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Induction of cell differentiation in human leukemia U-937 cells by 5-oxygenated-6,7-methylenedioxycoumarins from *Pterocaulon polystachyum*

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Abstract

The present study focused on the effect of a series of extracts and two 5,6,7-trioxygenated coumarins isolated from *Pterocaulon polystachyum* on the proliferation and differentiation of human promonocytic U-937 cells. The petroleum ether extract was the only extract that significantly reduced cell proliferation and induced cell differentiation. Treatment with pure 5-methoxy-6,7-methylenedioxycoumarin (C1) and 5-(3-methyl-2-butenyloxy)-6,7-methylenedioxycoumarin (C2), present in the petroleum ether extract, showed a time and concentration-dependent inhibition on cell proliferation. In addition, the coumarin derivatives were also able to induce CD88 functionality and NBT reduction, markers of monocytic cell differentiation.

These results suggest that C1 and C2 might have a potential therapeutic role in the management of leukemia.

Keywords: 5-oxygenated-6,7-methylenedioxycoumarins; *Pterocaulon polystachyum*; Cell differentiation; CD88; Leukemia

1. Introduction

Most chemotherapeutic agents currently used in cancer therapies display significant toxicity and are often nonspecific. A potential alternative to treat this prevalent disease includes agents that induce cell differentiation. This relative new approach is termed 'differentiation therapy'. This is based on the hypothesis that many neoplastic cell types exhibit

Abbreviations: ATRA, all *trans* retinoic acid; NBT, nitrobluetetrazolium; PMA, phorbol 12-myristate 13-acetate; dbcAMP, dibutyryl cAMP; CH_2Cl_2, dichloromethane; EtOAc, ethyl acetate; MeOH, methanol; DMSO, dimethyl sulfoxide; rhC5a, recombinant human C5a; HTMT, dimaleate, 6[2-(4-imidazolyl)ethylamino]-*N*-(4-trifluoromethylphenyl) heptanecarboxamide; IC_{50}, concentration that provokes an inhibition of 50%; CI_{95}, 95% confidence interval.

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reversible defects in the course of differentiation, which upon appropriate treatment, result in tumor reprogramming with a concomitant loss of proliferative capacity and induction of terminal differentiation or apoptosis [1].

Differentiation–induction as a therapeutic strategy has the greatest impact on hematopoietic malignancies, most notably on leukemia. Hematopoietic cells undergo a genetically-regulated program of maturation in which pluripotential stem cells, in response to both internal and external stimuli, gradually acquire the lineage specificity and functional characteristics of their more differentiated counterparts. This phenomenon is regularly accompanied by a reciprocal reduction in self-renewal capacity. Thus, the failure of leukemic cell to undergo differentiation represents a prototypical example of disregulated maturation as a fundamental hallmark of neoplastic transformation. The discovery that certain chemical compounds (e.g. phorbol esters) could restore a normal differentiation program in leukemic cells represented one of the first examples of differentiation therapy [2]. These have culminated in the successful introduction of all *trans* retinoic acid (ATRA) as a primary treatment in patients with acute promyelocytic leukemia [3]. Efforts to identify others and potentially more effective differentiation inducers for the treatment of leukemia have remained the focus of major interest.

The screening of plant extracts and natural products have shown that higher plants represent a potential source of new therapeutic agents, as well as of new drugs from natural products for primary lead compounds [4].

Direct antitumor effects have been reported for herbal extracts containing coumarins, demonstrating an inhibitory growth capacity for a number of malignant cell lines in vitro [5–7]. Coumarins comprise a very large class of natural compounds found throughout the plant kingdom. Natural coumarins, like other unsaturated lactones, may exert various effects on living organisms. Several important and comprehensive reviews deal with the occurrence, chemistry and biochemical properties of simple and complex natural coumarins [8–11]. Simple coumarins can be substituted at different positions on the aromatic nucleus and can be classified depending on their oxygenation pattern. However, up to date, most of the pharmacological and biochemical studies have been carried out on coumarin itself and on monohydroxy- and dihydroxycoumarins or methoxycoumarins [12,13]. Recent studies described a large number of biological effects for natural and synthetic coumarins such as, anti-bacterial [14], and anti-mutagenic properties [15], scavenging of reactive oxygen species [16], inhibition of human platelet aggregation [17] and anti-HIV-PR activity [18]. In sensitive tumors, coumarin and its derivatives cause significant changes in the regulation of the immune response, cell growth and differentiation [19,20]. More recently, several coumarins have been reported to be anti-tumourigenic [21–23] and synergistic with ATRA on the differentiation of human leukemic cells [24].

Our systematic studies on the chemical constituents of Argentine medicinal species of the *Pterocaulon* genus, deal with the isolation and structural determination of several 6,7-dioxygenated and 5,6,7-trioxygenated coumarins from *Pterocaulon polystachyum* [25]. In addition, different bioassays carried out with non-polar extracts of *P. polystachyum* suggested the presence of compounds that interact with cellular DNA [26].

The promonocytic U-937 cell line, derived from a histiocytic lymphoma is an appropriated model to study the mechanism of cell differentiation [27,28]. In this cell line, dibutyryl cAMP (dbcAMP), as other agents, induces monocyte maturation [29,30].

As part of our screening program to evaluate the potential chemotherapeutic effect of natural compounds, we have investigated the effect of crude extracts of *P. polystachyum* and two pure trioxygenated coumarins, 5-methoxy-6,7-methylenedioxycoumarin (C1) and 5-(3-methyl-2-butenyloxy)-6,7-methylenedioxycoumarin (C2) (Fig. 1), present as the main

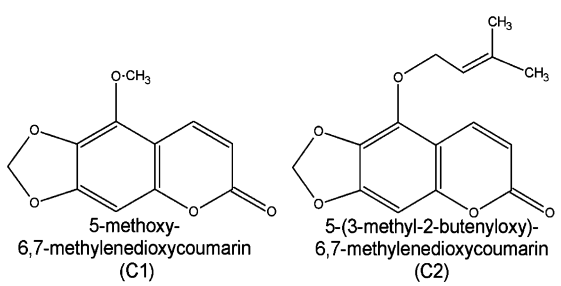

Fig. 1. Structure of pure coumarins isolated from the petroleum ether extract.

compounds in the petroleum ether extract, on U-937 cells. The 5-oxy-6,7-methylenedioxycoumarins, C1 and C2, are not common in nature or as synthetic products, and at present no biological activity has been reported.

2. Materials and methods

2.1. Chemicals

RPMI medium 1640, phosphate-buffered saline (PBS), Fura 2-AM, dbcAMP, recombinant human C5a (rhC5a), all-*trans* retinoic acid (ATRA), nitrobluetetrazolium (NBT), phorbol 12-myristate 13-acetate (PMA) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Fetal calf serum was purchased from Natocor (Argentina). All other chemicals were of analytical grade.

2.2. Plant extracts and 5,6,7-trioxygenated coumarins

Dried powder aerial parts of *P. polystachyum* L. DC (900 g) were successively extracted with petroleum ether (60–80 °C), CH_2Cl_2, EtOAc and MeOH in a Soxhlet apparatus and were concentrated to dryness on a rotary evaporator. For infusion preparation, 100 g of the dried powder plant material was extracted with 1000 ml of hot water, left standing for 20 min, filtered and lyophilized.

From the petroleum ether extract, 5-methoxy-6,7-methylenedioxycoumarin (C1) and 5-(3-methyl-2-butenyloxy)-6,7-methylenedioxycoumarin (C2) were isolated and identified on the basis of their physical and spectral data (UV, EI-MS, ^{1}H-NMR, ^{13}C-NMR, IR, m.p) [25]. The structure of each coumarin used in the present experiments is shown in Fig. 1.

Dried extracts and pure coumarins were dissolved in 0.01% (v/v) DMSO.

2.3. Cell culture

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

2.4. Assessment of cell viability

Cell viability was monitored by trypan blue exclusion test. Cells growing in exponential phase were seeded at 10^5 cells in 1 ml of RPMI 1640 in a 24 well culture plate and incubated in a 5% CO_2 atmosphere. Cells were exposed to different concentrations of the extracts from *P. polystachyum*, pure coumarins or DMSO (control group). The final concentration of DMSO in all cultures was 0.0001% (v/v). Every 24 h, for 5 days, an aliquot was collected and mixed with equal volumes of 0.4% trypan blue. After 5 min of incubation, the proportion of viable cells was estimated using a hemocytometer chamber. The assays were carried out by triplicate in at least three independent experiments.

2.5. Cell growth experiment

Cells (10^5 cells/ml) were seeded in 24 wells plates and treated with different plant extracts and trioxygenated coumarins from *P. polystachyum* at various concentrations, 400 μM dbcAMP (positive control), or 0.0001% (v/v) DMSO (control group), during 5 days. Cells were collected at the time indicated for each experiment and the number of cells was determined using a cellular meter Coulter Z-1. Cell density in culture did not exceed 1.5×10^6 cells/ml.

2.6. Intracellular Ca^{2+} measurements

Fura 2-AM was used as a fluorescent indicator. U-937 (1×10^6 cells/ml) were treated with different plant extracts or pure coumarins from *P. polystachyum*, 400 μM dbcAMP (positive control), or 0.0001% (v/v) DMSO (control group) for 48 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 12 H_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 2×10^6 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+} ($[Ca^{2+}]i$) levels were 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 HTMT dimaleate) 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). $[Ca^{2+}]i$ was calculated according to Grynkiewicz et al. [31].

2.7. Chemotaxis assay

The 'in vitro' locomotion of U-937 cells was assayed using the micropore filter technique (Transwell 3521, Costar Corp, Cambridge, MA). Briefly, 10^5 control or 3 days treated cells, were seeded 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 3 h at 37 °C in a 5% CO_2 atmosphere. Migrated cells were collected and counted using a cellular meter Coulter Z-1.

2.8. Nitrobluetetrazolium differentiation assays

U-937 (2×10^5 cells/ml) were treated with pure coumarins, 1 μM ATRA (positive control) or 0.0001% (v/v) DMSO (control group) for 72 h. Cells of each experimental group were washed and resuspended in 200 μl RPMI 1640 media containing 1 mg/ml NBT and 1 μg/ml PMA. After incubation at 37 °C for 30 min, cells were pelleted and resolved in 200 μl DMSO and their absorbance at 570 nm was determined.

2.9. Data analysis

Results are expressed as the mean ± SEM of at least three independent experiments. In cell growth experiments, results represent the mean ± SD of assay triplicates. Statistical significance was analyzed by one-way ANOVA. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Cytotoxicity of plant extracts and pure coumarins on U-937 cells

A screening of plant extracts and 5-oxygenated-6,7-methylenedioxycoumarins was carried out in order to determine those concentrations that did not display cytotoxicity for this cell line. Different concentrations of the extracts (10 μg/ml–0.1 μg/ml) and coumarins (20 μM–0.2 μM) were added to U-937 cells for 5 days, and cell viability was assayed by the trypan blue exclusion test. With concentrations below 1.6 μg/ml for the petroleum ether extract, 1.3 μg/ml for the CH_2Cl_2 extract, 1.1 μg/ml for the EtOAc extract, 1.1 μg/ml for the MeOH extract, 1.0 μg/ml for the infusion, 42.75 μM for C1, and 22.16 μM for C2, more than 90% of cell viability was achieved. The vehicle alone exhibited similar cell viability. Extracts and coumarins were then tested for their antiproliferative and differentiation-inducing activity exclusively in the non-toxic concentration range.

3.2. Effects of plant extracts on cell proliferation

According to the cytotoxicity assays, cells were treated with 1.6 μg/ml of petroleum ether extract, 1.3 μg/ml CH_2Cl_2 extract, 1.1 μg/ml EtOAc extract, 1.1 μg/ml MeOH extract, 1.0 μg/ml infusion, 400 μM dbcAMP (positive control), or 0.0001% (v/v) DMSO (vehicle) for 5 days. In the proliferation assay (Fig. 2) we observed that of the five different crude extracts of *P. polystachyum* screened for antiproliferative activity, the petroleum ether extract was the only extract that significantly reduced cell proliferation ($P < 0.01$). Similar results were obtained with dbcAMP (positive control).

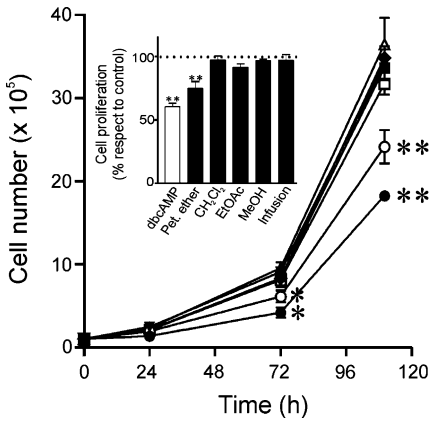

Fig. 2. Effect of plant extracts from *Pterocaulon polystachyum* on U-937 cell proliferation. Cells were treated with extracts from (○) 1.6 μg/ml petroleum ether, (◆) 1.3 μg/ml CH_2Cl_2, (□) 1.1 μg/ml EtOAc, (■) 1.1 μg/ml MeOH, (△) 1.0 μg/ml infusion, (●) 400 μM dbcAMP (positive control), or (▽) 0.0001% (v/v) DMSO for 24, 72, and 110 h as described in Section 2. The results represent the mean ± SD of assay triplicates. Similar results were obtained in at least three independent experiments. *$P < 0.05$ and **$P < 0.01$ vs. control group (DMSO treated cells). Inset. U-937 cell proliferation after 110 h of treatment with plant extracts from *Pterocaulon polystachyum*. Data are expressed as proliferation percentage respect to control group. Control group proliferation considered 100% is showed as a dotted line. Results represent the mean ± SEM ($n = 3$). **$P < 0.01$ vs. control.

3.3. Effects of plant extracts on cell differentiation

The C5a receptor (CD88) is a monocytic marker commonly used in differentiation protocols. This receptor is a G-protein coupled receptor associated with Ca^{2+} release from intracellular stores [32], and it is also involved in the chemotactic process [33]. Thus, we evaluated its expression by the measure of Ca^{2+}i release and the chemotaxis induced by rhC5a.

Pretreatment of U-937 cells with the different extracts for 2 days showed that those cells pretreated with the petroleum ether extract displayed a $[Ca^{2+}]$i spike induced by rhC5a, similar to that evoked by dbcAMP, that was used as a positive control.

Pretreatment of U-937 cells with the CH_2Cl_2 extract induced a minor $[Ca^{2+}]$i response (Fig. 3). No response was observed in vehicle pretreated cells, but when these cells were stimulated with HTMT dimaleate (H1 agonist), known to elevate $[Ca^{2+}]$i levels [34], they showed the typical spike indicating that these cells were able to evoke a Ca^{2+} response.

C5a chemotaxis is illustrated in Fig. 4 were the percentage of cell migration for each group is shown. Cells treated either with the petroleum ether extract, or with dbcAMP, exhibited significantly higher chemotactic response than control cells ($P < 0.01$). In this assay, the CH_2Cl_2 extract did not induce cell migration in response to rhC5a.

3.4. Effects of coumarins on cell proliferation

Due to the important antiproliferative and cell differentiating activity on the U-937 cell line displayed by the petroleum ether extract, 5-methoxy-6,7-methylenedioxycoumarin (C1) and 5-(3-methyl-2-butenyloxy)-6,7-methylenedioxycoumarin (C2), were studied in order to determine whether they were the active principles of this extract.

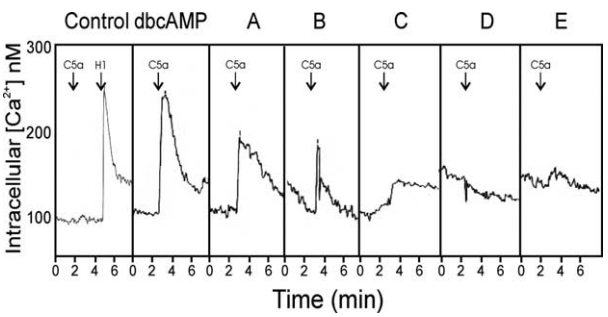

Fig. 3. Effect of extracts from *Pterocaulon polystachyum* on $[Ca^{2+}]$i response induced by rhC5a. U-937 cells were treated with (A) 1.6 μg/ml petroleum ether extract; (B) 1.3 μg/ml CH_2Cl_2 extract; (C) 1.1 μg/ml EtOAc extract; (D) 1.1 μg/ml MeOH extract; (E) 1.0 μg/ml infusion; 400 μM dbcAMP, or 0.0001% (v/v) DMSO, for 48 h. $[Ca^{2+}]$i was determined as described in Section 2. Arrows indicate the addition of rhC5a (C5a) or H1 agonist (H1). Similar results were obtained in at least three independent experiments.

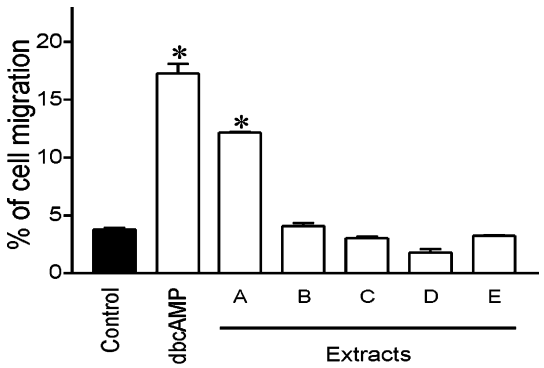

Fig. 4. C5a chemotactic responses of treated U-937 cells. U-937 cells were incubated with (A) 1.6 μg/ml petroleum ether extract; (B) 1.3 μg/ml CH_2Cl_2 extract; (C) 1.1 μg/ml EtOAc extract; (D) 1.1 μg/ml MeOH extract; (E) 1.0 μg/ml infusion; 400 μM dbcAMP, or 0.0001% (v/v) DMSO (vehicle) for 3 days, washed and exposed to 5×10^{-9} M rhC5a. Chemotactic assays were performed as detailed in Section 2. Results are expressed as means ± SEM ($n = 3$). *$P < 0.01$ vs. control group (DMSO treated cells).

C1 and C2 coumarins inhibited the growth of human leukemia U-937 cells in a time and concentration dependent manner (Figs. 5 and 6). This results showed that 0.85 μM C1 and 0.44 μM C2, significantly inhibited the growth of U-937 cells at 110 h ($P < 0.01$). At higher concentration of C1 and C2 (8.5 μM and 4.4 μM, respectively), the effect was observed after 72 h of treatment ($P < 0.05$) (Fig. 5).

Concentration-response studies, showed that C1 was more potent in inhibiting cell proliferation than C2, with an $IC_{50} = 2.2$ μM ($CI_{95} = 1.9$-2.5 μM) and $IC_{50} = 3.5$ μM ($CI_{95} = 2.8$-4.3 μM) for C1 and C2 respectively (Fig. 6).

3.5. Effects of coumarins on cell differentiation

Cells treated with coumarins C1 or C2 for 48 h showed phenotypic characteristics of differentiated cells. Pretreatment with C1 or C2, evoked a $[Ca^{2+}]i$ spike induced by rhC5a in U-937 cells similar to that induced by dbcAMP treated cells (Fig. 7). In addition, rhC5a significantly induced chemotaxis in U-937 cells pretreated with both pure coumarins ($P < 0.01$) (Fig. 8).

The burst oxidative capacity of differentiated U-937 cells was determined by NBT reduction assays. The rate of NBT reduction by differentiated U-937 cells was determined by the measure of formazan at 570 nm. A significant increase in the production of formazan was observed following 72 h exposure to 8.5 μM C1 and 4.4 μM C2, respectively, respect to control cells (DMSO treated cells) (Fig. 9). Similar results were obtained using 1 μM ATRA, control of cell differentiation.

4. Discussion

To our knowledge this is the first report that shows that 5-oxygenated-6,7-methylenedioxy-coumarins exhibit antiproliferative and differentiating properties.

Scientific interest in new natural compounds with potential therapeutic value for leukemia treatment and/or lead molecules that facilitate drug development have recently emerged, due to the importance of

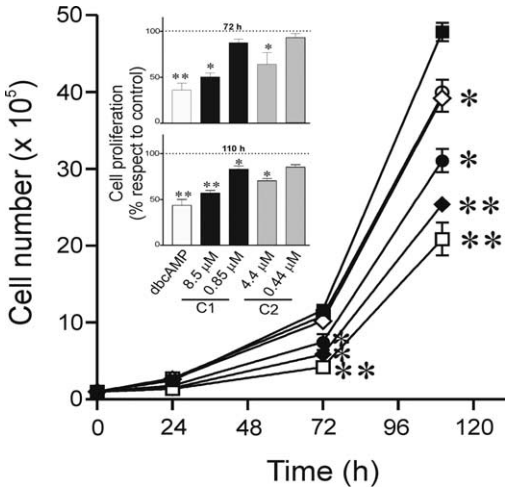

Fig. 5. Effect of pure coumarins on U-937 cell proliferation. Cells were treated with (◇) 0.85 μM C1, (○) 0.44 μM C2, (◆) 8.5 μM C1, (●) 4.4 μM C2, (□) 400 μM dbcAMP, or (■) 0.0001% (v/v) DMSO for 24, 72, and 110 h as described in Section 2. The results represent the mean ± SD of assay triplicates. Similar results were obtained in at least three independent experiments. *$P < 0.05$ and **$P < 0.01$ vs. control group (DMSO-treated cells). Inset. U-937 cell proliferation after 72 h and 110 h of treatment with 0.85 μM C1, 0.44 μM C2, 8.5 μM C1, 4.4 μM C2 or 400 μM dbcAMP. Data are expressed as proliferation percentage respect to control group. Control group proliferation considered 100% is showed as a dotted line. Results represent the mean ± SEM ($n = 3$). *$P < 0.05$ and **$P < 0.01$ vs. control.

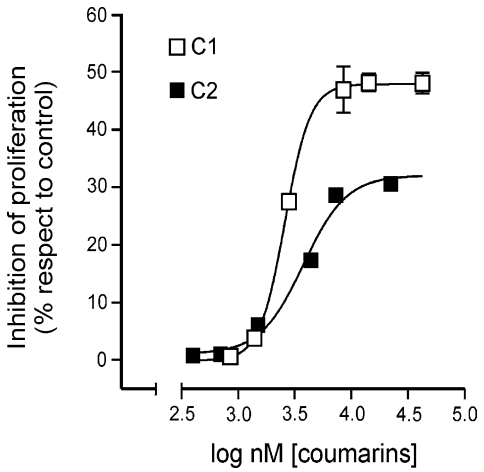

Fig. 6. Concentration-dependent inhibition of U-937 cell proliferation. U-937 cells were seeded in tissue culture plates and incubated for 3 days, with 0.0001% (v/v) DMSO (control group) or different concentrations of (□) C1 or (■) C2. At 72 h, cells were collected and the number of cells/ml was determined using a cellular meter Coulter Z-1. Data are expressed as inhibition of proliferation respect to control group. Results are expressed as mean ± SEM ($n = 4$).

the development of differentiating therapies with lower toxicity.

Imbalance between cell proliferation, apoptosis and differentiation leads to the development of malignant cells clones. Based on the understanding of tumor biology concerning the kinetics of cell populations, two new strategies, induction of differentiation and apoptosis, have recently emerged in the fields of cancer chemoprevention and chemotherapy [1]. Differentiation from malignant or premalignant cells into more mature or normal-like cells as well as apoptosis in multistep carcinogenesis are theoretically amenable as preventive cancer intervention. Thus, compounds capable of inducing differentiation are considered candidate agents for the prevention and/or treatment of cancer [35,36].

The U-937 cell line, derived from hystiocytic lymphoma is considered as an appropriated in vitro model system for studying the cellular and molecular events involved in the cell differentiation process. Any agent that possesses the ability to induce differentiation in U-937 cells, is commonly considered to have a potential therapeutic value. Terminal differentiation of U-937 cells can be monitored by changes of morphological, biochemical, and immunological properties [1].

The receptor for the anaphylactic portion of complement C5a (CD88), is not initially expressed in the monoblastic U-937 cell line [37], but it appears in differentiated U-937 cells. In this regard we analyzed CD88 expression by two different experimental approaches, the release of Ca^{2+} from intracellular stores and the chemotactic response.

In the present work, the petroleum ether extract from *P. polystachyum* exerted antiproliferative and differentiation activity on U-937 cells with no substantial cytotoxic effect at the concentration and time course tested (Figs. 2–4). Two trioxygenated coumarins isolated from this extract, 5-methoxy-6,7-methylenedioxycoumarin (C1) and 5-(3-methyl-2-butenyloxy)-6,7-methylenedioxycoumarin (C2) were analyzed to determine if they were the active compounds. C1 and C2 significantly inhibited the growth of U-937 cells in a time and concentration dependent manner (Figs. 5 and 6). U-937 cells pretreated with C1 and C2 displayed a $[Ca^{2+}]i$ spike and cell migration induction in response to rhC5a (Figs. 7 and 8). C1 and C2 pretreatment also increased the burst oxidative capacity of U-937 as measured by reduction of NBT (Fig. 9). Those results indicate the differentiating and antiproliferative activities of these novel coumarins on human leukemia U-937 cells.

On the other hand, the CH_2Cl_2 extract exhibited no effect on cell proliferation (Fig. 2). Concerning cell

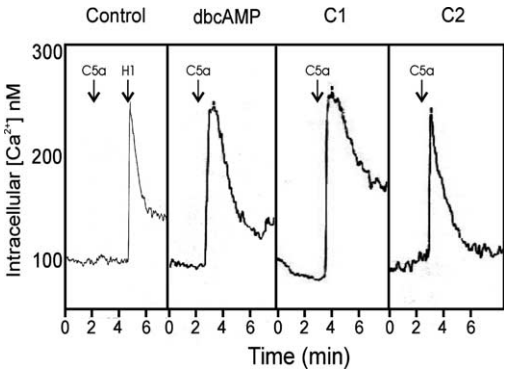

Fig. 7. Effect of coumarins on $[Ca^{2(}]i$ induction by rhC5a. U-937 cells were treated with 8.5 μM C1, 4.4 μM C2, 400 μM dbcAMP, or 0.0001% (v/v) DMSO (vehicle) for 48 h. $[Ca^{2+}]i$ was determined as described in Section 2. Arrows indicate the addition of rhC5a (C5a) or H1 agonist (H1). Similar results were obtained in at least three independent experiments.

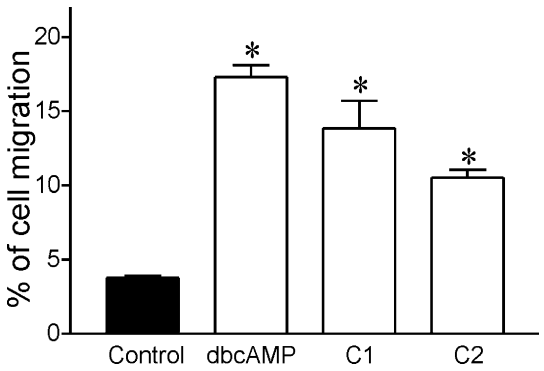

Fig. 8. C5a chemotactic responses of coumarins treated U-937 cells. U-937 cells were incubated with 8.5 μM C1, 4.4 μM C2, 400 μM dbcAMP, or 0.0001% (v/v) DMSO (vehicle) for 3 days and exposed to 5×10^{-9} M rhC5a. Chemotactic assays were performed as detailed in Section 2. Results are expressed as means ± SEM ($n = 3$). *$P < 0.01$ vs. control group (DMSO-treated cells).

differentiation (Figs. 3 and 4), a modest calcium release was observed with no change in cell migration. It is interesting to note that scopoletin was detected in CH_2Cl_2 extract [25] and this 6,7-dioxygenated coumarin may be responsible for the slight calcium increase. In the chemotaxis assay a significant effect was not observed, probably due to the limited sensitivity of this technique. Preliminary results indicate that pure scopoletin is able to induce inhibition of U-937 cell proliferation and differentiation (data not shown).

Treatment of U-937 cells with similar concentrations of the EtOAc and MeOH extracts as well as the infusion of *P. polystachyum* induced neither inhibition of cell proliferation nor terminal differentiation. In these plant extracts, there are other polyphenol compounds, different from coumarins [38].

The biological effects of coumarins appear to be based on the coumarin nucleus. It has been shown that the most active compounds have at least two polar functional groups positioned on the aromatic ring of coumarin, particularly at carbons 6 and 7 [39,40]. Since this is the first report on the antiproliferative and differenciating activity of 5-oxygenated-6,7-methylenedioxycoumarins, it could have a relevant value for further structure–activity considerations. As the results showed C1 has more potent antiproliferative and differentiating activity than C2, we are at present studying related coumarins, with a similar oxygenation pattern, in order to establish structure–activity relationships.

Evidences indicate that coumarins could exert their effects by several mechanisms of action. Some coumarins may act as cyclic nucleotide phosphodiesterases inhibitors [41,42], while 7,8-dihydroxycoumarins may act as protein kinase inhibitors [43]. The interaction of coumarins with signal transducing elements implies a modification in the propagation of the differentiating signal towards the nucleus. This kind of interference may account for various effects of coumarins reported in cells, such as downregulation of oncogene expression, induction of apoptosis on human leukemia HL-60 cells [44], inhibition of G1/S transition and decrease of cyclin D1 [45].

Present findings show that coumarins inhibit proliferation and induce differentiation in U-937 cells.

The mechanisms underlying the effect of coumarins in cell proliferation and differentiation are presently unknown but the characterization of those mechanisms will allow the identification of key molecular target for this group of compounds to facilitate the development of new pharmacological tools with potential therapeutic value for the management of leukemia.

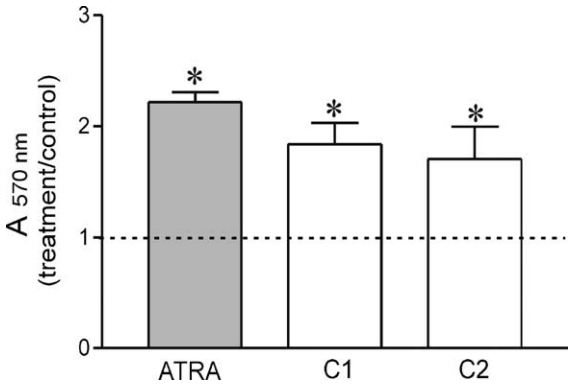

Fig. 9. Nitrobluetetrazolium reduction by U-937 cells. Cells were treated with 1 μM ATRA (positive control), 8.5 μM C1, 4.4 μM C2, or 0.0001% (v/v) DMSO (vehicle) for 72 h. Formazan production was determined as described in Section 2. Control group (DMSO-treated cells) absorbance considered 1 is showed as a dotted line. Results are expressed as means ± SEM ($n = 3$). *$P < 0.01$ vs. control group.

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