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Original article

Cell death triggered by synthetic flavonoids in human leukemia cells is amplified by the inhibition of extracellular signal-regulated kinase signaling

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ABSTRACT

A new class of methyl esters of flavonoids, with different substituents on the B ring were synthesized and evaluated for their antiproliferative activity against the human leukemia cell line HL-60. The presence of either a methyl group (**1f**) or a chlorine atom (**1o**) at position 2′ of the B ring played an important role in affecting antiproliferative activity. The cytotoxic effects of these compounds were accompanied by the concentration- and time-dependent appearance of DNA- and nuclear-fragmentation, increase in the percentage of sub-G_1 cells, and processing of multiple caspases and poly(ADP-ribose)polymerase cleavage. Pretreatment of cells with the specific mitogen-activated extracellular kinases (MEK) 1/2 inhibitor PD98059, together with **1f** and **1o**, resulted in an important enhancement of cell death, which might have clinical implications for the use of both compounds in combination with MEK 1/2 inhibitors as potential therapeutic agents.

1. Introduction

Flavonoids are phenylbenzo-γ-pyrones derivatives, which comprise a very large class of naturally-occurring polyphenol plant compounds [1]. Flavonoids are ubiquitous in plant foods and drinks and therefore consumed in our daily diet [2]. These polyphenolic compounds display a remarkable spectrum of biological activities and they are among the most promising anticancer agents [3]. A number of reports in different cell lines, animal models and human epidemiological trials have pointed out an association between consumption of fruits, vegetables and certain beverages and reduced risk of chronic disease, including cancer [4,5].

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is one of the best-studied flavonoids and it is widely distributed in nature. This compound potentiates the cytotoxic action of the chemotherapeutic agent 1-β-D-arabinofuranosylcytosine [6], inhibits cell invasion and induces apoptosis [7]. The flavone luteolin is another dietary flavonoid which shows a variety of anticancer effects, such as cell growth and kinase activity inhibition, apoptosis induction, differentiation, suppression of the secretion of matrix metalloproteinases, and a reduction in tumor cell adhesion, invasive behavior, metastasis and angiogenesis [8,9]. Some natural and semi-synthetic flavonoids display cytotoxic properties in cells that over-express the anti-apoptotic proteins Bcl-2 and Bcl-x_L, induce the activation of mitogen-activated protein kinases (MAPK) and show a selective growth inhibitory response in human tumor cells but not in normal cells [10–12]. A semi-synthetic flavonoid, flavopiridol, has been the first cyclin-dependent kinases (Cdks) inhibitor to enter in human clinical trials. This compound induces cell cycle arrest at either G_1 or G_2–M phases, in accordance with its ability to inhibit both Cdk2 and Cdk1, respectively [13]. Preclinical data indicate that flavopiridol inhibits the proliferation of neoplastic cells and induces apoptosis and also can potentiate the antitumor effects of many established chemotherapeutic agents [14].

The mechanisms involved in cell death triggered by a specific group of flavone-8-carboxylic acid derivatives are largely unexplored. In this article we describe the synthesis and cytotoxic evaluation of new methyl esters derivatives of flavonoids, some of which display cytotoxic activity in the human leukemia cell line HL-

Abbreviations: Cdk, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; ERK, extracelular signal-regulated kinase; IC_{50}, 50% inhibition of cell growth; JNK/SAPK, c-*jun* N-terminal kinases/stress-activated protein kinases; MAPK, mitogen-activated protein kinases; MEK, mitogen-activated extracellular kinases; *MLL*, mixed-lineage leukemia; MTT, 3-(4,5-dimethyl-2-thiazolyl-)-2,5-diphenyl-2*H*-tetrazolium bromide; PARP, poly(ADP-ribose)polymerase; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; $p38^{MAPK}$, p38 mitogen-activated protein kinases; SDS, sodium dodecyl sulfate; TRAIL, tumor necrosis factor-related apoptosis-induced ligand; wt, wild type.

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60. In this series, the A ring was kept unchanged because we were interested in investigating the influence of different substituents on the B ring (2-phenyl group) of the phenylbenzo-γ-pyrone core. Two selected methyl esters of flavonoids (**1f** and **1o**) were cytotoxic, induced G_1 arrest and were also potent apoptotic inducers in this cell line. The results also demonstrate that apoptosis induced by these compounds is mediated by caspase activation and is associated with cytochrome *c* release and with the activation of the ERK 1/2 (extracellular signal-regulated kinases 1 and 2), JNK/SAPK (c-*jun* N-terminal kinases/stress-activated protein kinases) and $p38^{MAPK}$ (p38 mitogen-activated protein kinases). Moreover, an important enhancement of apoptosis was observed with both flavones when ERK 1/2 signaling was inhibited.

2. Chemistry

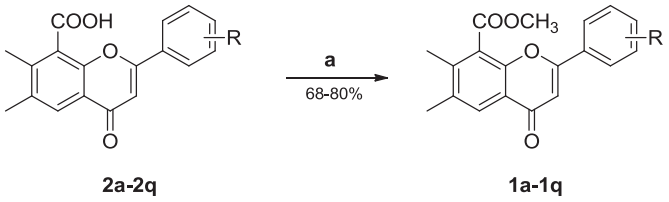
The flavonoid derivatives of type **2a**–**q** were prepared through a known procedure starting with Diels–Alder like-reaction employing 2-amino-3-cyano-4,5-dimethylfuran and methyl vinyl ketone. Workup of this adduct with aqueous hydrochloric acid led to the formation of 3-cyano-4,5-dimethyl-2-hydroxyacetophenone **3**. The esterification of **3** with the appropriate benzoyl chloride yielded the benzoates **4**. Treatment of compounds **4** with potassium hydroxide/pyridine under heat, followed by refluxing in glacial acetic acid/sodium acetate afforded the cyano-flavone derivatives **5**. Acid hydrolysis of the cyano derivative with sulfuric acid 80% or phosphoric acid yielded the amide derivatives, which were converted to the corresponding carboxyl acids **2** by treatment with nitrous acid. The synthetic route is shown in Scheme 1 [15,16].

Carboxylic acid flavones **2a**–**q**, then were converted to a methyl ester **1a**–**i** and **1l**–**q** with $TMSCHN_2$ in good yield according to the Scheme 2. In the case of the methylation of **2j**, two products have been isolated the methyl ester **1j1** and the methyl ester monomethylamine **1j2** in ratio (7:3). The rings B in the compounds **1a**–**q** are listed in the Table 1.

3. Pharmacology

3.1. Methyl esters of flavonoids *1f* and *1o* inhibit the viability of human myeloid leukemia cells

Recently, we have showed that the human myeloid leukemia HL-60 cell line was highly sensitive to the antiproliferative effect of some compounds with a phenylbenzo-γ-pyrone core structure

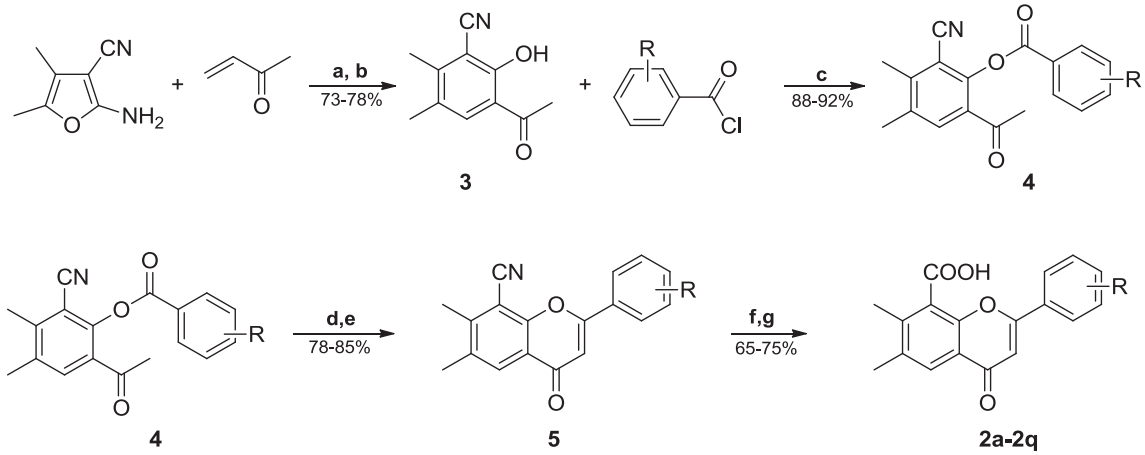


Scheme 2. Synthesis of compounds **1a**–**q**. Reagents and conditions: a) (trimethylsilyl) diazomethane, diethyl ether, dry methanol.

[10]. Here we have synthesized and analyzed the cytotoxicity of seventeen methyl esters of flavonoids against the HL-60 cell line. This is a useful model to study cell growth inhibition of leukemia cells by chemical, physical and physiological agents. We were interested in the effect on cytotoxic activity of electron-donating (methoxy or methyl) and electron-withdrawing (fluoro, chlorine or trifluoromethyl) groups and combinations of donating and withdrawing groups in different positions of the B ring. The effect of these compounds was investigated by MTT assay (Fig. 1A). Among them, two flavonoids containing either an atom of chlorine (**1f**) or a methyl group (**1o**) at position 2′ of the B ring showed significant growth inhibition of the HL-60 cell line (Fig. 1A).

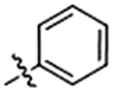
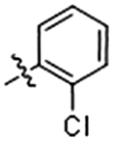
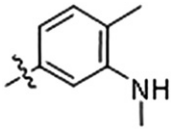
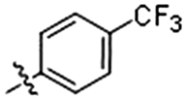
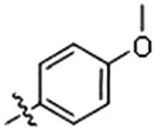
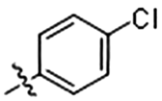
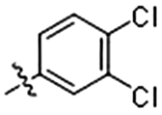
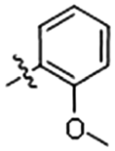
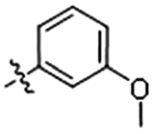
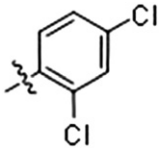
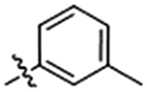
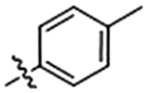
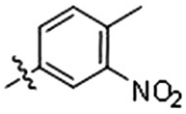
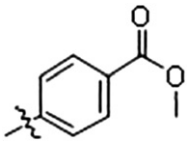
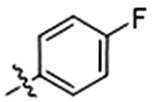
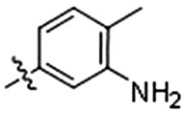
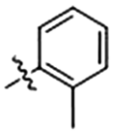
Antiproliferative studies indicate that both compounds display cytotoxic properties with IC_{50} values between 9 and 10 μM (Fig. 1B, Table 2). Interestingly, the compound containing the methyl group (**1o**) was more cytotoxic than **1f** in the mitoxantrone resistant HL-60/MX1 cell line ($IC_{50} = 18.9 \pm 3.7$ μM vs. $IC_{50} = 70.7 \pm 0.7$ μM). The cytotoxic-inducing capacity of flavonoids **1f** and **1o** in human leukemia cells raised the question whether they also affect healthy human peripheral blood mononuclear cells (PBMCs). Therefore, we also investigated whether these compounds were also cytotoxic for human PBMC. A very low cytotoxicity (up to 30 μM) to either fresh or proliferating PBMC growth was observed after treatment with **1f**. However, there was an important reduction in the proliferation of HL-60 cells, which were included in the experiment as a positive control (Fig. 1C). Although **1o** was slightly cytotoxic against normal lymphocytes, the sensitivity of HL-60 cells was higher than that of PBMC. Thus, the percentage of cell viability was 25 ± 3% and 75 ± 4% in HL-60 and PBMC, respectively, upon treatment with 30 μM of **1o** (Fig. 1D).

Previous studies have shown that natural flavonoids induce cleavage in the mixed-lineage leukemia (*MLL*) gene and may contribute to infant leukemia [17,18]. In order to assess the ability of



Scheme 1. Synthesis of compounds **2a**–**q**. Reagents and conditions: a) acetone, reflux, overnight; b) HCl, H_2O; c) triethylamine, dioxane; d) KOH, pyridine; e) acetic acid glacial, sodium acetate; f) sulfuric acid or phosphoric acid; g) sulfuric acid/H_2O and sodium nitrate or nitrous acid.

Table 1
Different substituent's in the ring B for compounds **1a**–**q**.

Ring B		Ring B		Ring B		Ring B	
1a		**1f**		**1j2**		**1p**	
1b		**1g**		**1l**		**1q**	
1c		**1h**		**1m**			
1d		**1i**		**1n**			
1e		**1j1**		**1o**			

these methyl esters of flavonoids to induce site-specific DNA cleavage in the breakpoint cluster region of the *MLL* gene, peripheral blood mononuclear cells were exposed to methyl esters **1f** and **1o** (30 μM) for 16 h, and the occurrence of *MLL* rearrangements were investigated by inverse PCR following the protocol described by Libura et al. [19]. Inverse PCR on the DNA isolated from control and treated samples generated one single band of approximately 1.8 kb, suggesting the amplification of the wt *MLL* only. There were not any bands additional to wt *MLL* in methyl esters-treated cells. However when the wt *MLL* was eliminated prior to PCR by enzymatic digestion with *Pvu* II a single band of approximately 0.65 kb was observed only in **1o**-treated cells, suggesting that this compound might induce *MLL* translocation (Fig. 1E).

3.2. Compounds *1f* and *1o* induce G_1 phase cell cycle arrest and apoptosis on human myeloid leukemia cells

When HL-60 cells were incubated with **1f** and **1o**, they displayed the morphological changes characteristic of apoptotic cells (condensed and fragmented chromatin) visualized by fluorescent microscopy (Fig. 2A) and the DNA showed the typical fragmentation patterns formed by internucleosomal hydrolysis of chromatin (Fig. 2B). The quantification of apoptotic cells by measurement of the number of hypodiploid cells by flow cytometry shows a 12-fold increase in response to **1f** and **1o** (Fig. 2C, Table 3). Evaluation of nuclear morphology (Fig. 2D) indicates that the percentage of apoptotic cells increased from 4.9 ± 1.0% to 30.6 ± 0.3% (6 times increase) and to 26.9 ± 3.5% (5 times increase) in **1f**- and **1o**-treated cells, respectively, after 24 h exposure at a concentration of 30 μM.

To assess whether **1f-** and **1o**-induced cell growth inhibition is mediated via alterations in cell cycle progression, we evaluated the effect of these compounds on cell cycle phase distribution by flow-cytometric studies. As shown in Fig. 2C and Table 3, consistent with growth inhibitory effects, both compounds caused a significant G_1 arrest at the expense of G_2–M phase cell population at 24 h of treatment. When the sub-diploid (sub-G_1) portions of the histograms were excluded from the analyses, the relative increases in the G_1 phase of the cell cycle in **1f**- and **1o**-treated HL-60 cells were 43% and 45%, respectively, when compared to the untreated control. The percentage of control cells in G_1 was ~49%, which increased to ~58% and ~70% after treatment with 10 μM and 30 μM of **1f**. The percentage of cells in G_1 increased to ~56% and ~71% after treatment with 10 μM and 30 μM of **1o**. This increase in G_1 cell population was accompanied with a concomitant decrease of cell number in G_2–M phase of the cell cycle.

Next we evaluated the expression of the cyclin D_1, a protein which is known to be operative in G_1 phase. The results indicate that both compounds induce the expression of this protein on HL-60 cells (Fig. 2E). Since the protein kinase activity of the cyclin-dependent kinases (Cdks) is controlled not only by their regulatory subunits but also by the cyclin-dependent kinases inhibitors (CKI), we assessed whether the expression of the CKI $p21^{Cip1}$ is modulated by the compounds under study. A dose–response experiment shows that the levels of $p21^{Cip1}$

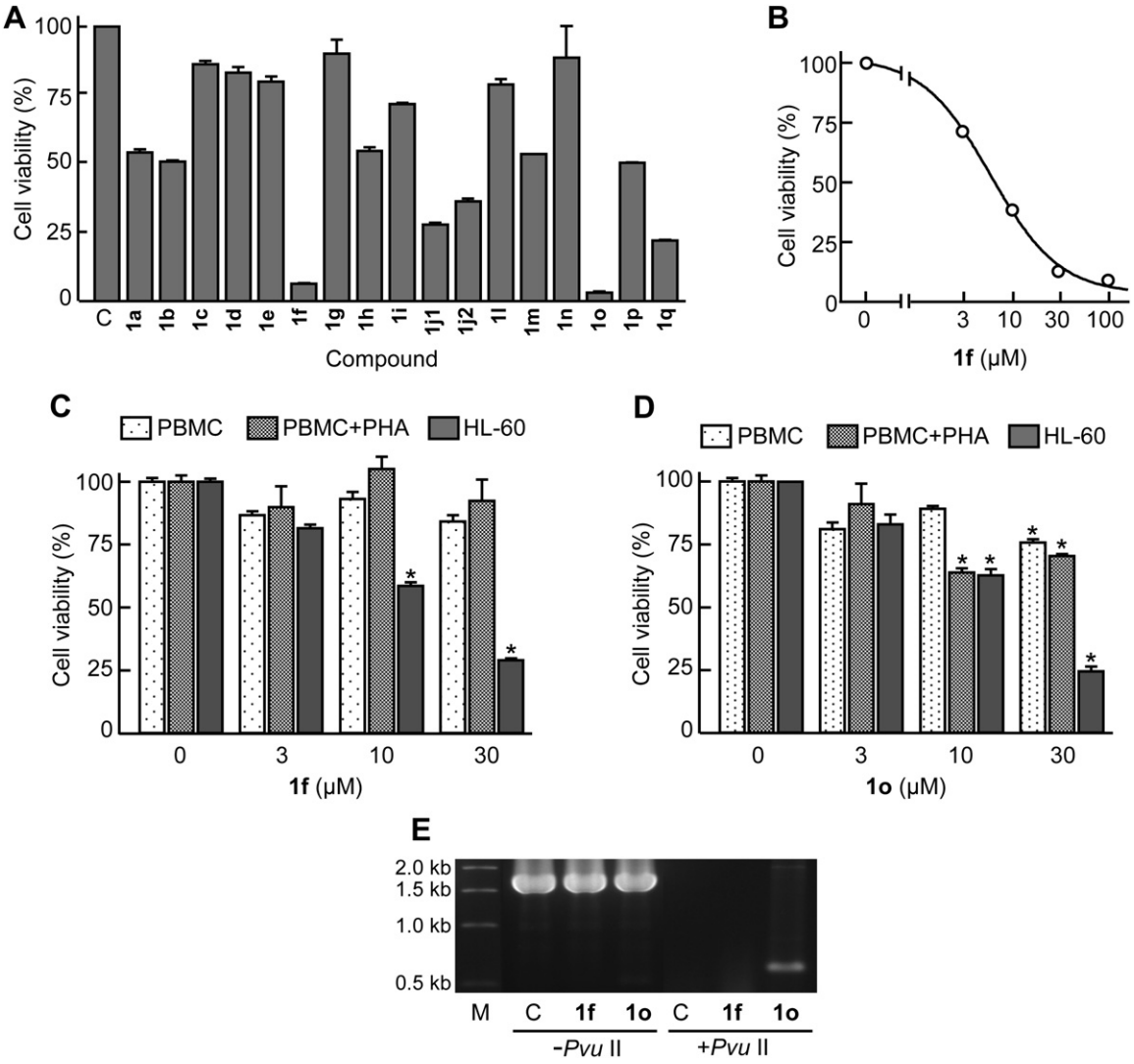


Fig. 1. (A) Analysis of cell viability in methyl ester of flavonoids-treated HL-60 cells by the MTT assay. Cells were plated into 96-well plates and treated with 100 μM of indicated compound for 72 h. Cell viability was determined by measuring the absorbance at a wavelength of 570 nm. (B) Effect of **1f** on human HL-60 cell viability. Cells were cultured in the presence of the indicated concentrations of **1f** for 72 h, and thereafter cell viability was determined by the MTT assay. The results of a representative experiment are shown. Each point represents the average of triplicate determinations. (C) Differential effect of **1f** and (D) **1o** on proliferation of normal peripheral blood mononuclear cells (PBMC) versus HL-60 cells. Proliferation of HL-60 cells, quiescent PBMCs and phytohemagglutinin (PHA)-activated healthy human PBMC cultured in presence of the indicated concentrations of **1f** and **1o** for 48 h. Values represent means ± SE of two independent experiments each performed in triplicate. $^{*}P < 0.05$, significantly different from untreated control. (E) Inverse PCR on genomic DNA of peripheral blood mononuclear cells. Genomic DNA was digested with *Xba* I restriction enzyme and circularized. Nested PCR reactions were carried out with MF1–MR1 primer pair, followed by MF2–MR2 primer pair. MF1, MF2, MR1 and MR2 are primers used for PCR (see Experimental part).

were increased after treatment with both compounds for 24 h (Fig. 2F).

3.3. *1f*- and *1o*-induced cell death is mediated by a caspase-dependent pathway

To determine whether caspases were involved in the response of the cells to **1f** and **1o**, we evaluated the effect of these flavonoids on poly(ADP-ribose) polymerase (PARP) cleavage, a hallmark of apoptosis that indicates activation of caspase. As shown (Fig. 3), hydrolysis of the 116 kDa PARP protein to the 85 kDa fragment was detected after 24 h exposure with both compounds.

Cytochrome *c* released from mitochondria binds to the adapter protein APAF-1 (Apoptotic Protease Activating Factor-1) and procaspase-9 to stimulate the auto-catalytic cleavage of the proenzyme to its active form, thereby initiating the so called intrinsic or mitochondrial pathway [20]. Caspase-9 then cleaves caspase-3 which recognizes multiple substrates including PARP. To determine whether **1f**- and **1o**-induced apoptosis involves the release of cytochrome *c* from mitochondria to cytosol, HL-60 cells were treated with these compounds and cytosolic preparations were analyzed by immunoblotting. As shown (Fig. 3), both compounds induce cytochrome *c* release. Next, we studied the effect on caspase-9 and found that both compounds stimulate the cleavage of inactive procaspase-9 to the active 35–37 kDa fragment (Fig. 3).

Table 2
Effects of synthetic flavonoids on the growth of human tumor cell lines.

Compound	IC_{50} (μM)	
	HL-60	HL-60/MX1
1f	9.3 ± 1.2	70.7 ± 0.7
1o	10.1 ± 1.1	18.9 ± 3.7

Cells were cultured for 72 h and the IC_{50} values were calculated as described in the Materials and methods section. The data shown represent the mean ± SEM of three independent experiments with three determinations in each.

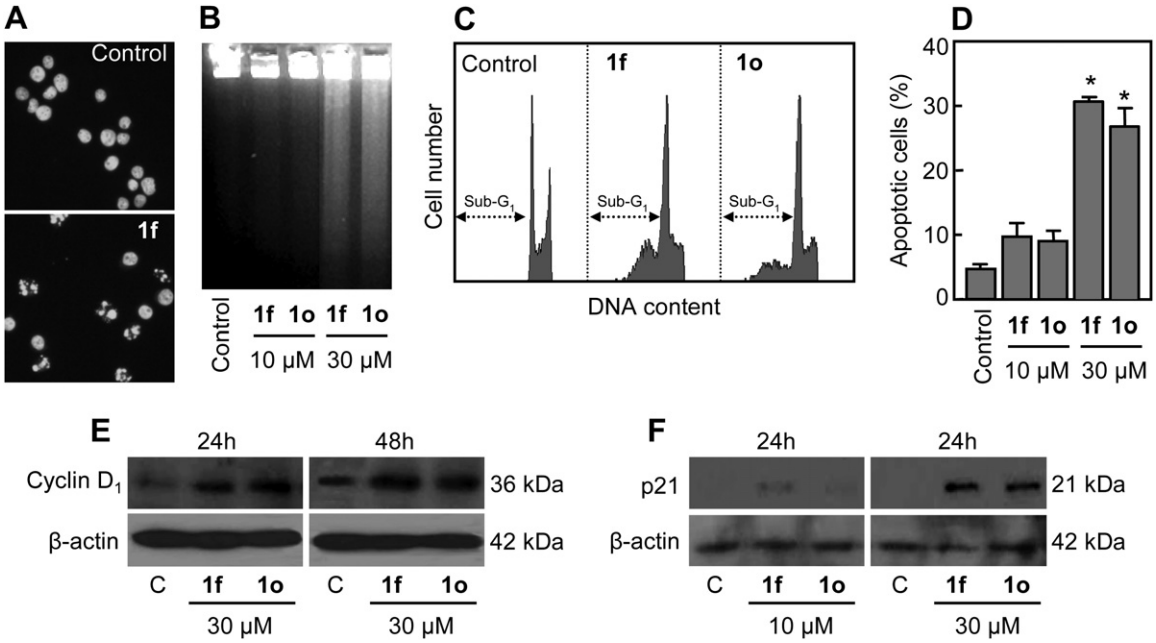


Fig. 2. Effects of **1f** and **1o** on apoptosis on human myeloid leukemia HL-60 cells. (A) Photomicrographs of representative fields of HL-60 cells stained with bisbenzimide trihydrochloride to evaluate nuclear chromatin condensation (i.e. apoptosis) after treatment with 30 μM of **1f** for 24 h. (B) Qualitative assessment of apoptotic DNA damage. Cells were treated with the indicated concentrations of **1f** and **1o** and genomic DNA was extracted, separated on an agarose gel and visualized under UV light by ethidium bromide staining. (C) Cells were incubated with 30 μM of compounds for 24 h and subjected to DNA flow cytometry. Hypodiploid cells (apoptotic cells) are shown in region marked with an arrow. (D) Cells were incubated with the indicated concentrations of flavonoids and apoptotic cells were quantitated by fluorescence microscopy. Values represent means ± S.E. of two independent experiments each performed in triplicate. $^*P < 0.05$, significantly different from untreated control. (E, F) Effect of **1f** and **1o** on the cell cycle regulatory protein expression levels in HL-60 cells. Cells were incubated in the presence of **1f** and **1o**, harvested at 24 h and whole cell lysates were assayed by immunoblotting. β-Actin was used as a loading control. A representative Western blot is shown; two additional experiments yielded similar results.

To determine the contribution of the extrinsic pathway, HL-60 cells were treated with **1f** and **1o** and the cell lysates were then subjected to immunoblot analysis. The results clearly demonstrate that both compounds significantly promote procaspase-8 hydrolysis after 24 h of treatment (Fig. 3). Proteolytic cleavage of the initiator caspase-8, which is demonstrated in response to **1f** and **1o**, typically occurs after triggering cell surface death receptors like the TRAIL (Tumor Necrosis factor-related Apoptosis-Inducing Ligand) receptors [21]. Active caspase-8 initiates the proteolytic activation of executioner caspases and in parallel may cleave the proapoptotic Bcl-2 family member Bid, which in the truncated form (tBid) promotes the release of mitochondrial factors that initiate caspase-9 activation and links the extrinsic pathway to the mitochondrial pathway of apoptosis. Therefore, we evaluated the effect of **1f** and **1o** on Bid cleavage by Western blot and found that the full-length Bid decreased after treatment with both compounds. This decrease implies a protein truncation and the transference of apoptotic signals from the extrinsic pathway to mitochondria [22]. As shown in Fig. 3, **1f** and **1o** also stimulate the proteolytic processing of executioner caspases-3, -6 and -7.

Table 3
Effects of selected compounds on cell cycle phase distribution of HL-60 cells.

	%Sub-G_1	%G_1	%S	%G_2–M
Control	2.3 ± 0.5*	48.0 ± 1.6*	16.7 ± 0.5*	33.2 ± 2.1*
10 μM **1f**	6.3 ± 0.6*	54.9 ± 1.7*	16.7 ± 0.6*	22.9 ± 2.2*
30 μM **1f**	28.1 ± 4.2*	47.2 ± 1.2*	7.8 ± 1.0*	13.5 ± 3.7*
10 μM **1o**	5.5 ± 0.6*	53.0 ± 1.0*	16.6 ± 1.3*	24.4 ± 0.7*
30 μM **1o**	28.5 ± 3.9*	48.1 ± 2.3*	7.6 ± 1.0*	11.8 ± 2.9*

Cells were cultured with the indicated concentration for 24 h and the cell cycle phase distribution was determined by flow cytometry. The values are means ± SE of two different experiments with three determinations in each. $^*P < 0.05$, significantly different from untreated control.

To determine whether **1f**- and **1o**-triggered apoptosis requires the activation of caspases, HL-60 cells were pretreated with the broad-spectrum caspase inhibitor z-VAD-fmk. The almost complete inhibition of apoptosis observed (Fig. 4A) suggests that both compounds induce a caspase-dependent mechanism. To identify which caspases were important in **1f**- and **1o**-induced apoptosis, the effect of selective caspase inhibitors was examined. The caspase-3 & -7 inhibitor (z-DEVD-fmk), the caspase-8 inhibitor (z-IETD-fmk) and the caspase-9 inhibitor (z-LEHD-fmk) partially blocked **1f**- and **1o**-induced apoptosis in HL-60 cells (Fig. 4B–D).

3.4. *1f and 1o activate mitogen-activated protein kinases (MAPK)*

In view of evidence that the MAPK play a critical role in cell fate, the effects of **1f** and **1o** on the activation of this pathway were examined. The results demonstrate that these compounds lead to a fast (30 min) phosphorylation of ERK 1/2, JNK/SAPK and $p38^{MAPK}$ in HL-60 cells (Fig. 5A). The level of phosphorylated $p38^{MAPK}$ was transient and returned to the control level at 2 h in **1f**- or 4 h in **1o**-treated cells, while phosphorylation of ERK 1/2 and JNK/SAPK remained elevated for at least 6 h. These results indicate that **1f** and **1o** lead to activation of ERK 1/2, JNK/SAPK and $p38^{MAPK}$ following different kinetics. To determine whether the phosphorylation of MAPK plays a key role in **1f**- and **1o**-induced apoptosis, we examined the effects of selective inhibitors for these protein kinases. Pretreatment with the mitogen-activated extracellular kinases 1/2 (MEK1/2) inhibitor PD98059 (10 μM) significantly enhances **1f**- and **1o**-mediated apoptosis (Fig. 5B). The percentage of apoptotic cells increased from 16% in **1f**-treated cells to 32% in presence of the inhibitor. A similar trend was observed with the combination of PD98059 and **1o**. The percentage of apoptotic cells increased from 15% in **1o**-treated cells to 25% with PD98059. The

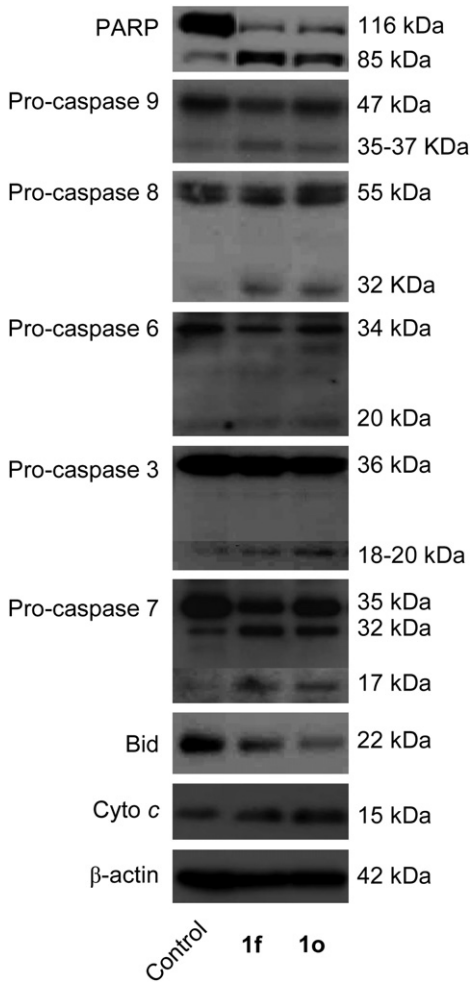


Fig. 3. Involvement of activation of caspases in the induction of apoptosis on human leukemia cells. HL-60 cells were incubated with 30 μM of the indicated compounds and harvested at 24 h and cell lysates (or cytosolic extracts in the case of cytochrome *c*) were assayed by immunoblotting for the cleavage of PARP, caspases and Bid, and for cytochrome *c* release. β-Actin was used as a loading control.

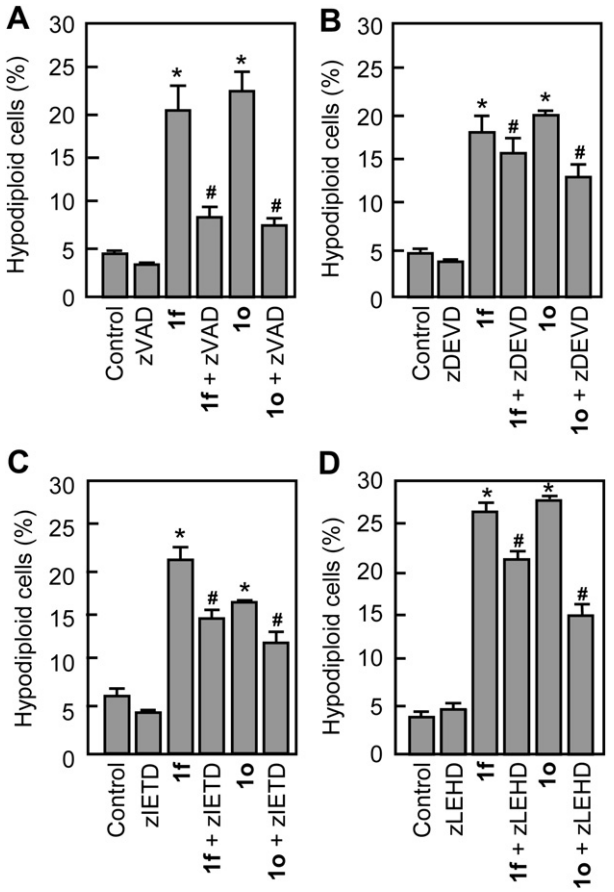


Fig. 4. (A) Effect of cell-permeable caspase inhibitors on **1f**- and **1o**-stimulated apoptosis. HL-60 cells were incubated with 30 μM of flavonoids for 24 h, in absence or presence of the broad-spectrum caspase inhibitor z-VAD-fmk (100 μM), the caspase-3 & -7 inhibitor z-DEVD-fmk (50 μM), the caspase-8 inhibitor z-IETD-fmk (50 μM) and the caspase-9 inhibitor z-LEHD-fmk (50 μM). The percentage of hypodiploid cells was determined and quantified by flow cytometry after staining with propidium iodide. Values represent means ± S.E. of three independent experiments each performed in triplicate. $^{*}P < 0.05$, significantly different from untreated control. $^{\#}P < 0.05$, significantly different from **1f** and **1o** treatments alone.

effect of the inhibitor on the phosphorylation status of ERK 1/2 in the combination treatment group was also confirmed by Western blot (results not shown). In HL-60 cells, **1f** and PD98059 combined caused almost two times more cell death than **1f** alone, and almost five times more cell death than PD98059 alone. These results suggest that this inhibitor of ERK 1/2 signaling may serve as sensitizer toward **1f**- and **1o**-mediated apoptosis in human leukemia cells.

Pretreatment of HL-60 cells with the specific JNK/SAPK inhibitor SP600125 (10 μM) or with the specific $p38^{MAPK}$ inhibitor SB203580 (2 μM) did not alter the rate of **1f**- and **1o**-mediated apoptosis. Both inhibitors had no influence on basal levels of apoptosis (results not shown). These data suggest that the activation of JNK/SAPK and $p38^{MAPK}$ is not involved in **1f**- and **1o**-mediated cell death.

4. Discussion

In the area of cancer chemotherapy, more than 60% of the effective drugs are of natural origin or can be traced back to a natural products source [23]. Most modern medicines currently available are very expensive, toxic and less effective in treating the disease. Therefore, the agents derived from natural and synthetic sources should be investigated further in detail for the prevention and treatment of cancer. Flavonoids are phenolic substances which have attracted a great deal of attention because they appear to have anticancer properties [1]. In this study, we have synthesized and analyzed the cytotoxicity of seventeen methyl esters of flavonoids **1a**–**q**. The evaluated compounds contain a common phenylbenzo-γ-pyrone core and a methyl ester functional group at C-8, with different substituents on the B ring. The introduction of the methyl group is essential for cytotoxicity, since the corresponding carboxylic acids did not show any antiproliferative activity against HL-60 cells (results not shown). It is possible that methyl esters of flavonoids can pass through biological membranes more easily and therefore reach a higher intracellular concentration than the corresponding carboxylic acids. The introduction of a substituent at the C-4′ position had no any effect (**1b**, **1p**) or decreased the potency (**1d**, **1e**, **1g**, **1n**) with respect to **1a** against cell growth inhibition. Similar results were obtained by the introduction of a substituent in C-3′ position. The 3′-methoxy derivative **1c** was less active than **1a**, but the 3′-methyl derivative **1m** displayed similar cell growth inhibition as **1a**. The structural requirement for the antiproliferative action of these compounds seems to be the mono substitution at position C-2′. The introduction of an atom of chlorine (**1f**) as well as a methyl group (**1o**) on position 2′ improves the antiproliferative activity with respect to **1a**. When a methoxy

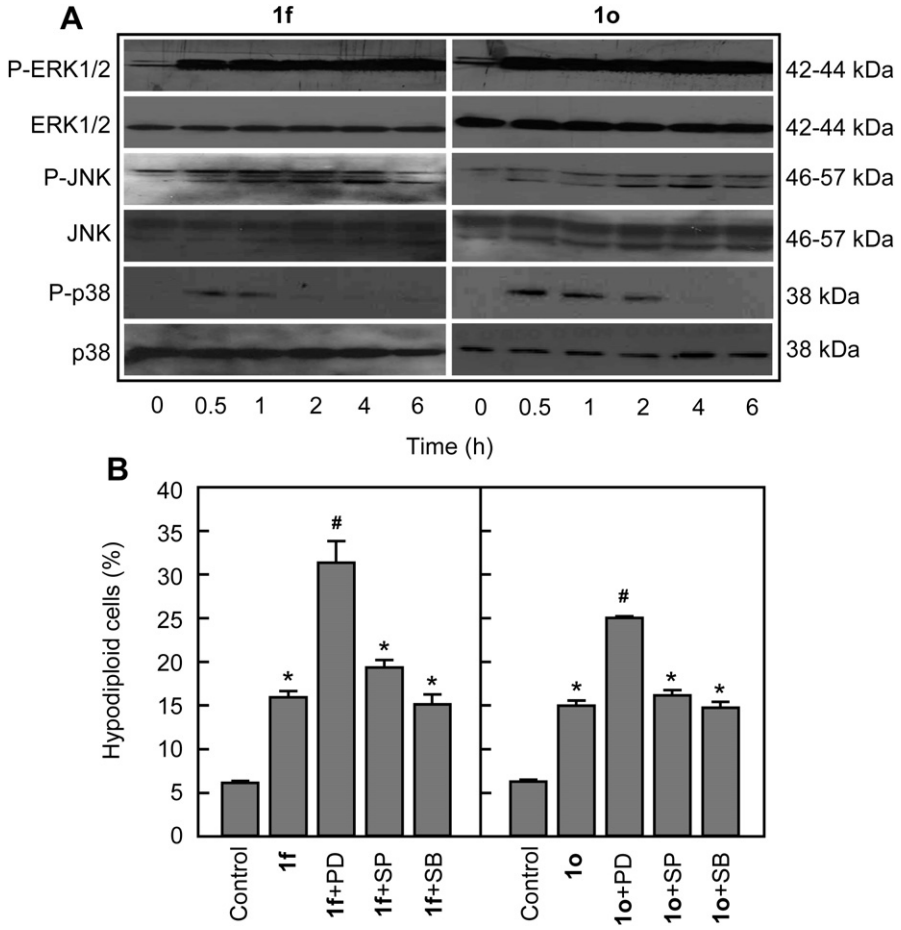


Fig. 5. Effects of **1f** and **1o** on MAPK pathway. (A) Representative Western blots show the time-dependent phosphorylation of ERK 1/2, JNK/SAPK and $p38^{MAPK}$ by **1f** and **1o**. HL-60 cells were incubated with both flavonoids for the indicated time points and protein extracts were prepared and analyzed on Western blots. Membranes were stripped and reprobed with total ERK 1/2, JNK/SAPK or $p38^{MAPK}$ antibodies as loading controls. (B) HL-60 cells were preincubated with PD98059 (PD, 10 μM), SP600125 (SP, 10 μM) or SB203580 (SB, 2 μM) for 1 h and then treated with **1f** and **1o**. Apoptosis was quantified by flow cytometry as described in Materials and methods. Bars represent the mean ± S.E. of three independent experiments each performed in triplicate. $^{*}P < 0.05$, significantly different from untreated control. $^{\#}P < 0.05$, significantly different from **1f** and **1o** treatments alone.

group is placed in the same position, the resulting compound (**1q**) also displays higher antiproliferative activity than **1a**, but is significantly less cytotoxic than **1f** and **1o**. These results suggest that both the C-2′ position and the substituent seem to play a key role in determining the potency of these compounds on cell viability. The lesser cytotoxic activity of compound **1q** with respect to **1f** and **1o** could be explained by the fact that the CH_3 of the methoxy group can be oriented in such way that it imposes a steric restriction and/or the methoxy oxygen atom might accept a hydrogen bond from the side chain of specific aminoacids residues of the target proteins which might be accompanied by an alteration of the compound binding mode. However, there are probably additional factors that play important roles in determining the final biological response in this particular tumor cell line. The antiproliferative activity of **1f** was reduced by the introduction of an additional atom of chlorine in position 4′ (**1h**), and led to similar growth inhibition as **1a**.

One of the most potent derivatives **1f** contains chlorine at the same position as in the semi-synthetic flavonoid flavopiridol a well known cyclin-dependent kinase inhibitor. Moreover, the 2′-chloro substituent of the flavopiridol is important for the Cdk inhibitory activity [24]. Further studies are needed to determine whether the different cytotoxicity between **1f** and **1a** could be explained by Cdk inhibition. Although **1f** and **1o** show similar potency in HL-60 cells, the latter was more cytotoxic against the mitoxantrone resistant HL-60/MX1. This result is relevant because these cells display atypical multidrug resistance and cross resistance to etoposide and the anthracyclines daunorubicin and doxorubicin [25]. The effects of **1f** and **1o** against human PBMCs were also determined since selectivity toward cancer cells is an important criterion in the study of the agents used or developed for cancer treatment. Dose-response studies revealed that quiescent PBMC and proliferating PBMC were more resistant than HL-60 cells toward **1f** and **1o**.

The inverse PCR approach demonstrate that cells exposed to the methyl ester **1f** showed no abnormal PCR products indicating that this compound does not induce *MLL* translocations in the experimental conditions assayed. However the inverse PCR method generated the amplification of a smaller fusion product in **1o**-treated cells suggesting that this compound might induce *MLL* translocation.

Flow cytometry studies in HL-60 cells indicate that both compounds induce cell cycle arrest at G_1 phase and increase the percentage of cells in the sub-G_1 region which is considered as a marker of cell death by apoptosis. Although other targets are possible, the changes in the expression and/or activity of G_1 cell cycle regulators might also be involved in the antiproliferative activity of these methyl esters. The G_1 cell cycle arrest was associated with induction of cyclin D_1 and accumulation of the cyclin-dependent kinases inhibitor $p21^{Cip1}$ which can induce a G_1 arrest through its interactions with cyclin/Cdk complexes. The

up-regulation of $p21^{Cip1}$ involves a p53-independent pathway since HL-60 cells lack functional p53. These results might have important implications for developing **1f** and **1o** as chemopreventive or as chemotherapeutic agents in treatment of cancer, because approximately 50% of human cancers harbor p53 mutations [26,27].

Apoptotic hallmarks such as DNA fragmentation and apoptotic bodies formation were also observed in **1f**- and **1o**-treated cells. Apoptosis induction was accompanied by PARP cleavage which indicates involvement of this protein as a key regulator of cell death [28]. The experiments shown here demonstrate proteolytic processing of the effector caspases-3, -6 and -7, and the initiator caspases-8 and -9, suggesting that both compounds activate these proteases in HL-60 cells. Our results indicate that the cell death was dependent on caspase activation since apoptosis was pharmacologically inhibited by the general caspase inhibitor z-VAD-fmk.

Previous studies have demonstrated that many cytotoxic agents induce mitochondrial cytochrome *c* release [29], which triggers a caspase-dependent assembly of the apoptosome [30]. Here we have shown that both methyl esters **1f** and **1o** induce the release of cytochrome *c* from mitochondria into the cytosol, and thus the activation of caspase-9 and caspase-3. Moreover, the use of selective caspase inhibitors against caspase-3 (z-DEVD-fmk), caspase-8 (z-IETD-fmk) and caspase-9 (z-LEHD-fmk) reduced the percentage of hypodiploid cells induced by **1f** and **1o** which suggest that the intrinsic and the extrinsic apoptotic pathways appear to be involved.

We have previously demonstrated on HL-60 cells an effective blockage of betuletol 3-methyl ether induced-apoptosis by z-IETD-fmk but not by z-LEHD-fmk, supporting a caspase-8 mediated mechanism [10]. Therefore, different apoptotic pathways are activated in this cell line in response to the phenylbenzo-γ-pyrones **1f** and **1o** and betuletol 3-methyl ether. We also evaluated whether **1f** and **1o** could induce cleavage of Bid since it is known that this Bcl-2 family protein is a substrate for caspase-8, and observed a reduction of this protein after 24 h of treatment, in accordance with the results of caspase-8 activation. Further studies are needed to determine the effect of **1f** and **1o** on expression and/or activation of Fas, tumor necrosis factor receptor 1 and TRAIL receptors, which are usually involved in the extrinsic pathway activation.

Mitogen-activated protein kinases are essential parts of the signal transduction machinery and play central roles in cell growth, differentiation and cell death. The JNK/SAPK and $p38^{MAPK}$ have generally been associated with pro-apoptotic actions, whereas ERK 1/2 in most cases exerts cytoprotective effects [31]. The ability of ERK 1/2 inhibitors has been reported to potentiate the antitumor effects of cytotoxic agents such as 1-β-D-arabinofuranosylcytosine in a substantial increase in release of cytochrome *c* [32]. Previous studies have shown that quercetin treatment results in high and sustained activation of ERK 1/2 in A549 lung cancer epithelial cells [33]. However, the inhibition of MEK-ERK activation utilizing PD98059 abolishes quercetin-induced apoptosis [33]. Treatment of ovarian carcinoma cells and the C8161 melanoma cell line with cisplatin caused ERK 1/2 activation and cell death, and this latter effect was potentiated by ERK 1/2 inhibitors, indicating that ERKs behave as survival-inducing kinases in these cells [34–36].

One of the findings described here is that **1f** and **1o** also induce activation of the MEK/ERK pathway, which is expected to increase cell proliferation and survival, and may compromise the efficacy of both compounds in potential cancer treatment. Our data indicate that the MEK 1/2 inhibitor PD98059 potentiates the apoptotic effects of **1f** and **1o**. This finding is in agreement with previous work that has shown that inhibition of MEK–ERK activation with U0126 or PD98059 enhances cell death induced by the acetyl derivative of the flavonoid quercetin 3-methyl ether [11]. The potential use of low dose in chemotherapy is important, because lower dosages are more attainable during cancer therapy and likely to be less toxic to patients. These results might have important clinical implications for the use of these methyl esters of flavonoids as potential therapeutic agents in combination of MEK 1/2 inhibitors.

Although the JNK/SAPK pathway has been shown to be closely linked to apoptosis [37], its exact role seems to depend on the cell type and stimulus. Here we show that **1f** and **1o** induce JNK/SAPK activation in HL-60 cells and its inhibition by SP600125 did not influence **1f**- and **1o**-induced apoptosis indicating that this protein kinase is not required for cell death.

The $p38^{MAPK}$ signaling has been shown to promote cell death, but also to enhance survival, cell growth and differentiation. Therefore, the role of $p38^{MAPK}$ in apoptosis is dependent on cell types and stimuli [38]. In the present study we show that although $p38^{MAPK}$ is activated prior to caspase processing, this protein kinase is not required for **1f**- and **1o**-induced apoptosis since SB203580 was unable to block cell death. Activation of p38 ^{MAPK} has been reported to be involved in cadmium-induced apoptosis on U937 cells [39] and resveratrol-induced apoptosis of human malignant B cells [40], while it is not involved in UV-induced apoptosis in U937 cells [41] and in Fas-induced apoptosis in Jurkat T cells [42].

In this paper we describe that these compounds modulate multiple biochemical signals that control tumor cell proliferation and apoptosis, which suggests the existence of many substrates. However the direct molecular targets of these novel anti-proliferative compounds are unknown. Previous studies have shown that the direct targets of natural occurring flavonoids such as quercetin are key anti-apoptotic protein kinases, such as, phosphatidylinositide-3-kinase (PI3K) [43], Raf and MEK [44]. Among the different potential targets, methyl esters of flavonoids could interact with cellular receptors such as receptor tyrosine kinases involved in cell proliferation. The results shown here demonstrate that these compounds display an effect on the phosphorylation of ERK, which suggests that they could block other proteins involved in survival such as the PI3K pathway perhaps by direct inhibition of PI3K and/or the serine threonine protein kinase B (AKT) in these cells, although direct inhibition of additional upstream tyrosine kinases can not be ruled out. Further studies will be needed to determine the effect of these compounds on these potential targets.

5. Conclusion

In summary, our results show that **1f** and **1o** display cytotoxic properties, induce G_1 phase cell cycle arrest and apoptosis on human myeloid leukemia HL-60 cells. Since these cells are p53 null, our results clearly demonstrate that apoptosis induced by **1f** and **1o** could occur independently of p53-mediated cellular events. Given the fact that about 50% of human cancers harbor p53 mutations [26,27], these results have important implications for developing these flavonoid derivatives as chemopreventive or chemotherapeutic agents. **1f**- and **1o**-induced apoptosis is accompanied by the activation of multiple caspases, mitochondrial release of cytochrome *c*, PARP cleavage and DNA fragmentation. Although cleavage of procaspase-8, -9, -3, -6 and -7 is detected in **1f**- and **1o**-treated cells by immunoblotting, the inhibition data indicate that these caspases do not fully account for cell death. These results, when considered together with the almost complete blockage of apoptosis by z-VAD-fmk, suggest that additional caspases may contribute to apoptosis. One possibility is that other executioner caspases, such as caspase-6 is involved in this process as it is shown in this paper. **1f** and **1o** induce the activation of the MEK/ERK pathway, and the combination with the inhibitor of MEK 1/2 kinases leads to enhanced cell death. The findings of this study

suggest that these synthetic flavonoids could be useful in the development of novel anticancer agents.

6. Experimental protocols

6.1. Chemistry

6.1.1. Materials and methods

Purity of all tested compounds was >95.0% at 254 nm as determined by high-performance liquid chromatography. HPLC analysis was obtained on a Waters W2690/5 using a Phenomenex Luna C18 column (150 × 4.60 mm, 5 μm particle size) with a 1.0 mL/min flow rate using a gradient of 3%–100% [0.1% formic acid in acetonitrile] in [0.1% formic acid in water] over 37 min. ^{1}H and ^{13}C NMR spectra were obtained on Bruker model AMX 500 and 400 NMR spectrometers with standard pulse sequences, operating at 500 MHz in ^{1}H and 125 MHz in ^{13}C. $CDCl_3$ was used as solvent, and TMS was used as internal standard. IR spectra were recorded using a Perkin–Elmer model spectrum-100 spectrophotometer. High-resolution mass spectra (HRMS) were recorded on a Micromass Q-Tof Micro mass spectrometer with a lock spray source. Column chromatography was carried out on silica gel (70–230 mesh, Merck) and fractions were monitored by TLC (silica gel 60 F254). Preparative TLC was carried out on silica gel 60 PF254 + 366 plates (20 × 20 cm, 1 mm thick). All products reported showed ^{1}H and ^{13}C NMR spectra in agreement with the assigned structures.

6.1.2. Experimental part

General procedure for the synthesis of compounds **2a**–**q** have already been described [15,16].

6.1.3. General procedure for the preparation of compounds *1a–i* and *1l–q*

To a solution of the respective flavonoid (15 mg) in dehydrated MeOH (5 mL), excess of dissolution of $TMSCHN_2$ in diethyl ether 2.0 M (0.5 mL) was added and stirred at room temperature for 24 h, the mixture was concentrated under vacuum, and then the resulting residue was chromatographed over silica gel with *n*-hexane–EtOAc (8:2) to give the methyl ester flavonoids.

*6.1.3.1. Methyl 6,7-dimethyl-4-oxo-2-phenyl-4H-chromene-8-carboxylate (**1a**).* This compound was prepared from **2a** using the procedure described above. (0.040 mmol, 12.5 mg, 80%) yellow solid; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.06 (1H, s, H-5), 7.86–7.84 (2H, m, H-2′, H-6′), 7.54–7.52 (3H, m, H-3′, H-4′, H-5′), 6.82 (1H, s, H-3), 4.09 (3H, s, OCH_3), 2.53 (3H, s, CH_3-11), 2.40 ppm (3H, s, CH_3-12); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.7 (C, C-4), 167.1 (C, C-13), 162.9 (C, C-2), 151.5 (C, C-9), 141.3 (C, C-7), 134.7 (C, C-6), 131.7 (C, C-1′), 131.6 (CH, C-4′), 129.1 (2× CH, C-3′ and C-5′), 126.8 (CH, C-5), 126.2 (2× CH, C-2′ and C-6′), 123.8 (C, C-8), 121.6 (C, C-10), 107.4 (CH, C-3), 52.7 (CH_3, OCH_3), 19.8 (CH_3, C-11), 17.5 ppm (CH_3, C-12); IR (KBr): ν_{max} = 2960, 1715, 1650, 1429, 1368, 1336, 1259, 1058, 769 cm^{-1}; HRMS (ESI$^+$): *m*/*z* [M + H]$^+$ calcd for $C_{19}H_{17}O_4$: 309.1127, found: 309.1189.

*6.1.3.2. Methyl 2-(4-methoxyphenyl)-6,7-dimethyl-4-oxo-4H-chromene-8-carboxylate (**1b**).* This compound was prepared from **2b** using the procedure described above. (0.035 mmol, 12.0 mg, 76%) yellow solid; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.04 (1H, s, H-5), 7.79 (2H, d, *J* = 8.0 Hz, H-2′, H-6′), 7.01 (2H, d, *J* = 8.0 Hz, H-3′, H-5′), 6.72 (1H, s, H-3), 4.09 (3H, s, OCH_3), 3.89 (3H, s, OCH_3–C-4′), 2.41 (3H, s, CH_3-11), 2.39 ppm (3H, s, CH_3-12); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.4 (C, C-4), 167.2 (C, C-13), 163.0 (C, C-2), 162.4 (C, C-4′), 151.4 (C, C-9), 141.0 (C, C-7), 134.5 (C, C-6), 127.9 (2× CH, C-2′ and C-6′), 126.8 (CH, C-5), 123.9 (C, C-1′), 123.6 (C, C-8), 121.5 (C, C-10), 114.5 (2× CH, C-3′ and C-5′), 106.0 (CH, C-3), 56.0 (CH_3, OCH_3–C-4′), 52.7 (CH_3, OCH_3), 19.8 (CH_3, C-11), 17.5 ppm (CH_3, C-12); IR (KBr): ν_{max} = 2962, 1724, 1651, 1514, 1434, 1370, 1261, 1058, 800 cm^{-1}; HRMS (ESI$^+$): *m*/*z* [M + H]$^+$ calcd for $C_{20}H_{19}O_5$: 339.1232, found: 339.1295.

*6.1.3.3. Methyl 2-(3-methoxyphenyl)-6,7-dimethyl-4-oxo-4H-chromene-8-carboxylate (**1c**).* This compound was prepared from **2c** using the procedure described above. (0.034 mmol, 11.7 mg, 75%) yellow solid; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.00 (1H, s, H-5), 7.38–7.37 (2H, m, H-5′, H-6′), 7.34 (1H, d, *J* = 1.0 Hz, H-2′), 7.03 (1H, dd, *J* = 8.3, 5.4 Hz, H-4′), 6.76 (1H, s, H-3), 4.06 (3H, s, OCH_3), 3.86 (3H, s, OCH_3–C-3′), 2.37 (3H, s, CH_3-11), 2.36 ppm (3H, s, CH_3-12); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.6 (C, C-4), 167.0 (C, C-13), 162.6 (C, C-2), 159.8 (C, C-3′), 151.4 (C, C-9), 141.3 (C, C-7), 134.7 (C, C-6), 132.8 (C, C-1′), 130.1 (CH, C-5′), 126.7 (CH, C-5), 123.7 (C, C-8), 121.5 (C, C-10), 118.5 (CH, C-6′), 117.4 (CH, C-4′), 111.48 (CH, C-2′), 107.5 (CH, C-3), 55.3 (CH_3, OCH_3–C-3′), 52.7 (CH_3, OCH_3), 19.8 (CH_3, C-11), 17.5 ppm (CH_3, C-12); IR (KBr): ν_{max} = 2951, 1728, 1648, 1607, 1492, 1365, 1274, 1251, 1211, 1051, 846, 782 cm^{-1}; HRMS (ESI$^+$): *m*/*z* [M + H]$^+$ calcd for $C_{20}H_{19}O_5$: 339.1232, found: 339.1291.

*6.1.3.4. Methyl 6,7-dimethyl-4-oxo-2-p-tolyl-4H-chromene-8-carboxylate (**1d**).* This compound was prepared from **2d** using the procedure described above. (0.040 mmol, 13.0 mg, 83%) white solid; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.07 (1H, s, H-5), 7.75 (2H, t, *J* = 8.2 Hz, H-2′, H-6′), 7.34 (2H, d, *J* = 8.2 Hz, H-3′, H-5′), 6.79 (1H, s, H-3), 4.09 (3H, s, OCH_3), 2.46 (3H, s, CH_3–C-4′), 2.43 (3H, s, CH_3-11), 2.41 ppm (3H, s, CH_3-12); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.8 (C, C-4), 167.2 (C, C-13), 163.2 (C, C-2), 151.5 (C, C-9), 142.3 (C, C-4′), 141.1 (C, C-7), 134.6 (C, C-6), 129.8 (2× CH, C-3′ and C-5′), 128.9 (C, C-1′), 126.8 (CH, C-5), 126.2 (2× CH, C-2′ and C-6′), 123.7 (C, C-8), 121.6 (C, C-10), 106.8 (CH, C-3), 52.7 (CH_3, OCH_3), 21.5 (CH_3, CH_3–C-4′), 19.8 (CH_3, C-11), 17.5 ppm (CH_3, C-12); IR (KBr): ν_{max} = 2964, 1731, 1638, 1260, 1027, 799 cm^{-1}; HRMS (ESI$^+$): *m*/*z* [M + H]$^+$ calcd for $C_{20}H_{19}O_4$: 323.1283, found: 323.1319.

*6.1.3.5. Methyl 2-(4-fluorophenyl)-6,7-dimethyl-4-oxo-4H-chromene-8-carboxylate (**1e**).* This compound was prepared from **2e** using the procedure described above. (0.037 mmol, 12.4 mg, 79%) yellow solid; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.01 (1H, s, H-5), 7.80 (2H, dd, *J* = 1.9, 8.6 Hz, H-2′, H-6′), 7.17 (2H, t, *J* = 8.6 Hz, H-3′, H-5′), 6.71 (1H, s, H-3), 4.06 (3H, s, OCH_3), 2.38 (3H, s, CH_3-11), 2.37 ppm (3H, s, CH_3-12); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.5 (C, C-4), 167.0 (C, C-13), 164.7 (C, J_{FC} = 251 Hz, C-4′), 161.9 (C, C-2), 151.4 (C, C-9), 141.4 (C, C-7), 134.8 (C, C-6), 127.7 (C, J_{FC} = 3.7 Hz, C-1′), 128.4 (2× CH, J_{FC} = 8.7 Hz, C-2′ and C-6′), 126.8 (CH, C-5), 123.7 (C, C-8), 121.4 (C, C-10), 128.9 (C, C-1′), 116.2 (2× CH, J_{FC} = 22.5 Hz, C-3′ and C-5′), 107.1 (CH, C-3), 52.7 (CH_3, OCH_3), 19.8 (CH_3, C-11), 17.5 ppm (CH_3, C-12); IR (KBr): ν_{max} = 3001, 2956, 1715, 1651, 1605, 1510, 1432, 1367, 1238, 1164, 1017, 836, 748 cm^{-1}; HRMS (ESI$^+$): *m*/*z* [M + H]$^+$ calcd for $C_{19}H_{16}FO_4$: 327.1033, found: 327.1070.

*6.1.3.6. Methyl 2-(2-chlorophenyl)-6,7-dimethyl-4-oxo-4H-chromene-8-carboxylate (**1f**).* This compound was prepared from **2f** using the procedure described above. (0.038 mmol, 13.3 mg, 85%) white solid; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.06 (1H, s, H-5), 7.62 (1H, dd, *J* = 9.3, 1.6 Hz, H-6′), 7.52 (1H, dd, *J* = 9.3, 2.4 Hz, H-3′), 7.46 (1H, td, *J* = 9.3, 2.3 Hz, H-4′), 7.40 (1H, td, *J* = 9.2, 1.9 Hz, H-5′), 6.70 (1H, s, H-3), 3.97 (3H, s, OCH_3), 2.41 (3H, s, CH_3-11), 2.37 ppm (3H, s, CH_3-12); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.4 (C, C-4), 167.0 (C, C-13), 162.1 (C, C-2), 151.7 (C, C-9), 141.3 (C, C-7), 134.9 (C, C-6), 132.9 (C, C-2′), 131.8 (CH, C-4′), 131.6 (C, C-1′), 131.0 (CH, C-3′), 130.8 (CH, C-6′), 127.2 (CH, C-5′), 126.6 (CH, C-5), 124.1 (C, C-8), 121.4 (C, C-10),

112.9 (CH, C-3), 52.7 (CH_3, OCH_3), 19.8 (CH_3, C-11), 17.5 ppm (CH_3, C-12); IR (KBr): ν_{max} = 2962, 1732, 1648, 1435, 1363, 1302, 1259, 1162, 1058, 799 cm^{-1}; HRMS (ESI^+): m/z $[M + H]^+$ calcd for $C_{19}H_{16}ClO_4$: 343.0737, found: 343.0780.

*6.1.3.7. Methyl 2-(4-chlorophenyl)-6,7-dimethyl-4-oxo-4H-chromene-8-carboxylate (**1g**).* This compound was prepared from **2g** using the procedure described above. (0.038 mmol, 13.3 mg, 85%) white solid; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.00 (1H, s, H-5), 7.72 (2H, t, J = 9.0 Hz, H-2′, H-6′), 7.45 (2H, d, J = 9.0 Hz, H-3′, H-5′), 6.73 (1H, s, H-3), 4.07 (3H, s, OCH_3), 2.39 (3H, s, CH_3-11), 2.37 ppm (3H, s, CH_3-12); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.4 (C, C-4), 167.0 (C, C-13), 161.7 (C, C-2), 151.3 (C, C-9), 141.5 (C, C-7), 137.9 (C, C-4′), 135.0 (C, C-6), 130.0 (C, C-1′), 129.4 (2× CH, C-3′ and C-5′), 127.4 (2× CH, C-2′ and C-6′), 126.8 (CH, C-5), 123.7 (C, C-8), 121.4 (C, C-10), 107.4 (CH, C-3), 52.7 (CH_3, OCH_3), 19.9 (CH_3, C-11), 17.6 ppm (CH_3, C-12); IR (KBr): ν_{max} = 2963, 1716, 1661, 1435, 1366, 1259, 1058, 827, 799 cm^{-1}; HRMS (ESI^+): m/z $[M + H]^+$ calcd for $C_{19}H_{16}ClO_4$: 343.0737, found: 343.0778.

*6.1.3.8. Methyl 2-(2,4-dichlorophenyl)-6,7-dimethyl-4-oxo-4H-chromene-8-carboxylate (**1h**).* This compound was prepared from **2h** using the procedure described above. (0.033 mmol, 12.4 mg, 80%) white solid; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.02 (1H, s, H-5), 7.56 (1H, d, J = 8.3 Hz, H-6′), 7.51 (1H, d, J = 1.8 Hz, H-3′), 7.37 (1H, dd, J = 1.8, 8.3 Hz, H-5′), 6.67 (1H, s, H-3), 3.96 (3H, s, OCH_3), 2.39 (3H, s, CH_3-11), 2.35 ppm (3H, s, CH_3-12); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.2 (C, C-4), 166.9 (C, C-13), 161.0 (C, C-2), 151.6 (C, C-9), 141.5 (C, C-7), 137.4 (C, C-4′), 135.1 (C, C-6), 133.7 (C, C-2′), 131.5 (CH, C-6′), 130.9 (CH, C-3′), 130.0 (C, C-1′), 127.6 (CH, C-5′), 126.6 (CH, C-5), 124.0 (C, C-8), 121.3 (C, C-10), 113.0 (CH, C-3), 52.7 (CH_3, OCH_3), 19.9 (CH_3, C-11), 17.5 ppm (CH_3, C-12); IR (KBr): ν_{max} = 2962, 1729, 1656, 1439, 1359, 1259, 1058, 827, 801 cm^{-1}; HRMS (ESI^+): m/z $[M + H]^+$ calcd for $C_{19}H_{15}Cl_2O_4$: 377.0347, found: 377.0386.

*6.1.3.9. Methyl 6,7-dimethyl-2-(4-methyl-3-nitrophenyl)-4-oxo-4H-chromene-8-carboxylate (**1i**).* This compound was prepared from **2i** using the procedure described above. (0.031 mmol, 11.4 mg, 73%) white solid; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.46 (1H, d, J = 1.0 Hz, H-2′), 8.00 (1H, s, H-5), 7.90 (1H, dd, J = 1.2, 7.9 Hz, H-6′), 7.48 (1H, d, J = 8.0 Hz, H-5′), 6.80 (1H, s, H-3), 4.14 (3H, s, OCH_3), 2.68 (3H, s, CH_3—C-4′), 2.40 (3H, s, CH_3-11), 2.39 ppm (3H, s, CH_3-12); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.3 (C, C-4), 166.9 (C, C-13), 160.0 (C, C-2), 151.3 (C, C-9), 149.5 (CH, C-3′), 142.1 (C, C-7), 137.2 (C, C-4′), 135.3 (C, C-6), 133.8 (C, C-5′), 130.7 (C, C-1′), 129.7 (CH, C-6′), 126.7 (CH, C-5), 123.7 (C, C-8), 122.4 (CH, C-2′), 121.4 (C, C-10), 107.8 (CH, C-3), 53.1 (CH_3, OCH_3), 20.71 (CH_3, CH_3—C-4′), 19.9 (CH_3, C-11), 17.6 ppm (CH_3, C-12); IR (KBr): ν_{max} = 2962, 1725, 1656, 1532, 1438, 1368, 1260, 1165, 1033, 800, 701 cm^{-1}; HRMS (ESI^+): m/z $[M + H]^+$ calcd for $C_{20}H_{18}NO_6$: 368.1134, found: 368.1172.

*6.1.3.10. Methyl 2-(3,4-dichlorophenyl)-6,7-dimethyl-4-oxo-4H-chromene-8-carboxylate (**1l**).* This compound was prepared from **2l** using the procedure described above. (0.028 mmol, 10.6 mg, 68%) white solid; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.02 (1H, s, H-5), 7.91 (1H, d, J = 1.8 Hz, H-2′), 7.63 (1H, dd, J = 1.9, 8.4 Hz, H-6′), 7.57 (1H, d, J = 8.4 Hz, H-5′), 6.75 (1H, s, H-3), 4.11 (3H, s, OCH_3), 2.41 (3H, s, CH_3-11), 2.40 ppm (3H, s, CH_3-12); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.3 (C, C-4), 166.9 (C, C-13), 160.2 (C, C-2), 151.3 (C, C-9), 142.1 (C, C-7), 135.9 (C, C-4′), 135.2 (C, C-6), 133.7 (C, C-3′), 131.5 (C, C-1′), 131.1 (CH, C-5′), 128.0 (C, C-2′), 127.6 (CH, C-5′), 126.9 (CH, C-5), 125.1 (CH, C-6′), 123.6 (C, C-8), 121.4 (C, C-10), 107.9 (CH, C-3), 52.9 (CH_3, OCH_3), 20.0 (CH_3, C-11), 17.6 ppm (CH_3, C-12); IR (KBr): ν_{max} = 2961, 1725, 1642, 1435, 1355, 1257, 1160, 1025, 869, 846, 799, 750 cm^{-1}; HRMS (ESI^+): m/z $[M + H]^+$ calcd for $C_{19}H_{15}Cl_2O_4$: 377.0347, found: 377.0378.

*6.1.3.11. Methyl 6,7-dimethyl-4-oxo-2-m-tolyl-4H-chromene-8-carboxylate (**1m**).* This compound was prepared from **2m** using the procedure described above. (0.026 mmol, 10.1 mg, 65%) white solid; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.00 (1H, s, H-5), 7.61 (2H, s br, H-2′, H-6′), 7.35 (1H, t, J = 7.0 Hz, H-5′), 7.29 (1H, d, J = 1.8 Hz, H-4′), 6.75 (1H, s, H-3), 4.07 (3H, s, OCH_3), 2.41 (3H, s, OCH_3—C-3′), 2.36 ppm (6H, s, CH_3-11, CH_3-12); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.6 (C, C-4), 167.1 (C, C-13), 163.0 (C, C-2), 151.4 (C, C-9), 141.3 (C, C-7), 138.7 (C, C-3′), 134.7 (C, C-6), 132.4 (CH, C-4′), 131.5 (C, C-1′), 129.0 (CH, C-5′), 126.8 (CH, C-2′), 126.7 (CH, C-5), 123.7 (C, C-8), 123.3 (CH, C-6′), 121.5 (C, C-10), 107.2 (CH, C-3), 52.7 (CH_3, OCH_3), 21.5 (CH_3, OCH_3—C-3′), 19.9 (CH_3, C-11), 17.5 ppm (CH_3, C-12); IR (KBr): ν_{max} = 2950, 1731, 1645, 1436, 1362, 1302, 1247, 1196, 1062, 999, 943, 879, 848, 782, 756, 696 cm^{-1}; HRMS (ESI^+): m/z $[M + H]^+$ calcd for $C_{20}H_{19}O_4$: 323.1283, found: 323.1324.

*6.1.3.12. Methyl 2-(4-(methoxycarbonyl)phenyl)-6,7-dimethyl-4-oxo-4H-chromene-8-carboxylate (**1n**).* This compound was prepared from **2n** using the procedure described above. (0.041 mmol, 15.1 mg, 93%) white solid; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.12 (2H, d, J = 8.3 Hz, H-3′, H-5′), 8.00 (1H, s, H-5), 7.56 (2H, d, J = 8.3 Hz, H-2′, H-6′), 6.82 (1H, s, H-3), 4.08 (3H, s, OCH_3), 3.95 (3H, s, $COOCH_3$—C-4′), 2.38 (3H, s, CH_3-11), 2.37 ppm (3H, s, CH_3-12); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.4 (C, C-4), 167.0 (C, C-13), 166.0 (CH_3, $COOCH_3$—C-4′), 161.4 (C, C-2), 151.4 (C, C-9), 141.0 (C, C-7), 135.5 (C, C-1′), 135.0 (C, C-6), 132.6 (C, C-4′), 130.2 (2× CH, C-3′ and C-5′), 126.8 (CH, C-5), 126.1 (2× CH, C-2′ and C-6′), 123.7 (C, C-8), 121.5 (C, C-10), 108.5 (CH, C-3), 52.7 (CH_3, OCH_3), 52.4 (CH_3, $COOCH_3$—C-4′), 19.9 (CH_3, C-11), 17.6 ppm (CH_3, C-12); IR (KBr): ν_{max} = 2960, 1724, 1716, 1655, 1569, 1432, 1368, 1328, 1285, 1259, 1206, 1189, 1163, 1080, 1022, 975, 857, 801 cm^{-1}; HRMS (ESI^+): m/z $[M + H]^+$ calcd for $C_{21}H_{19}O_6$: 367.1182, found: 367.1226.

*6.1.3.13. Methyl 6,7-dimethyl-4-oxo-2-o-tolyl-4H-chromene-8-carboxylate (**1o**).* This compound was prepared from **2o** using the procedure described above. (0.036 mmol, 11.6 mg, 74%) white solid; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.05 (1H, s, H-5), 7.48 (1H, dd, J = 8.3, 1.6 Hz, H-6′), 7.38 (1H, td, J = 7.5, 1.4 Hz, H-4′), 7.28 (2H, m, H-3, H-5′), 6.46 (1H, s, H-3), 3.94 (3H, s, OCH_3), 2.45 (3H, s, CH_3—C-2′), 2.38 (3H, s, CH_3-11), 2.35 ppm (3H, s, CH_3-12); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.5 (C, C-4), 167.0 (C, C-13), 165.8 (C, C-2), 151.6 (C, C-9), 141.0 (C, C-7), 136.9 (C, C-2′), 134.7 (C, C-6), 132.4 (C, C-1′), 131.3 (CH, C-3′), 130.8 (CH, C-4′), 129.4 (CH, C-6′), 126.6 (CH, C-5), 126.2 (CH, C-5′), 124.0 (C, C-8), 121.4 (C, C-10), 111.9 (CH, C-3), 52.6 (CH_3, OCH_3), 20.5 (CH_3, C-2′), 19.8 (CH_3, C-11), 17.5 ppm (CH_3, C-12); IR (KBr): ν_{max} = 2959, 1733, 1648, 1435, 1361, 1302, 1253, 1161, 1061, 850, 801, 770 cm^{-1}; HRMS (ESI^+): m/z $[M + H]^+$ calcd for $C_{20}H_{19}O_4$: 323.1283, found: 323.1353.

*6.1.3.14. Methyl 6,7-dimethyl-4-oxo-2-(4-(trifluoromethyl)phenyl)-4H-chromene-8-carboxylate (**1p**).* This compound was prepared from **2p** using the procedure described above. (0.036 mmol, 11.6 mg, 76%) white solid; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.99 (1H, s, H-5), 7.91 (2H, d, J = 8.0 Hz, H-2′, H-6′), 7.73 (2H, d, J = 8.0 Hz, H-3′, H-5′), 6.80 (1H, s, H-3), 4.07 (3H, s, OCH_3), 2.38 (3H, s, CH_3-11), 2.37 ppm (3H, s, CH_3-12); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.3 (C, C-4), 166.9 (C, C-13), 161.9 (C, C-2), 151.3 (C, C-9), 141.8 (C, C-7), 135.1 (C, C-6), 135.0 (C, C-1′), 133.4 (C, J_{FC} = 32.5 Hz, C-4′), 126.8 (CH, C-5), 126.4 (2CH, C-2′ and C-6′), 126.0 (2CH, J_{FC} = 3.5 Hz, C-3′ and C-5′), 123.7 (C, C-8), 123.5 (C, J_{FC} = 271.0 Hz, CF_3), 121.4 (C, C-10), 108.5 (CH, C-3), 52.7 (CH_3, OCH_3), 19.8 (CH_3, C-11), 17.5 ppm (CH_3, C-12); IR (KBr): ν_{max} = 3074, 3006, 2954, 1730, 1647, 1574, 1437, 1367, 1324,

1253, 1234, 1167, 1120, 1069, 1016, 924, 843, 758 cm^{-1}; HRMS (ESI^+): m/z $[M + H]^+$ calcd for $C_{20}H_{16}F_3O_4$: 377.1001, found: 377.1044.

*6.1.3.15. Methyl 2-(2-methoxyphenyl)-6,7-dimethyl-4-oxo-4H-chromene-8-carboxylate (**1q**).* This compound was prepared from **2q** using the procedure described above. (0.035 mmol, 11.9 mg, 76%) white solid; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 8.00$ (1H, s, H-5), 7.78 (1H, d, $J = 7.7$ Hz, H-6′), 7.41 (1H, t, $J = 7.9$ Hz, H-4′), 7.15 (1H, s, H-3), 7.05 (1H, t, $J = 7.5$ Hz, H-5′), 6.99 (1H, d, $J = 8.3$ Hz, H-3′), 4.00 (3H, s, OCH_3), 3.90 (3H, s, OCH_3—C-2′), 2.36 (3H, s, CH_3-11), 2.33 ppm (3H, s, CH_3-12); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 178.1$ (C, C-4), 167.2 (C, C-13), 160.2 (C, C-2), 158.1 (C, C-2′), 151.6 (C, C-9), 140.9 (C, C-7), 134.6 (C, C-6), 132.4 (CH, C-4′), 129.1 (CH, C-6′), 126.5 (CH, C-5), 123.7 (C, C-8), 121.3 (C, C-10), 120.8 (CH, C-5′), 120.4 (C, C-1′), 112.4 (CH, C-3), 111.7 (CH, C-3′), 55.6 (CH_3, OCH_3—C-2′), 52.6 (CH_3, OCH_3), 19.8 (CH_3, C-11), 17.4 ppm (CH_3, C-12); IR (KBr): $\nu_{max} = 2953$, 1726, 1648, 1617, 1567, 1492, 1454, 1433, 1362, 1310, 1255, 1228, 1202, 1165, 1066, 1014, 856, 801, 756 cm^{-1}; HRMS (ESI^+): m/z $[M + H]^+$ calcd for $C_{20}H_{19}O_5$: 339.1232, found: 339.1270.

*6.1.3.16. Methylation of compound **2j**.* To a solution of the respective flavonoid (15 mg) in dehydrated MeOH (5 mL), excess of dissolution of $TMSCHN_2$ in diethyl ether 2.0 M (0.5 mL) was added and stirred at room temperature for 24 h, the mixture was concentrated under vacuum, and then the resulting residue was subjected to chromatographic separation over silica gel with *n*-hexane–EtOAc (8:2) to furnish **1j1** (9 mg) and **1j2** (4 mg) in a ratio of (7:3).

*6.1.3.17. Methyl 2-(3-amino-4-methylphenyl)-6,7-dimethyl-4-oxo-4H-chromene-8-carboxylate (**1j1**).* Yellow solid; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 8.04$ (1H, s, H-5), 7.17 (2H, s br, H-5′, H-6′), 7.11 (1H, s br, H-2′), 6.73 (1H, s, H-3), 4.08 (3H, s, OCH_3), 2.40 (3H, s, CH_3-11), 2.39 (3H, s, CH_3-12), 2.23 ppm (3H, s, CH_3—C-4′); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 177.8$ (C, C-4), 167.3 (C, C-13), 163.5 (C, C-2), 151.5 (C, C-9), 145.1 (CH, C-3′), 141.0 (C, C-7), 134.6 (C, C-6), 131.0 (C, C-5′), 130.3 (C, C-1′), 126.7 (CH, C-5), 126.5 (C, C-4′), 123.7 (C, C-8), 121.6 (C, C-10), 116.5 (CH, C-6′), 112.1 (CH, C-2′), 106.8 (CH, C-3), 52.7 (CH_3, OCH_3), 19.9 (CH_3, C-11), 17.5 ppm ($2CH_3$, C-12 and CH_3—C-4′); IR (KBr): $\nu_{max} = 3359$, 2925, 2855, 1729, 1631, 1568, 1511, 1435, 1365, 1292, 1250, 1164, 1063, 982, 846, 808, 756, 657 cm^{-1}; HRMS (ESI^+): m/z $[M + H]^+$ calcd for $C_{20}H_{20}NO_4$: 338.1392, found: 338.1423.

*6.1.3.18. Methyl 6,7-dimethyl-2-(4-methyl-3-(methylamino)phenyl)-4-oxo-4H-chromene-8-carboxylate (**1j2**).* Yellow solid; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 8.05$ (1H, s, H-5), 7.16 (2H, s br, H-5′, H-6′), 7.05 (1H, s br, H-2′), 6.81 (1H, s, H-3), 4.07 (3H, s, OCH_3), 3.00 (3H, s, $NHCH_3$), 2.41 (3H, s, CH_3-11), 2.39 (3H, s, CH_3-12), 2.21 ppm (3H, s, CH_3—C-4′); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 177.9$ (C, C-4), 167.2 (C, C-13), 164.1 (C, C-2), 151.5 (C, C-9), 147.5 (CH, C-3′), 140.9 (C, C-7), 134.5 (C, C-6), 130.4 (C, C-5′), 130.5 (C, C-1′), 126.7 (CH, C-5), 126.1 (C, C-4′), 123.8 (C, C-8), 121.6 (C, C-10), 115.0 (CH, C-6′), 106.8 (CH, C-3), 106.2 (CH, C-2′), 52.7 (CH_3, OCH_3), 30.7 (CH_3, $NHCH_3$), 19.8 (CH_3, C-11), 17.5 ppm ($2CH_3$, C-12 and CH_3—C-4′); IR (KBr): $\nu_{max} = 3401$, 2962, 1731, 1642, 1571, 1523, 1437, 1365, 1259, 1162, 1030, 845, 800, 758, 705 cm^{-1}; HRMS (ESI^+): m/z $[M + H]^+$ calcd for $C_{21}H_{22}NO_4$: 352.1549, found: 352.1580.

6.2. Pharmacology

6.2.1. Reagents

Stock solutions of the different compounds at 100 mM were made by dissolving them in dimethyl sulfoxide (DMSO), and aliquots were frozen at −20 °C. Antibodies for poly(ADP-ribose) polymerase (PARP), caspase-3, caspase-8 and caspase-9 were purchased from Stressgen (Victoria, British Columbia, Canada). Antibodies for cytochrome *c* and caspase-7 were purchased from BD PharMingen (San Diego, CA, USA). Anti-caspase-6 monoclonal and anti-$p21^{Cip1}$ antibodies were from Medical & Biological Laboratories (Nagoya, Japan) and Millipore (Temecula, CA, USA), respectively. Anti-cyclin D1, anti-JNK/SAPK, anti-p44/42 MAP Kinase, anti-Phospho-p44/42 MAP Kinase (T202/Y204), anti-$p38^{MAPK}$ and a phosphorylated form (T180/Y182) of $p38^{MAPK}$ antibodies were purchased from New England BioLabs (Cell Signaling Technology, Inc., Beverly, MA, USA). JNK/SAPK (phosphor T183 + Y185) antibody was purchased from Abcam (Cambridge, UK). Secondary antibodies were from GE Healthcare UK Limited (Little Chalfont, UK). All other chemicals were obtained from Sigma (Saint Louis, MO, USA).

6.2.2. Cell culture

HL-60 cells (DSMZ No.: ACC3, DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and HL-60/MX1 (ATCC, American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Human peripheral blood mononuclear cells (PBMCs) were isolated from heparin-anticoagulated blood of healthy volunteers by centrifugation with Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). PBMCs were also stimulated with phytohemagglutinin (PHA, 2 μg mL^{-1}) for 48 h before experimental treatment.

6.2.3. Cytotoxicity of methyl esters of flavonoids on human myeloid leukemia cells

The cytotoxicity of methyl esters of flavonoids on HL-60 and HL-60/MX1 cells was analyzed by colorimetric 3-(4,5-dimethyl-2-thiazolyl-)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay as described [45]. Concentrations inducing a 50% inhibition of cell growth (IC_{50}) were determined graphically using the curve-fitting algorithm of the computer software Prism 4.0 (GraphPad). Values are means ± S.E. from three independent experiments, each performed in triplicate.

6.2.4. Inverse PCR assay

Genomic DNA was isolated using the QIAamp DNA micro kit (Qiagen, Hilden, Germany), incubated with shrimp alkaline phosphatase (Takara, Shiga, Japan) for 1 h at 37 °C and subsequently inactivated at 75 °C for 30 min. Next, DNA was digested with 24 U *Xba* I (Takara, Shiga, Japan) for 4 h at 37 °C. After 1 h heat inactivation at 75 °C, the DNA was circularized overnight at 4 °C using 3 U T4 DNA ligase (Takara) in a final volume of 50 μL. A fraction of the ligation product was heat inactivated and digested with *Pvu* II (Takara) to abolish amplification of the wild-type *MLL*. Approximately 40 ng of the circularized DNA (either *Pvu* II treated or untreated) was used for the first PCR. The PCR was carried out using conditions and primers described by Libura et al. [19]. The two sets of nested primers used to amplify *MLL* and putative fusion partners were: MF1-5′TCTACAAGTGCCAGGGGTCT3′; MF2-5′AATAGCATGCTGCCTGCACTGCA-CTCCTAA3′; MR1-5′CCCGACG TGGATT TTCTTTA3′; MR2-5′GATCGTAGGATATGTCCCTTATAAA TGACAAACTACTG CTTCC3′. The nested PCR products were separated by electrophoresis on a 1.8% agarose gel and visualized under UV light by ethidium bromide staining.

6.2.5. Evaluation of apoptosis

The rate of apoptotic cell death was analyzed by fluorescent microscopy and by flow-cytometric analysis of propidium iodide (PI)-stained nuclei as described [45].

6.2.6. Analysis of DNA fragmentation

DNA isolation and gel electrophoresis were performed as described previously [12].

6.2.7. Western blot analysis

HL-60 cells (1×10^6 mL^{-1}) were treated in the absence or presence of methyl esters of flavonoids, **1f** and **1o** (10–30 μM) for various time periods as indicated and harvested by centrifugation. Cell pellets were resuspended in lysis buffer [20 mM Tris–HCl (pH 7.4), 2 mM EDTA, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM tetrasodium pyrophosphate, 20 mM sodium β-glycerophosphate, 10 mM sodium fluoride, 2 mM sodium orthovanadate], supplemented with protease inhibitors phenylmethylsulfonyl fluoride (PMSF, 1 mM), leupeptin, aprotinin and pepstatin A (5 μg mL^{-1} each) for 15 min at 4 °C. Lysates were homogenized by a sonifier (five cycles) and centrifuged at 11,000× *g* for 10 min at 4 °C. Protein concentration of supernatants was measured by the Bradford method and samples containing equal amounts of proteins were boiled in sodium dodecyl sulfate (SDS)-sample buffer for 5 min before loading on an SDS-polyacrylamide gel (7.5% for PARP, 10% for MAPK and 12.5% for caspases). Proteins were electrotransferred to poly(vinylidene difluoride) (PVDF) membranes and detected as described [45].

6.2.8. Detection of cytochrome c release from mitochondria

Release of cytochrome *c* from mitochondria was detected by Western blot analysis as described [45].

6.2.9. Statistical analysis

Statistical significance of differences between control and treated samples were calculated using Student's *t*-test. *P* values of <0.05 were considered significant.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejmech.2012.07.028.

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