang, R.; Nilsson, K.; Hellman, L. *Leukemia* **1994**, *8*, 1579.
23. Drach, J.; McQueen, T.; Engel, H.; Andreeff, M.; Robertson, K. A.; Collins, S. J.; Malavasi, F.; Mehta, K. *Cancer Res.* **1994**, *54*, 1746.
24. Witcher, M.; Ross, D. T.; Rousseau, C.; Deluca, L.; Miller, W. H., Jr. *Blood* **2003**, *102*, 237.
25. Asada, M.; Yamada, M. T.; Ichijo, H.; Delia, D.; Miyazono, K.; Fukumuro, K.; Mizutani, S. *EMBO J.* **1999**, *18*, 1223.
26. Kawaii, S.; Tomono, Y.; Katase, E.; Ogawa, K.; Yano, M. *Anticancer Res.* **2000**, *20*, 2505.
27. Kawaii, S.; Tomono, Y.; Ogawa, K.; Sugiura, M.; Yano, M.; Yoshizawa, Y. *Anticancer Res.* **2001**, *21*, 917.
28. Zhang, J. X.; Fong, W. F.; Wu, J. Y.; Yang, M.; Cheung, H. Y. *Planta Med.* **2003**, *69*, 223.
29. Burg, M.; Martin, U.; Rheinheimer, C.; Kohl, J.; Bautsch, W.; Böttger, E. C.; Klos, A. *J. Immunol.* **1995**, *155*, 4419.
30. Brodsky, A.; Davio, C.; Shayo, C.; Lemos Legnazzi, B.; Barbosa, M.; Lardo, M.; Morelli, A.; Baldi, A.; Sanchez Avalos, J. C.; Rivera, E. *Eur. J. Pharmacol.* **1998**, *350*, 121.
31. Zhang, H. Y.; Wang, L. F. *J. Mol. Struct.* **2004**, *673*, 199.
32. Vizirianakis, I. S.; Chatzopoulou, M.; Bonovolias, I. D.; Nicolaou, I.; Demopoulos, V. J.; Tsiftsoglou, A. S. *J. Med. Chem.* **2010**, *53*, 6779.
33. Stagos, D.; Spanou, C.; Margariti, M.; Stathopoulos, C.; Mamuris, Z.; Kazantzoglou, G.; Magiatis, P.; Kouretas, D. *J. Agric. Food Chem.* **2007**, *55*, 5246.
34. Bhat, S. H.; Azmi, A. S.; Hadi, S. M. *Toxicol. Appl. Pharmacol.* **2007**, *218*, 249.
35. Abdel-Wahab, O.; Levine, R. L. *J. Exp. Med.* **2010**, *207*, 677.
36. Chuluyan, H. E.; Issekutz, A. C. J. *Clin. Invest.* **1993**, *92*, 2768.
37. Wu, Q.; Dewald, H. D. *Electroanalysis* **2000**, *13*, 45.