



Apoptosis and cell cycle disturbances induced by coumarin and 7-hydroxycoumarin on human lung carcinoma cell lines

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Summary Coumarin and 7-hydroxycoumarin have anti-tumour actions in vitro and in vivo. There are no previous reports on the cytostatic and apoptotic actions of coumarin and 7-hydroxycoumarin in non-small cell lung carcinoma (NSCLC) cell lines. Here we report on: (1) the inhibition of cell proliferation, (2) the phase in which cell cycle arrest occurs, and (3) the induction of apoptosis. Inhibition of cell proliferation was determined by ³H-thymidine incorporation. The effects on cell cycle phases were determined at 100 μg/ml of coumarin or 7-hydroxycoumarin using propidium iodide and flow cytometry. Higher concentrations were used to study apoptosis, detected by: (1) morphological cell changes, (2) subG₁ peak detection and (3) Annexin-V assay. Peripheral blood mononuclear cells (PBMC) stimulated with phytohemagglutinin were used as controls. The actions of these compounds depended on drug concentrations and on histological cell type. Coumarin and 7-hydroxycoumarin inhibited cell growth by inducing cell cycle arrest in the G₁ phase in all the lung carcinoma cell lines. Apoptosis required large concentrations of the coumarin compounds and was observed in adenocarcinomas. Apoptosis was not associated with intra-nucleosomal DNA fragmentation. Apoptosis was not observed in squamous lung carcinoma cell lines, but an increase in G₁ cell cycle arrest was detected. In PBMC, only large concentrations of the coumarin compounds elicited a cystostatic action. Coumarins in combination with other anti-neoplastic drugs might increase the effectiveness of NSCLC treatments.
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1. Introduction

Lung cancer is the most common fatal cancer worldwide and its incidence is increasing. Non-small cell lung carcinoma (NSCLC) accounts for approximately 75% of the cases and the overall 5-year survival rate

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is <15%. The main obstacles to improve NSCLC patient survival are the lack of effective methods of early detection, and inefficient strategies for advanced tumour treatment [1]. It is, therefore, necessary to search for new and better treatments [2].

Several authors have reported on the use of coumarin (1,2-benzopyrone), or its metabolite 7-hydroxycoumarin, for the treatment of some human carcinomas [3–6]. No adverse effects of coumarin have been reported in humans using doses up to 7 g daily, after two weeks of continued treatment [7,8].

There are several reports indicating that some coumarin compounds, including coumarin and 7-hydroxycoumarin, inhibit cell growth of cell lines of various types of cancer [9–11]. The aim of this study was to learn about the events associated with the anti-proliferative actions of coumarin and 7-hydroxycoumarin in NSCLC cell lines. We determined: (1) the inhibition of cell proliferation, (2) the phase in which cell cycle arrest occurs, and (3) the contribution of apoptosis to the anti-proliferative effect. Our results indicate that the cytostatic and apoptotic actions of these compounds depend on the histological type of lung carcinoma and on the concentration used. Coumarin and 7-hydroxycoumarin may become new options in the treatment of NSCLC. Clinical studies will be necessary to assess if the *in vitro* effects also occur *in vivo*.

2. Materials and methods

2.1. Reagents

Coumarin and 7-hydroxycoumarin with 99% purity were obtained from Aldrich Chemical Co. (Milwaukee, WI). Coumarin or 7-hydroxycoumarin was dissolved in absolute ethanol (Merck, Darmstadt, Germany) and the solutions were diluted in RPMI-1640 medium in a proportion 37.5/62.5 (v/v). The final concentration of these compounds was 2.5 mg/ml and was stored at 4°C. 7-Hydroxycoumarin (the less soluble compound) remained dissolved at this concentration. Dilutions were prepared using medium supplemented with 10% fetal calf serum (FCS) before use. Phytohemagglutinin (PHA) was obtained from GIBCO BRL (Gaithersburg, MD). Propidium iodide and DNase free RNase were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell lines

Seven human NSCLC lines were employed. The squamous carcinoma cell lines SK-MES-1 and Calu-1, and

the adenocarcinoma cell lines A-427 and SK-LU-1 were obtained from the American Type Culture Collection (ATCC), Rockville, MD. The adenocarcinoma cell lines 1.3.11 and 1.3.15 were established by us, as has been previously reported [12]. The 3A5A cell line was obtained from a pleural malignant effusion of a treatment-free patient with a diagnosis of lung adenocarcinoma. Under light microscopy, spherical cells with abundant mucus-containing vacuoles were observed, suggesting a signet-ring cell variant of adenocarcinoma. This cell line showed weak immunocytochemical staining with antibodies against the following antigens: BerEP-4, epithelial membrane antigen (EMA) and high and low cytokeratins. The cells were negative with respect to mesothelial and vimentin antigens.

All cell lines employed were cultured in RPMI-1640 supplemented with 10% of heat-inactivated FCS and 100 U/ml of penicillin and 100 µg/ml of streptomycin. Cell lines grown in monolayers were harvested by trypsinisation. To maintain tumour cells in exponential growth, we used different cell densities. For 24 h experiments, the following initial cell densities were used: 1.5×10^5 cells/ml for A-427 and SK-LU-1 cell lines; 2×10^5 cells/ml for 3A5A, SK-MES-1 and Calu-1 cell lines and 3.5×10^5 cells/ml for 1.3.11 and 1.3.15 cell lines. For 48 h experiments, half of initial cell densities for 24 h experiments were used. Cells were incubated in a humid chamber at 37°C with 5% CO₂. Cell viability was always >92%.

Peripheral blood mononuclear cells (PBMC) from four healthy donors were isolated by density gradient centrifugation using Ficoll-Hypaque (Nycomed Pharma, Oslo, Norway). The initial cell density for PBMC was 1×10^6 cells/ml.

2.3. Cell growth inhibition by coumarin and 7-hydroxycoumarin

Tumour cell suspensions (100 µl) were seeded in each well of 96-well plates and 100 µl of dilutions of coumarin or 7-hydroxycoumarin were added. The final concentration range of these compounds was 10–160 µg/ml. As controls, ethanol (final concentration range: 0.15–2.4% (v/v)) or medium were included in each experiment. The plates were incubated for 24 or 48 h.

PBMC suspended in 100 µl of medium containing 2 µg/ml of phytohemagglutinin (PHA) were seeded and in the same manner as for the tumour cells, the coumarin compounds and ethanol were diluted. In contrast to the tumour cell lines, the PBMC were incubated for 72 h.

³H-thymidine (1 µCi) was added to each well, 18 h before the end of the incubation period. The

radioactivity incorporated into DNA was detected (counts per minute) using a beta counter LS 6000SE from Beckman Instruments (Fullerton, CA). Assays were done at least three times and each determination was performed in quadruplicate. The percentage of inhibition was estimated with respect to the corresponding ethanol-treated cells.

2.4. Effects of coumarin and 7-hydroxycoumarin on cell cycle phases

To 1.2 ml of cell suspensions of either tumour cell lines or PBMC stimulated with PHA were added: (1) medium, (2) ethanol (final concentration: 1.5% (v/v)), and (3) coumarin or 7-hydroxycoumarin (final concentration: 100 µg/ml). The cell cultures were incubated at 37 °C with 5% CO₂ in 24-well plates. The tumour cell cultures and the PBMC cultures were incubated for 24 and 40 h, respectively. The assays were performed at least three times and each treatment was done in triplicate. The procedure for cell cycle analysis is described below.

2.5. Apoptosis induced by coumarin and 7-hydroxycoumarin

The same procedure described above for cell cycle phases was used for studying apoptosis. The concentrations were higher than those for studying the cytostatic effects; 2.4% (v/v) for ethanol, and 160 µg/ml for coumarin or 7-hydroxycoumarin. The cell cultures were monitored repeatedly from two to 24 h.

In order to assess whether the induction of apoptosis by the coumarin compounds was the result of inhibition of cell adhesion, in some experiments the coumarin compounds were added after cell adhesion had taken place.

Apoptotic cells were identified by: (i) characteristic morphological changes associated with apoptosis, (ii) subG₁ peak detection, and (iii) phosphatidylserine exposure on the outer membrane of the cells. Each assay was performed in duplicate and each experiment was done at least three times.

2.6. Cell cycle analysis and subG₁ peak detection

Cellular DNA was stained with propidium iodide (PI) and quantified by flow cytometry according to Nicoletti's procedure [13]. Briefly, cells were collected after treatment, washed in PBS, fixed with 70% (v/v) cold aqueous ethanol (−20 °C) and stored at 4 °C for at least 24 h. The cells were washed in PBS, after cell centrifugation, cell pellets were stained with PI staining solution contain-

ing 10 µg/ml PI, 5 Kunitz units of RNase, and 0.1% (v/v) Triton X-100. The cell suspension was incubated in the dark at room temperature for 30 min. DNA content was determined using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA.). A total of 10,000 events were acquired. ModFIT LT software (Becton Dickinson), for both cell cycle analysis of the cytostatic effect and for subG₁ peak detection of the apoptotic effect was used.

2.7. Apoptosis detection using Annexin-V staining

After 2–4 h treatment with the coumarin compounds, cells were removed by extremely gentle mechanic repeat aspirations and washed in PBS. When cells were treated for more hours or cells were seeded for 24 h, and later exposed to the coumarin compounds, both floating and adherent cells were collected. The floating cells were collected by centrifugation, whereas adherent cells were harvested by trypsinisation, both cell suspensions were pooled.

After centrifugation, cell pellets were suspended in 100 µl of staining solution of the Annexin-V-Fluos Staining Kit (Roche Diagnostics, Mannheim). After incubation at room temperature for 15 min, 400 µl of HEPES buffer were added, and cells were immediately analysed by flow cytometry. Early apoptotic cells were localised in the lower right quadrant of a dot-plot graph using Annexin-V-fluorescein versus PI.

2.8. Statistical analysis

Linear regression of the concentration of the tested compounds versus percentage of inhibition of proliferation was calculated, and the 50% inhibitory concentration (IC₅₀) ± S.E.M. for each cell line was estimated.

Changes in the percentage of cells in different phases of the cell cycle as well as the percentage of cells in apoptosis were analysed using one-way analysis of variance (ANOVA). To compare several groups, Tukey's test was applied. The software SigmaStat (Version 2.0, Jandel Scientific) was used. *P*-values smaller than 0.05 were considered statistically significant.

3. Results

3.1. Cytostatic effect of coumarin and 7-hydroxycoumarin

Coumarin and 7-hydroxycoumarin at 10–160 µg/ml induced a dose-dependent growth-inhibition in all

Table 1 Growth inhibition induced by coumarins

Histological type	Cell Line	IC ₅₀ ± S.E.M. (µg/ml)	
		Coumarin	7-OH-coumarin
Adenocarcinoma	A-427	>160	121 ± 6
	SK-LU-1	160 ± 4	74 ± 5
	1.3.11	160 ± 4	136 ± 8
	1.3.15	103 ± 8	78 ± 4
	3A5A	115 ± 5	50 ± 2
Squamous cell carcinoma	Calu-1	>160	74 ± 4
	SK-MES-1	121 ± 6	59 ± 3

7-OH-coumarin: 7-hydroxycoumarin.

lung carcinoma cell lines. At high concentrations, morphological changes were observed in some cell lines. The cell lines exhibited different sensitivities, but were always more sensitive to the cytostatic effect of 7-hydroxycoumarin. The IC₅₀ values for coumarin and 7-hydroxycoumarin after a 24 h exposure are shown in Table 1. The IC₅₀ values for a 48 h exposure to the coumarin compounds were similar and are not shown. The proliferation rate of PBMC stimulated with PHA was not modified by the addition of the coumarin compounds (data not shown). Large concentrations of solvent exposure (ethanol 1.5–2.4% (v/v)) affected cell proliferation rate of some tumour cell lines, but not PHA-stimulated PBMC (data not shown).

3.2. Cell cycle effect of coumarin and 7-hydroxycoumarin

Exposure to coumarin or 7-hydroxycoumarin at 100 µg/ml for 24 h produced cell cycle arrest in all tested tumour cell lines in G₁ phase (see Table 2 and Fig. 1). Ethanol (1.5% (v/v)) induced cell arrest in G₁ phase in some cell lines (1.3.11, 1.3.15, 3A5A and SK-MES-1), but not in A-427, SK-LU-1 and Calu-1

cell lines (see Table 2 and Fig. 1). Several authors have reported that ethanol induces this effect in cell lines of other histopathological types [14]. The effects of coumarin and 7-hydroxycoumarin were greater than those of ethanol, and the differences were statistically significant (see Table 2).

Kubbies et al. [15] reported that PBMC exposed for 40 h to PHA, allowed the cells to distribute in the different phases of the cell cycle. In our study, using the same procedure, neither ethanol (1.5% (v/v)) nor the coumarin compounds (100 µg/ml) affected the distribution of PBMC in the different phases of the cell cycle, see Table 3. These results are consistent with those obtained by us in cell proliferation assays.

3.3. Apoptotic effect of coumarin and 7-hydroxycoumarin

As was indicated in cytostatic effects of coumarin and 7-hydroxycoumarin, morphological changes were observed in some cell lines when exposed to high concentrations of coumarin compounds. Thus, for apoptosis induction 160 µg/ml of coumarin or

Table 2 Distribution of cells in phases of the cell cycle

Treatment (concentration)	Percentage of cells ± S.D.								
	1.3.15			Calu-1			SK-MES-1		
	G ₁	S	G _{2M}	G ₁	S	G _{2M}	G ₁	S	G _{2M}
Medium	66 ± 2	22 ± 1	12 ± 1	36 ± 1	47 ± 1	17 ± 1	56 ± 1	41 ± 2	3 ± 2
Ethanol (1.5% (v/v))	71 ± 1 ⁺	20 ± 1 ⁺	9 ± 1	40 ± 3	46 ± 1	14 ± 1	75 ± 1 ⁺	24 ± 2 ⁺	1 ± 1
Coumarin (100 µg/ml)	77 ± 1 [*]	13 ± 1 [*]	10 ± 1	54 ± 2 [*]	38 ± 2 [*]	8 ± 1 [*]	88 ± 1 [*]	10 ± 1 [*]	2 ± 1
7-OH-coumarin (100 µg/ml)	76 ± 1 [*]	12 ± 1 [*]	12 ± 1	72 ± 3 [*]	21 ± 1 [*]	7 ± 2 [*]	87 ± 3 [*]	10 ± 5 [*]	3 ± 2

Results from three independent assays. Significant differences ($P < 0.05$) with respect to medium⁺. Significant differences ($P < 0.05$) with respect to medium and ethanol^{*}.

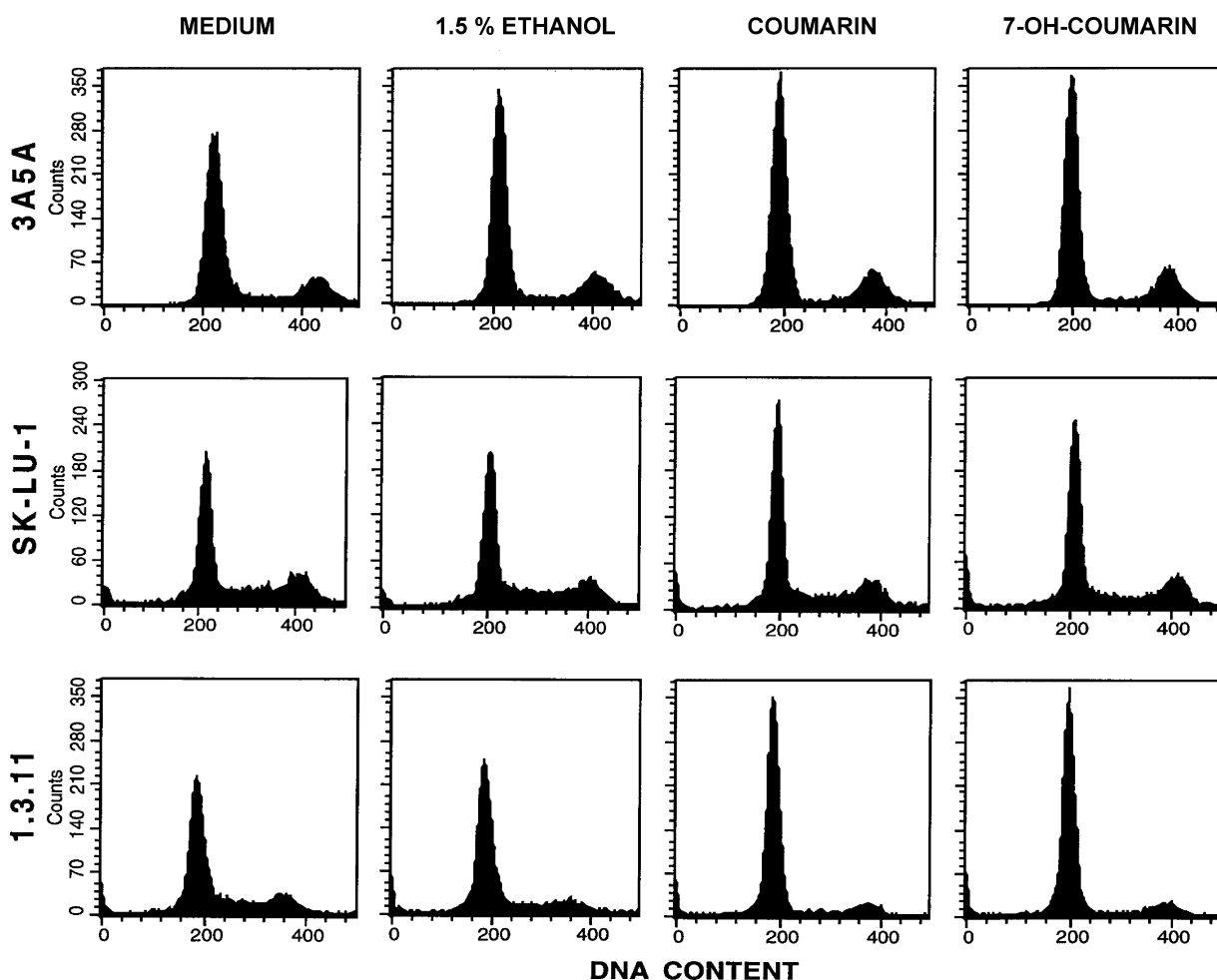


Fig. 1 Cell cycle distribution of cells from lung carcinoma cell lines with medium, 1.5% (v/v) ethanol, 100 $\mu\text{g/ml}$ of coumarin or 7-hydroxycoumarin (7-OH-coumarin) after 24 h exposure.

7-hydroxycoumarin and 2.4% of ethanol (solvent control for this concentration of coumarin compounds) were used. The kinetics of apoptotic cells generation was estimated (data not shown). The greatest percentages of apoptotic cells observed

and detected using the Annexin-V assay occurred during 4–6 h of exposure. In PBMC, the coumarin compounds produced arrest in G_0/G_1 phase but did not induce apoptotic changes through out 40 h of incubation (see Table 3).

Table 3 Percentage of PBMC in cell cycle phases

Treatment (concentration)	G_0/G_1	S	G_2/M
Medium	70 \pm 3	20 \pm 2	10 \pm 4
Low concentration			
Ethanol (1.5% (v/v))	71 \pm 5	18 \pm 5	11 \pm 4
Coumarin (100 $\mu\text{g/ml}$)	75 \pm 4	16 \pm 6	9 \pm 5
7-OH-coumarin (100 $\mu\text{g/ml}$)	76 \pm 5	15 \pm 6	9 \pm 5
High concentration			
Ethanol (2.4% (v/v))	72 \pm 3	19 \pm 5	9 \pm 3
Coumarin (160 $\mu\text{g/ml}$)	79 \pm 5*	11 \pm 3*	10 \pm 3
7-OH-coumarin (160 $\mu\text{g/ml}$)	85 \pm 6*	6 \pm 2*	9 \pm 5

PBMC from four healthy volunteers. Significant differences $P < 0.05$ with respect to medium and ethanol*.

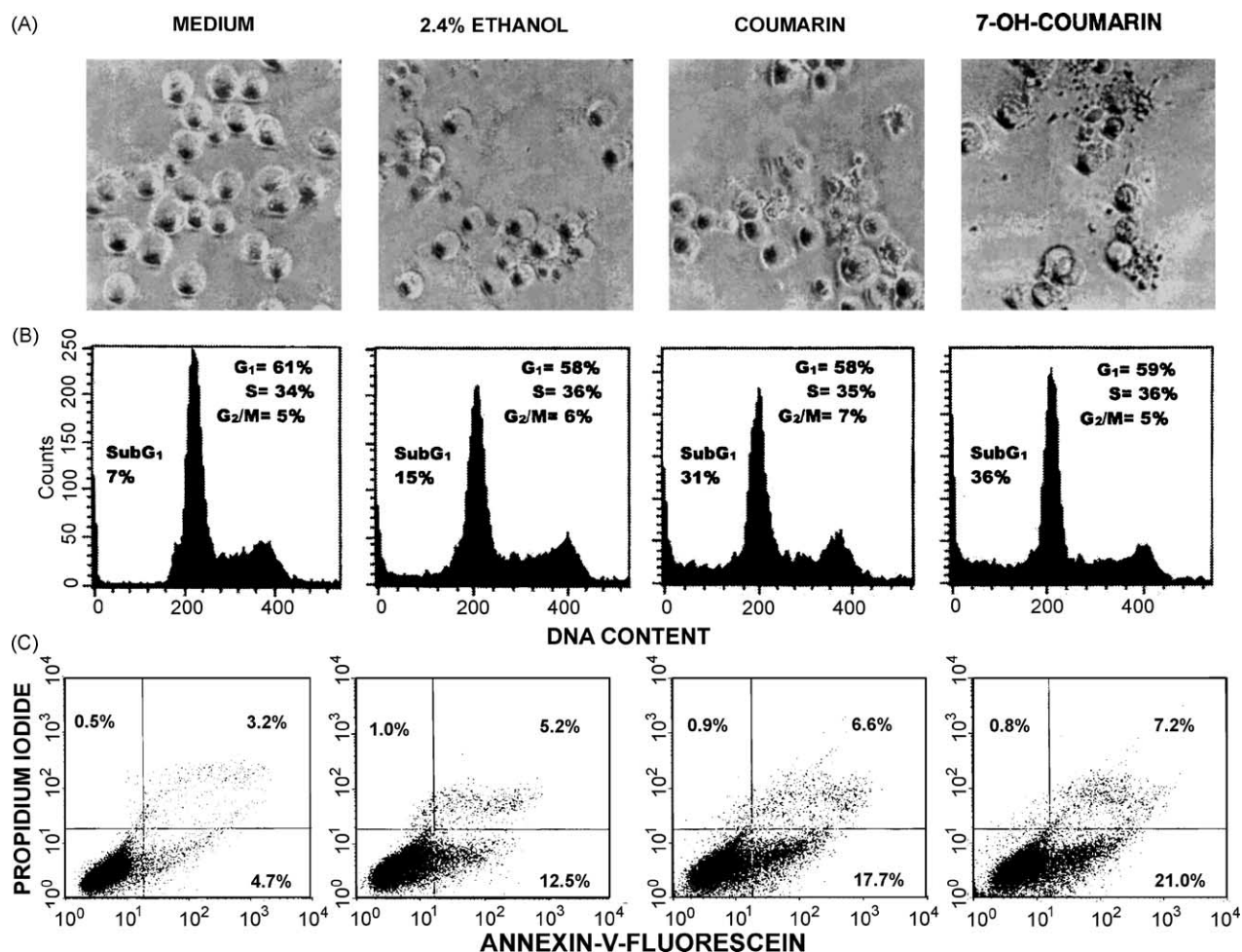


Fig. 2 Apoptosis of the adenocarcinoma A-427 cell line. (A) Morphological changes (magnification: 400×). (B) DNA histogram showing cell cycle phases and subG₁ peak. (C) Dot-plot of Annexin-V-fluorescein vs. PI, apoptotic cells (%) are localised in the lower right quadrant. Cells were treated with medium, 2.4% (v/v) ethanol, 160 µg/ml of coumarin or 7-OH-coumarin for 4 h.

3.3.1. Morphological features

In those tumour cell lines where apoptosis occurred, the following changes in some cells were observed: (i) delay in complete cell adhesion, (ii) cell shrinkage, (iii) membrane blebbing, and (iv) apoptotic bodies. In four adenocarcinoma cell lines (A-427, SK-LU-1, 1.3.11, 1.3.15) but not in the 3A5A cell line, 7-hydroxycoumarin caused the above indicated morphological changes. With coumarin or ethanol (2.4% (v/v)) apoptotic changes occurred in some cells of only the A-427 and 1.3.11 cell lines. Similar changes were observed when tumour cell lines were seeded for 24 h, and were subsequently exposed to the coumarin compounds for 4–6 h (data not shown). Morphological changes in the A-427 cell line at 4 h are shown in Fig. 2A. The two squamous cell carcinoma cell lines were not affected by the coumarin treatments (data not shown). In the PBMC, no morphological alterations were observed even after 40 h of drug exposure (data not shown).

3.3.2. SubG₁ peak

Cells in the subG₁ region were detected only in the adenocarcinoma A-427 cell line. In this region, 15% were found in the ethanol treated cells. This percentage was increased to 30 and 36% in the coumarin and 7-hydroxycoumarin treated cells, respectively. The cells in the subG₁ region did not derive from any particular phase, as the proportion of cells in each phase did not vary appreciably (see Fig. 2B). In the SK-LU-1, 1.3.11 and 1.3.15 cell lines, some apoptotic cells were observed under light microscopy but no subG₁ peak was detected (data not shown).

3.3.3. Annexin-V assay

When tumour cell lines were seeded for 24 h and subsequently exposed to the coumarin compounds for 4–6 h, the results were similar to those obtained when the tumour cell lines were exposed from the beginning to the coumarin compounds (data not shown).

Table 4 Apoptosis in adenocarcinoma cell lines

Treatment (concentration)	Percentage of Annexin-V positive cells \pm S.D.			
	A-427	SK-LU-1	1.3.11	1.3.15
Medium	5 \pm 1	20 \pm 2	14 \pm 1	16 \pm 2
Ethanol (2.4% (v/v))	12 \pm 1 ⁺	23 \pm 1	23 \pm 3 ⁺	16 \pm 1
Coumarin (160 μ g/ml)	18 \pm 1 [*]	20 \pm 1	29 \pm 1 [*]	19 \pm 2
7-OH-coumarin (160 μ g/ml)	22 \pm 2 [*]	31 \pm 2 [*]	32 \pm 3 [*]	22 \pm 1 [*]

Results obtained from three independent assays. Significant differences ($P < 0.05$) with respect to medium⁺. Significant differences ($P < 0.05$) with respect to medium and ethanol^{*}.

shown). In any case, 7-hydroxycoumarin induced apoptosis in some cells of the four adenocarcinoma cell lines (A-427, 1.3.11, SK-LU-1 and 1.3.15) but not in the signet-ring cell variant of the adenocarcinoma 3A5A cell line. In the adenocarcinoma A-427 and 1.3.11 cell lines, coumarin and ethanol caused apoptosis in a smaller proportion of cells. Significant differences were found between treatments of the coumarin compounds and ethanol. Results are shown in Table 4. For the A-427 cell line, see Fig. 2C. No apoptosis was detected either in the squamous carcinoma cell lines or in PBMC.

In summary, in this study we found that the effects induced by the coumarin compounds depend on the cell type and the concentration tested. (i) In the PBMC, only concentration of 160 μ g/ml or higher, induced cytostasis by G_0/G_1 arrest. (ii) In the squamous cell carcinoma cell lines, cytostatic effect was observed in proportion to the concentration of the coumarin compounds used; this effect was induced by blocking G_1 phase. (iii) In all the adenocarcinomas cell lines, the coumarin compounds at 100 μ g/ml or lower concentrations arrested the cells in the G_1 phase. In four of the five adenocarcinomas tested at 160 μ g/ml, the coumarin compounds induced apoptosis in some cells.

4. Discussion

The cytostatic effect of the coumarin compounds in NSCLC cell lines is consistent with the cell growth inhibition observed both in vitro and in vivo in no pulmonary tumour cell lines [4,9,11,16]. Our results also showed a greater cytostatic action of 7-hydroxycoumarin than coumarin [9,17]. Recently, Kawai et al. [11] and Wang et al. [18] have reported that esculetin (6,7-dihydroxycoumarin) inhibits cell growth and cell cycle progression by inducing arrest in the G_1 phase in leukaemia HL-60, and CCRF-HSB-2 cell lines. Seliger and Petters-

son [17] and Kahn et al. [19] have reported that coumarin and 7-hydroxycoumarin reduce the expressions of Ras and Myc, two proteins associated with cell proliferation, in ras- or myc-transfected murine fibroblasts. Furthermore, Kahn et al. [19] have shown a G_1 phase arrest. Recently, we reported that the percentage of cells expressing cyclin D1 in the lung adenocarcinoma cell line A-427 is reduced by exposure to 7-hydroxycoumarin. In contrast, the mRNA of cyclin D1 is not affected [20]. Wang et al. [18] have reported that esculetin downregulates cyclin D1, up-regulates *p27*, and hypophosphorylates Rb in the leukaemia cell line HL-60. The cytostatic effect of the coumarin compounds is probably *p53*-independent, as in Calu-1 cell line, which has been shown to have homozygous deletion of the *p53* gene [21], the coumarin compounds inhibited cell growth. Further studies will be required to define the signalling pathway that leads to the cell cycle arrest in the G_1 phase.

With respect to PBMC, there are contradictory reports regarding the actions of several coumarin compounds on mitogen-induced proliferation of PBMC. Berkarda [22] and Marshall et al. [23] have reported that coumarin increases PHA-induced mitogenesis of PBMC. In contrast, Roskopf et al. [24] have reported anti-proliferative effects of several coumarin compounds also in PBMC, suggesting that these effects are the result of cytotoxic actions. Our observations with PBMC exposed to high concentrations of the coumarin compounds agree with those obtained by Roskopf et al. [24], but do not support the notion that the cell growth inhibition is mediated by a cytotoxic action, as no cell death was detected.

The apoptotic effect occurred only in the adenocarcinoma cell lines and in large concentrations of the coumarin compounds. The apoptosis cannot be attributed to lack of cell-adhesion; as similar results were observed when the coumarin compounds were added after the adhesion had taken place. As no cells in the sub G_1 region were detected,

apoptosis is not associated with intra-nucleosomal DNA fragmentation, and therefore, in epithelial cell lines, some authors [25–27] using several known pro-apoptotic agents, have not observed cells in the subG₁ region. Marshall et al. [9] using DNA fragmentation as the only criterion for detection of apoptosis, have reported that among the various tumour cell lines, the coumarin compounds induced apoptosis only in the HL-60 cell line. As indicated above, apoptosis may occur in the absence of intra-nucleosomal DNA fragmentation. Thus it is likely that Marshall's group underestimated the occurrence of apoptosis. Chu et al. [28] have reported that in the HL-60 cell line esculetin induces apoptosis by the mitochondrial pathway. Based on the structural similarity between esculetin (6,7-dihydroxycoumarin) and 7-hydroxycoumarin, it is likely that they induce apoptosis by the same pathway. Our group is currently studying the pathway leading to apoptosis elicited by 7-hydroxycoumarin in the adenocarcinoma cell lines.

As apoptosis is induced in adenocarcinoma cell lines and is not induced in the squamous cell carcinoma cell lines, differences in the expression of pro-apoptotic and anti-apoptotic molecules in these cell lines may clarify the processes leading to apoptosis.

The apoptotic action of 7-hydroxycoumarin might further increase the efficacy of lung adenocarcinoma therapies. Most of the anti-neoplastic drugs in current use block the cell cycle in the S or G₂/M phases; in contrast, 7-hydroxycoumarin blocks the cell cycle in the G₁ phase or it induces apoptosis. An association of 7-hydroxycoumarin with currently used drugs might possibly lead to improve therapies of NSCLC.

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