



Research article

In vitro activity of some flavonoid derivatives on human leukemic myeloid cells: evidence for aminopeptidase-N (CD13) inhibition, antiproliferative and cell death properties

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Abstract: Leukemia cells from patients with acute myeloid leukemia (AML) display high proliferative capacity and are resistant to death. Membrane-anchored aminopeptidase-N/CD13 is a potential drug target in AML. Clinical research efforts are currently focusing on targeted therapies that induce death in AML cells. We previously developed a non-cytotoxic APN/CD13 inhibitor based on flavone-8-acetic acid scaffold, the 2',3-dinitroflavone-8-acetic acid (**1**). In this context, among the variously substituted 113 compounds further synthesized and tested for evaluation of their effects on APN/CD13 activity, proliferation and survival in human AML U937 cells, eight flavonoid derivatives emerged: 2',3-dinitro-6-methoxy-flavone-8-acetic acid (**2**), four compounds (**3–6**) with the 3-chloro-2,3-dihydro-3-nitro-2-phenyl-4*H*-1-benzopyran-4-one structure, and three (**7–9**) with the 3-chloro-3,4-dihydro-4-hydroxy-3-nitro-2-phenyl-2*H*-1-benzopyran framework. Different structure-activity relationships were observed between APN/CD13 activity and growth/survival processes. We showed that compound **2**, but not benzopyran derivatives **3–9**, inhibited APN activity (although to a less degree than **1**). Both **1** and **2** did not affect AML cell proliferation and survival, indicating that

CD13's APN activity is not required for these processes. In contrast, benzopyran compounds **3–9** inhibited in a concentration-dependent manner the growth of U937 cells by inducing death as evidenced by phosphatidylserine externalization. Cell death was associated with the presence of geminal nitro group and chlorine at the 3-position of the 2*H*-1-benzopyran scaffold. The presence of other substituents such as CH₂COOH or CH₂CH=CH₂ groups at the 8-position, NO₂ or I substituents at the 2'- or 3'-position, OCH₃ or OCH₂C₆H₅ groups at the 4'-position did not affect cell death. Importantly, the inhibitory effects evidenced with compounds **7–9** were not due to their potential decomposition into the corresponding (*Z*)-(2chloro-2-nitroethenyl)benzene and salicylaldehyde. Based on these preliminary data, the 3-chloro,3-nitro-2*H*-1-benzopyran derivatives could be classified as a new group of compounds with promising antitumor properties; this study therefore provides the opportunity to explore their potential efficiency in AML patients' cells *ex vivo*.

Keywords: aminopeptidase-N; apoptosis; benzopyran; CD13; cell death; flavone; leukemia; metalloproteinase; proliferation; survival

1. Introduction

Acute myeloid leukemia (AML) is a clinically and genetically heterogeneous hematopoietic cancer characterized by the clonal expansion and accumulation of immature myeloid precursors in the marrow and blood [1,2]. Leukemia cells are unable to undergo growth arrest, terminal differentiation and death in response to appropriate environmental stimuli [1,2]. The conventional chemotherapeutic approach to treatment of AML patients is based on a combination of an anthracycline with cytarabine [1,2]. However, AML remains a challenging disease in the clinic because patients are either refractory to front-line therapy or subsequently relapse [2]. Thus, the development of new compounds directed against leukemia-specific targets is needed to increase the cure rate in AML patients exhibiting chemoresistance and poor outcomes. A range of drug candidates (including tyrosine kinase inhibitors, farnesyltransferase inhibitors, histone deacetylase inhibitors and deoxyadenosine analogues) are now in clinical development [1,2]. Other approaches are based on the identification of compounds capable of inducing cell death, which is impaired in AML cells.

A survey of the literature of natural compounds indicates that flavonoids may play a critical role in cancer progression by interfering with cell proliferation and survival [3-9]. Flavonoids can be classified into several classes including flavones [6,10]. The synthetic flavone flavopiridol has been shown to induce death in human AML cell lines [11-13] and has been evaluated in Phase I clinical trials in relapsed/refractory AML [14-17]. Modest antileukemic activity has been observed, although most patients experience side effects such as short-duration neutropenia, diarrhoea, cytokine release syndrome and fatigue [15-17]. Novel active compounds with less side-effects are thus required.

We previously reported the synthesis of novel flavone-8-acetic acid derivatives [18,19]. The 3,3'-diamino-4'-methoxyflavone was shown to induce caspase-dependent apoptosis of AML cell lines [20]. As a novel proteasome inhibitor, 3,3'-diamino-4'-methoxyflavone targets Bax activation and the degradation of caspase-3 substrate P70S6K during AML apoptosis [20]. We also identified the 2',3'-dinitro flavone-8-acetic acid (compound **1**) as a non-cytotoxic inhibitor of aminopeptidase-N (APN) activity [18] carried by the antigen CD13 in human AML cell lines [21,22]. CD13 is a useful marker in AML, being strongly expressed on stem cells and blasts in all AML subtypes [23,24].

Studies highlighted CD13 as a potential drug target in AML [22,25,26]. In contrast to 3,3'-diamino-4'-methoxyflavone, compound **1** did not affect AML U937 cell survival thus suggesting that substitutions with amine and methoxy groups in 3,3'-diamino-4'-methoxyflavone are important for conferring its pro-apoptotic activity [20].

With the above in mind, we undertook a study to evaluate the *in vitro* efficacy of a large series of new flavonoid derivatives (related to **1**) (113 molecules collected in the chemical library of Curie Institute, Paris, France) on human cell lines representative of AML. We sought to determine whether these molecules have potential drug activity in AML disease based on differential effects observed on APN/CD13 inhibition, antiproliferative and cell death properties. We report herein the effects of 8 flavonoids selected on these tumour-related processes of AML cells.

2. Materials and methods

2.1. Chemistry

Compounds **1** and **5** were synthesized as described in [18,27]. Compounds **9** and **10** were prepared according to a previously published procedure [28-31]. The synthesis of the *gem*-chloronitro derivative **6** was reported in [31]. Analytic and preparative separation of the two enantiomers of the racemic (\pm) **9** were carried out by HPLC using Chiralpak IA as the stationary phase and a mixture chloroform/hexane (9/1) as eluent. Products were detected by UV at 254 nm and circular dichroism at 254 nm. Salicylaldehyde was purchased from Sigma (St Louis, MO, USA). The novel carboxylic acids **2–4** were prepared as a racemic (2R*, 3S*), (2S*, 3R*) mixture on a 2 mmol scale starting from the convenient 8-allyl derivative by oxidation with sodium periodate in the presence of ruthenium(III) chloride hydrate according to a formerly described methodology [18,27]. The so far unknown chloronitrochromanol **8** was synthesized on a 5 mmol scale as a racemic (2R*, 3R*, 4R*), (2S*, 3S*, 4S*) mixture conforming to an already reported procedure [18,27] starting from the hitherto undescribed (*Z*)-4-Benzyloxy-1-(2-chloro-2-nitroethenyl)-3-iodobenzene (see physicochemical description below) and salicylaldehyde. The structures of the tested compounds are detailed in Figure 1.

Melting points were measured on a Köfler hot stage apparatus and are uncorrected. Infrared spectra were obtained using a Perkin-Elmer spectrum 65 FT-IR spectrometer. The ¹H-NMR (300 MHz) were recorded on a Varian AC 300 spectrometer. Chemical shifts are expressed as parts per million downfield from tetramethylsilane. Splitting patterns have been designated as follows: s (singlet), d (doublet), dd (doublet of doublet), t (triplet), dt (doublet of triplet), m (multiplet), br. (broad signal). Coupling constants (*J* values) are listed in hertz (Hz). Mass spectra were obtained with a Nermag-Ribermag R10-10C spectrometer applying a desorption chemical ionization technique using ammonia as the reagent gas. The purity of the tested products was greater than 97% as judged by HPLC analysis.

2-[6-Methoxy-3-nitro-2-(2-nitrophenyl)-4-oxo-4*H*-chromen-8-yl]acetic acid (**2**):

Yield 73%; mp 216–217 °C recrystallized from toluene as pale yellow microcrystals. IR ν_{\max} (cm⁻¹): 1711, 1669, 1532, 1516, 1474, 1342, 1191, 1142. ¹H NMR (DMSO-*d*₆) δ : 3.77 (s, 2H), 3.92 (s, 3H), 7.51 (d, 1H, *J* = 2.3 Hz), 7.56 (d, 1H, *J* = 2.3 Hz), 7.92–8.08 (m, 3H), 8.44 (d, 1H, *J* = 7.5 Hz), 12.50 (s, 1H, exchangeable with D₂O). MS (*m/z*): 401 (M + H)⁺, 418 (M + NH₄)⁺.

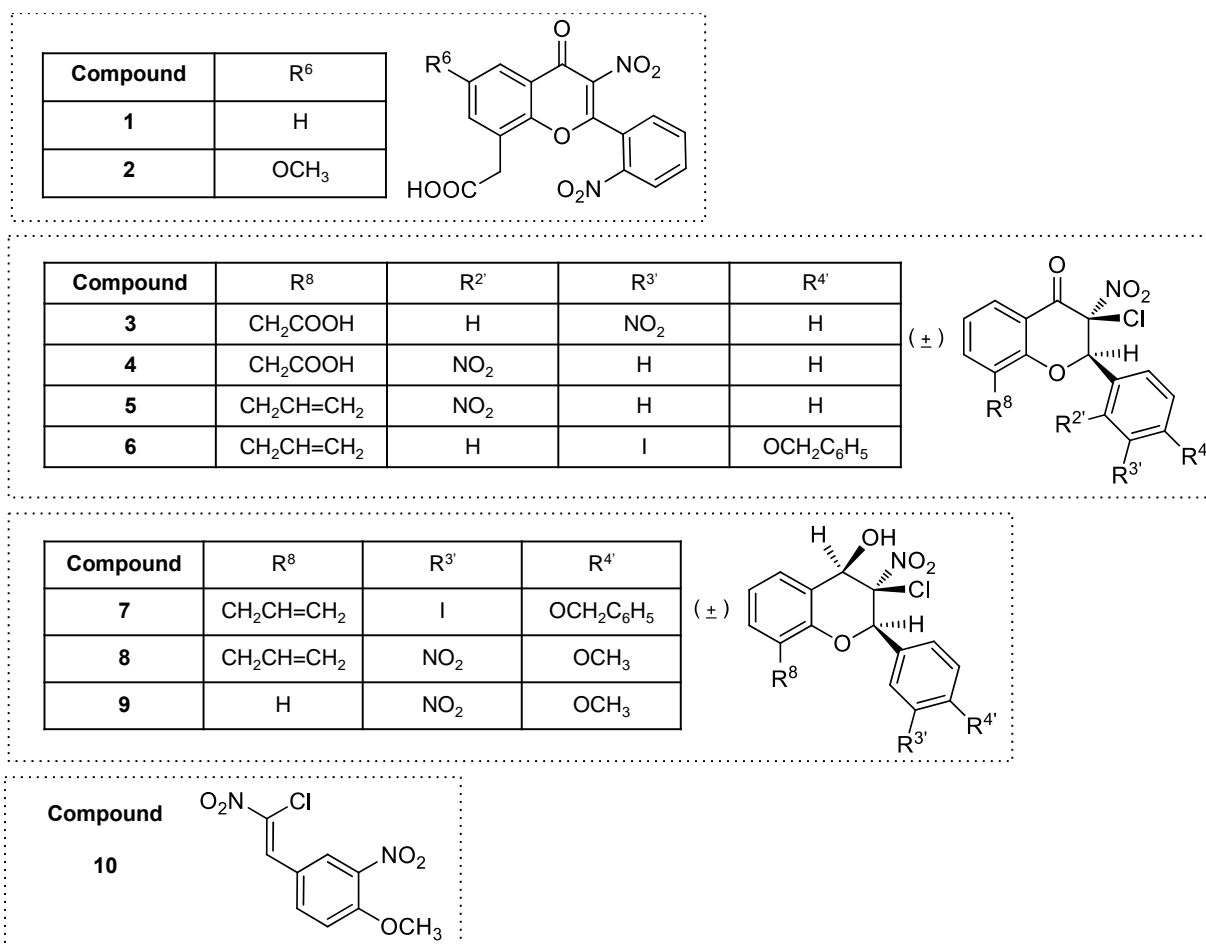


Figure 1. Chemical structures of tested compounds 1–10. All compounds were stored frozen in DMSO.

[3-Chloro-2-(3-nitrophenyl)-3-nitro-4-oxo-chroman-8-yl]acetic acid (3):

Yield 79%; mp 217–219 °C recrystallized from toluene as white microcrystals. **IR** ν_{\max} (cm⁻¹): 1709, 1577, 1533, 1477, 1455, 1351, 1279, 1223, 1215, 1189, 1073. **¹H NMR (DMSO-d₆)** δ : 3.68 (d, 1H, J = 16.7 Hz), 3.77 (d, 1H, J = 16.7 Hz), 7.13 (s, 1H), 7.36 (t, 1H, J = 7.7 Hz), 7.81–7.92 (m, 3H), 7.96 (dd, 1H, J = 8.0 and 1.5 Hz), 8.26 (br. s, 1H), 8.41 (dt, 1H, J = 7.5 and 2.0 Hz), 12.49 (s, 1H, exchangeable with D₂O). **MS** (*m/z*): 407–409 (M + H)⁺.

[3-Chloro-2-(2-nitrophenyl)-3-nitro-4-oxo-chroman-8-yl]acetic acid (4):

Yield 81%; mp 186–188 °C recrystallized from toluene as white microcrystals. **IR** ν_{\max} (cm⁻¹): 1716, 1572, 1532, 1471, 1456, 1353, 1282, 1225, 1190, 1069. **¹H NMR (DMSO-d₆)** δ : 3.66 (d, 1H, J = 16.8 Hz), 3.74 (d, 1H, J = 16.8 Hz), 7.26 (s, 1H), 7.35 (t, 1H, J = 7.7 Hz), 7.82 (d, 1H, J = 7.7 Hz), 7.84 (dt, 1H, J = 8.0 and 1.2 Hz), 7.94 (br. d, 1H, J = 8.0 Hz), 7.95 (dt, 1H, J = 7.8 and 1.0 Hz), 8.06 (dd, 1H, J = 8.0 and 1.2 Hz), 8.07 (dd, 1H, J = 7.7 and 1.2 Hz), 12.45 (s, 1H, exchangeable with D₂O). **MS** (*m/z*): 407–409 (M + H)⁺.

8-Allyl-2-[4-(benzyloxy)-3-iodophenyl]-3-chloro-3-nitrochroman-4-ol (7):

Yield 71%; mp 149–150 °C, recrystallized from a benzene/heptane mixture as pale yellow

crystals. **IR** ν_{\max} (cm^{-1}): 3601, 1596, 1560, 1497, 1491, 1452, 1388, 1336, 1313, 1293, 1280, 1257, 1209, 1154, 1108, 1092, 1056, 1044, 1023, 902. ^1H NMR (CDCl_3) δ : 2.41 (d, 1H, $J = 12.2$ Hz exchangeable with D_2O), 3.19–3.34 (m, 2H), 5.03 (dd, 1H, $J = 17.0$ and 1.5 Hz), 5.10 (dd, 1H, $J = 10.1$ and 1.5 Hz), 5.17 (s, 2H), 5.72 (s, 1H), 5.87–6.00 (m, 1H), 5.99 (d, 1H $J = 12.2$ Hz), 6.83 (d, 1H, $J = 8.6$ Hz), 7.07 (t, 1H, $J = 7.6$ Hz), 7.21 (br. d, 1H, $J = 7.6$ Hz), 7.31 (dd, 1H, $J = 8.6$ and 2.1 Hz), 7.33–7.45 (m, 4H), 7.48 (br. t, 2 H, $J = 7.0$ Hz), 7.69 (d, 1H, $J = 2.1$ Hz). MS m/z : 578–580 ($\text{M} + \text{H}$)⁺, 595–597 ($\text{M} + \text{NH}_4$)⁺.

(Z)-4-Benzyloxy-1-(2-chloro-2-nitroethenyl)-3-iodobenzene:

Yield 62%; mp 134–135 °C, recrystallized from a benzene/heptane mixture as yellow crystals. **IR** ν_{\max} (cm^{-1}): 1615, 1584, 1525, 1482, 1455, 1382, 1296, 1259, 1207, 1163, 1038, 974. ^1H NMR (CDCl_3) δ : 5.26 (s, 2H), 6.94 (d, 1H, $J = 8.7$ Hz), 7.32–7.53 (m, H), 7.85 (dd, 1H, $J = 2.1$ and 8.7 Hz), 8.27 (s, 1H), 8.36 (d, 1H, $J = 2.1$ Hz). MS m/z : 416–418 ($\text{M} + \text{H}$)⁺, 433–435 ($\text{M} + \text{NH}_4$)⁺.

The tested compounds were stored at -20 °C as DMSO stock solutions (100 mM).

2.2. Human AML cell lines and cell culture

The AML cell lines U937 (CRL-1593.2), THP-1 (202-TIB) and HL-60 (240-CCL) cells were purchased from American Type Culture Collection (ATCC). NB4 cells were obtained from Dr. Michel Lanotte's laboratory (Hôpital Saint-Louis, Paris, France) [32]. According to the French-American-British (FAB) Committee's cytological criteria, these AML cell lines were subdivided into FAB subgroups: M2/myeloblastic with maturation (HL-60), M3/promyelocytic (NB4) and M5/monoblastic (U937, THP-1). The cell lines were phenotyped for CD11b, CD11c, CD15, CD44, CD13, tested for mycoplasma every 4 months, and used within 3–10 passages. Cells were cultured in complete RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum (FCS, Gibco, Paisley, Scotland, LPS levels < 0.1 ng/mL) or 10% (THP-1), 2 mM L-glutamine, 1 mM sodium pyruvate and 40 $\mu\text{g}/\text{mL}$ gentamycin (Gibco) in a 5% CO_2 humidified atmosphere at 37 °C [33]. For every experiment, cells were harvested in log-phase proliferation. Cells ($1 \times 10^5/\text{mL}$) were treated with various concentrations (10^{-7} – 10^{-3} M) of flavonoid derivatives for 3 days. In negative control experiments, cells were treated with the appropriate volume of DMSO (vehicle) used to dissolve these molecules in the corresponding experiments.

2.3. Cell viability and proliferation

The proliferation of cell lines was evaluated by counting the number of viable cells (with diameters ranging from 9 to 14 μm) and dead cells (diameters ranging from 4 to 9 μm) in a Coulter Multisizer (Beckman-Coulter, Villepinte, France). All cultures were performed in triplicates.

2.4. Detection of cell death by flow cytometry

Cell death was assessed by using the Annexin-V-FITC/propidium iodide (PI) detection cell death kit (Beckman-Coulter), according to the manufacturer's instructions. Phosphatidylserine externalization was quantified by the specific binding of FITC-conjugated annexin-V, and cell membrane disruption was quantified by PI labelling. Stained cells (20,000) were analyzed with a flow cytometer (Beckman-Coulter).

2.5. APN activity assay

APN activity at the surface of intact cells ($3\text{--}5 \times 10^6/\text{mL}$) was assayed as previously described [34] using Ala-para-nitroanilide (Ala-pNA, Sigma, Chemical Co, St Louis, MO) (1 mg/mL) as a substrate of APN hydrolytic activity. Formation of pNA was recorded at 405 nm. Results were expressed as nmoles of pNA formed per 10^5 cells for 30 min at 37 °C. The remaining activity in the presence of tested compounds was expressed as the percentage of the control activity with DMSO.

2.6. Statistics

Data are presented as the mean \pm SD from N independent experiments. Statistical analysis was performed using the ANOVA test to compare test and control groups. The threshold for statistical significance was set to $p < 0.05$.

3. Results

3.1. Effects of compounds on APN/CD13 activity of U937 cells

Surface CD13 is overexpressed on AML (monoblastic M5) U937 cells [24]. Our previous study evidenced compound **1** as a specific inhibitor of APN/CD13 in U937 cells, and emphasized the importance of the NO₂ group in the 3-position and the CH₂COOH group in the 8-position of the benzyl ring for efficient inhibition of APN activity [18]. Herein, we first assessed the ability of compounds **1–9** (100 μM) to inhibit APN/CD13 enzymatic activity expressed by intact U937 cells (Figure 2A). Our results indicated that compound **2** (with a methoxy group in the 6-position) was still capable of inhibiting APN activity (Figure 2A). In contrast, compounds **3–6** which possess geminal NO₂ and Cl group in the 3-position of the 2*H*-1-benzopyran ring, were without inhibitory effect on APN activity (Figure 2A) confirming the importance of the NO₂ group at the 3-position. The other substitutions in the 8-, 2'-, 3'- and 4'-positions (compounds **3–6**) did not affect APN activity (Figure 2A). Similarly, compounds **7–9** (also built on the 2*H*-1-benzopyran scaffold) exhibited no significant effects on APN activity (Figure 2A). The IC₅₀ values of APN activity inhibition for compounds **1** and **2** are about 50 μM and 100 μM respectively (Figure 2B), emphasizing the importance to keep unsubstituted the 6-position. The other AML cell lines HL-60 (myeloblastic, M2), NB4 (promyelocytic, M3) and THP-1 (monoblastic, M5) express surface CD13 [26]; compounds **1** and **2** were also capable of inhibiting APN/CD13 activity at the surface of all cell lines (data not shown).

3.2. Inhibitory effects of compounds on U937 cell proliferation

The effects of compounds were evaluated on the proliferation of U937 cells cultured in the presence of a range of concentrations (1–100 μM) of compounds or with vehicle (DMSO). When compared with untreated cells, DMSO did not affect cell proliferation. A treatment period of 3 days was selected since DMSO-treated cells were still in the linear growth phase at this time. The flavones **1** and **2** (up to 100 μM) did not affect the proliferation of U937 cells after 3 days of treatment when compared with vehicle (Figure 3A). In contrast, cell proliferation was dose-dependently reduced in cell samples treated with the 2,3-dihydro-2-phenyl-4*H*-1-benzopyran-4-ones **3** and **4** (Figure 3A).

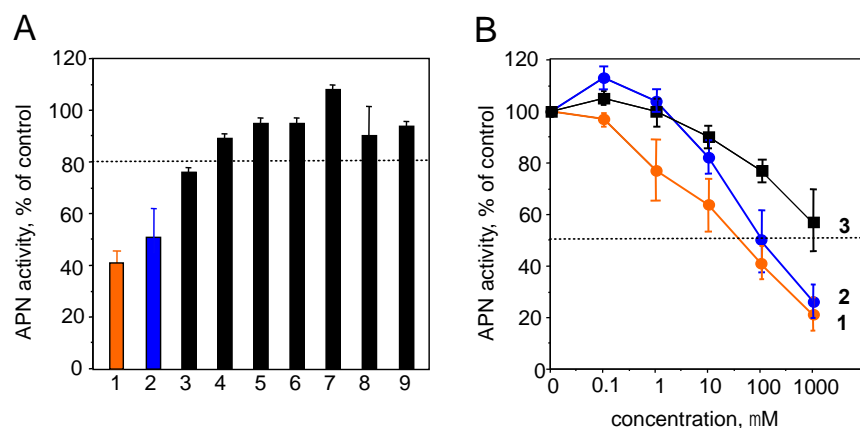


Figure 2. Effects of compounds on APN/CD13 activity of U937 cells. A) Cells were assayed for APN activity using the chromogenic substrate Ala-pNA, in the presence of DMSO (control) or compounds 1–9 (100 μ M), as described in the Materials and Methods section. Results are expressed as percent APN activity relative to APN activity of control cells (100% values were 12.5 ± 1.1 nmol/30 min/ 10^5 cells at 37 $^{\circ}$ C). Data represent the means of 3 independent determinations \pm SD. B) Dose-response inhibition of APN activity by compounds 1–3. Values correspond to the mean of 3 independent experiments \pm SD.

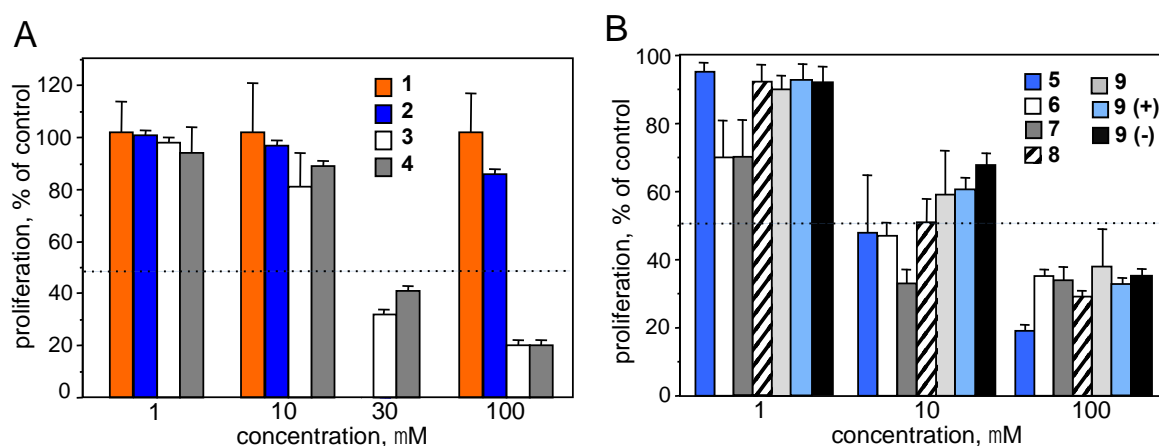


Figure 3. Dose-dependent effects of compounds on U937 cell proliferation. Cells (10^5 /mL) were cultured for 3 days in the presence of DMSO (control) or increasing concentrations (1–100 μ M) of compounds 1–9 and enantiomers 9 (+) and 9 (–). After 3 days, living cells were counted with a cell Coulter as described in Methods. Results are expressed as percent cell proliferation relative to the proliferation of control cells. Data represent the means of 3 independent determinations \pm SD.

The IC_{50} values of growth inhibition for both isomers were about 20 μ M. Moreover, cell proliferation was inhibited almost to the same level by the other 2-phenylbenzopyran derivatives 5–9 in a dose dependent-manner (Figure 3B). The IC_{50} values of growth inhibition for these compounds

were about 10 μM . The comparison with compounds **1** and **2** which do not show any influence on U937 cell proliferation in the same range of concentration (up to 100 μM), suggests that the NO_2 and Cl substituents in the 3-position of the 2,3-dihydro-2-phenyl-4*H*-1-benzopyran-4-one scaffold (compounds **3–6**) or in the 3-position of the 3,4-dihydro-4-hydroxy-2-phenyl-2*H*-1-benzopyran scaffold (compounds **7–9**) play a critical role in cell growth arrest.

Compounds **3–9** were synthesized as racemic (\pm) molecules. Whether (+) and/or (–) enantiomers were involved in the antiproliferative action of these compounds is unknown. Following enantioseparation of compound **9**, we evaluated the effects of compounds **9** (+) and **9** (–) on U937 cell proliferation. As shown in Figure 3B, both enantiomers retained the ability to block cell growth at the same level. Additional studies are warranted to investigate the inhibitory effects of enantiomers of compounds **3–8**.

3.3. Inducing lethal effects of compounds in U937 cells

Whether cell growth arrest induced by these molecules was related to cell death was next investigated. Cell death was assessed by determining phosphatidylserine (PS) exposure at the cell surface (using annexin-V-FITC binding) and cell membrane disruption (using propidium iodide/PI labelling). Figure 4 shows representative experiments in which U937 cells were cultured for 3 days in the absence or presence of all compounds at the dose of 10 μM (and 30 μM for isomers **3** and **4**). U937 cells treated with DMSO (Figure 4A, control) or 10 μM of compounds **1–4** (Figure 4A) were found weakly positive for Annexin V (gate L4) and for both Annexin V and PI (gate L2). In contrast, after treatment with compounds **5–9** at the dose of 10 μM , U937 cells became strongly positive for Annexin V (L4, dying cells) and for both Annexin V and PI (L2, dying cells which become secondary necrotic cells) (Figure 4B). Moreover, the isomers **3** and **4** at the dose of 30 μM induced cell death (Figure 4C). The lethal effects of all compounds were both time- and dose-dependent (data not shown).

In a weakly basic aqueous medium, compounds **7–9** could be quantitatively decomposed into the corresponding salicylaldehydes and (*Z*)-4-(2-chloro-2-nitroethenyl)benzenes (retro-reaction). Here, we showed, in the particular case of compound **9**, that the methoxy derivative **10** (10 μM) and salicylaldehyde neither affected the proliferation nor survival of U937 cells after 3 days of treatment (Figure 5). This result indicates that the lethal effects of 3-chloro-3,4-dihydro-4-hydroxy-3-nitro-2-phenyl-2*H*-1-benzopyran compounds (**7–9**) on U937 cells were specific and not related to degradation products.

Caspases are important mediators of cell death [35,36]. In order to establish whether caspases are activated during compound-mediated cell death, we examined the effects of the broad-spectrum caspase inhibitor Z-VAD-fmk on cell death (as determined by annexin-V-FITC binding). Compound **6**-mediated cell death was markedly blocked by 50 μM zVAD-fmk (>60% inhibition). However, Z-VAD-fmk did not block cell death mediated by the other molecules tested (compounds **3–5** and **7–9**).

We also determined the effects of compound **9** at concentrations 20 and 40 μM on the viability and proliferation of HL-60, NB4 and THP-1 cells compared with U937 cells. Compound **9** consistently blocked at day 3 proliferation of all AML cell lines at 20 and 40 μM compared with vehicle (Figure 6A) resulting in cell death (over 40% at 40 μM) (Figure 6B). As expected, compounds **1** and **2** did not affect the proliferation and survival of these cell lines (data not shown).

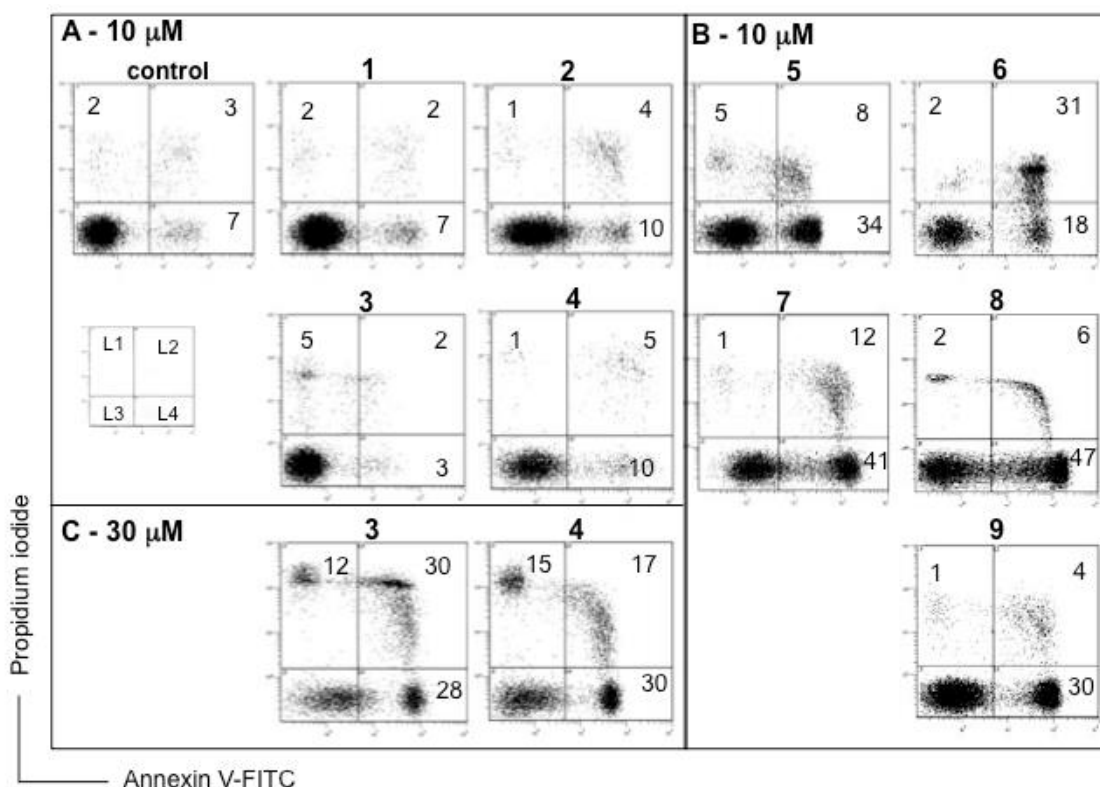


Figure 4. Representative histograms showing flow cytometry analysis of Annexin V-FITC/PI stained U937 cells before or after treatment with compounds. Cells ($10^5/\text{mL}$) were cultured in the presence of DMSO (control) or compounds 1–9 (A and B, $10\ \mu\text{M}$; C, $30\ \mu\text{M}$) on U937 cells for 3 days. Results are expressed as log propidium iodide fluorescence intensity (y-axis) vs log Annexin V fluorescence intensity (x-axis). L1, PI positive cells; L2, Annexin-V/PI positive cells; L3, intact cells; L4, Annexin-V positive cells. The quoted values correspond to the percentage of positive cells. One of 3 representative experiments is shown.

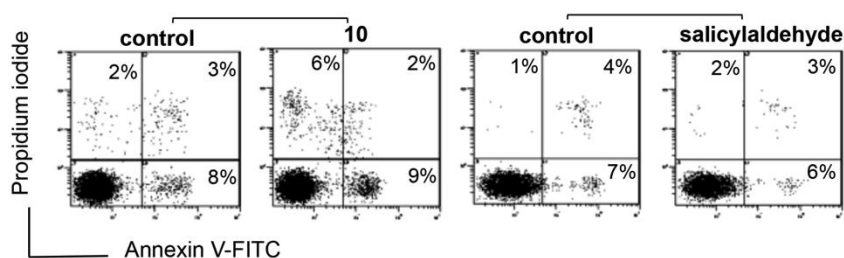


Figure 5. Representative histograms showing flow cytometry analysis of Annexin V-FITC/PI stained U937 cells before or after treatment with salicylaldehyde and compound 10. Cells ($10^5/\text{mL}$) were cultured in the presence of DMSO (control) or salicylaldehyde or compound 10 ($10\ \mu\text{M}$) on U937 cells for 3 days. Results are expressed as log propidium iodide fluorescence intensity (y-axis) vs log Annexin V fluorescence intensity (x-axis). L1, PI positive cells; L2, Annexin-V/PI positive cells; L3, intact cells; L4, Annexin-V positive cells. The quoted values correspond to the percentage of positive cells.

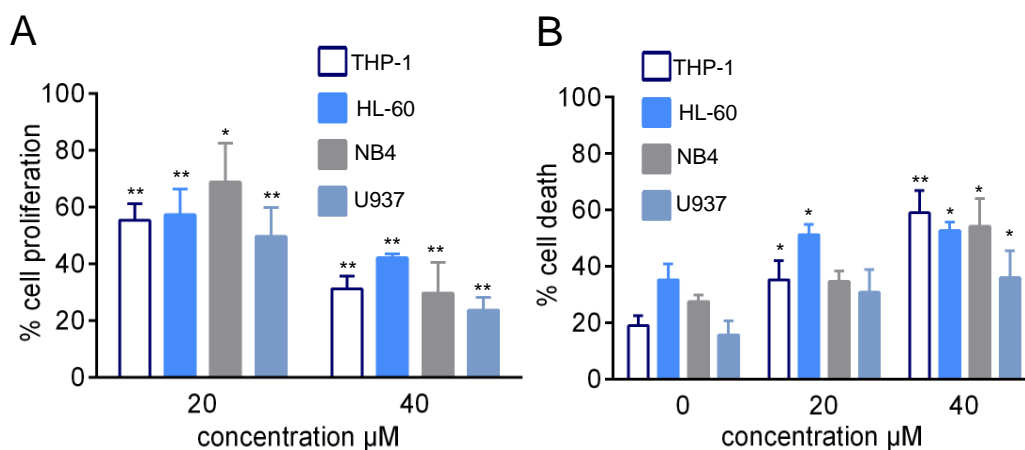


Figure 6. Comparative effects of compound 9 on the growth and survival of U937, NB4, HL-60 and THP-1 cell lines. Cells ($10^5/\text{mL}$) were cultured in the presence of DMSO (control) or compound 9 (20 μM) on the AML cell lines U937, THP-1, NB4 and HL-60 for 3 days. Results are expressed as A) percent cell proliferation relative to control cells, and B) percent cell death relative to total (control or compound-treated) cells. Data represent the means of 3 independent determinations \pm SD. * $p < 0.05$ and ** $p < 0.01$ compared with control cells.

4. Discussion

Our present study provides information about the influence of novel flavonoid derivatives in human AML cell lines which are representative of primary AML cells accordingly to the FAB classification. These are HL-60 (FAB M2), NB4 (FAB M3), U937 and THP-1 (FAB M5) cell lines. The flavonoid derivatives 1–9 have backbones close to that of 2',3-dinitroflavone-8-acetic acid (1) previously identified by our group as a non-cytotoxic inhibitor of APN/CD13 activity in AML U937 cells [18]. Our results are summarized in Table 1, and a model for the action of compounds 1–9 is proposed in Figure 7.

We first report the capacity of compound 2 to inhibit, like 1, the enzymatic activity of APN/CD13 expressed by AML cell lines. Our previous study emphasized on the importance of the presence, in the flavone-8-acetic scaffold, of a CH_2COOH group in the 8-position and of two NO_2 substituents in the 2'- and 3-positions, respectively [18]. Herein, we show that insertion of the OCH_3 group in the 6-position leads to a lowered inhibitory effect against APN activity, emphasizing the importance to keep unsubstituted the 6-position. Furthermore, we showed that compound 2, like compound 1 [18], is a reversible competitive inhibitor of APN/CD13 (data not shown).

Previous studies suggested that APN/CD13 can influence cell growth and survival in various cellular systems [21,37-39]. CD13's involvement in these processes has been demonstrated by APN inhibitors [21,38-40]. However, these effects remain controversial because high doses of the inhibitors used might induce cytotoxicity in a non-specific manner [21,40]. For example, interaction of APN/CD13 with doses $\geq 200 \mu\text{M}$ of bestatin or actinonin (the most frequently used APN inhibitors) induces death in various cell types including AML cell lines [21,41]. It is noteworthy that the inhibitory effects observed with high doses of these inhibitors likely reflect their intracellular effects [42]. Moreover, bestatin and actinonin may inhibit other aminopeptidase activities [21,42].

Table 1. Summary of the effects of flavonoid derivatives on the growth and survival of U937 cells, and associated APN/CD13 enzymatic activity.

Compound	A) APN/CD13 activity inhibition (IC ₅₀ , μM)	B) Cell growth inhibition (IC ₅₀ , μM)	C) Cell death induction
DMSO	–	–	–
1	+ (50)	–	–
2	+ (100)	–	–
3	–	+ (20)	+
4	–	+ (20)	+
5	–	+ (10)	+
6	–	+ (10)	+
7	–	+ (<10)	+
8	–	+ (10)	+
9	–	+ (10)	+
10	–	–	–
Salicylaldehyde	–	–	–

A) Inhibition of APN activity expressed by U937 cells in the presence of tested compounds (10^{-7} – 10^{-3} M). IC₅₀ values were calculated from inhibition curves in Figure 2B and 2C) U937 cells were cultured for 3 days in the presence of DMSO (control) or increasing concentrations (10^{-6} – 10^{-4} M) of compounds. IC₅₀ values were calculated from inhibition curves in Figure 3. Cell death was assessed from flow cytometry analysis of Annexin V-FITC/PI as shown in Figures 4 and 5. +, effect; –, no effect.

When used at a concentration of 100 μM which inhibits APN activity (> 50%), compounds **1** and **2** did not alter cell growth and survival—suggesting that CD13's APN activity is not required for these processes. In accordance, the study of Winnicka et al showed that CD13 was not involved in the modulation of hematopoietic cell growth in the mouse [43]. In the hematopoietic system, CD13 is expressed specifically on the earliest cell committed to the myeloid lineage and all of its differentiated progeny, as well as on subsets of peripheral blood progenitor cells [21]. Winnicka et al showed an indistinguishable distribution of cells of the myeloid, B and T lineages in wild-type and CD13 null adult mice, as well as no difference in total colony-forming ability of precursors from the wild-type versus CD13 null animals [43]. Thus, we describe the ability of compounds **3–9** (which do not inhibit APN activity) to inhibit in a concentration-dependent manner the growth of AML U937 cells *in vitro*. Flavones (natural and synthetic) may exhibit lethal actions on various models of human cancers [6,10]. Our experiments indicate that U937 cell growth arrest mediated by compounds **3–9** is accompanied by induction of cell death. The presence of geminal NO₂ and Cl substituents at the 3-position of the 3-chloro-2,3-dihydro-3-nitro-2-phenyl-4H-1-benzopyran-4-ones **3–6** or in the 3-position of the 3-chloro-2,3-dihydro-3-nitro-2-phenyl-4H-1-benzopyran-4-ones **7–9** appeared associated with cell death. In contrast, replacement of the CH₂COOH group at the 8-position by a CH₂CH=CH₂ group (compounds **4–7**), the absence of the NO₂ group in the 2'-position (compounds **3, 6–9**) or the presence of a NO₂ group at the 3'-position (compounds **3, 8** and **9**) did not affect the death process. Importantly, we showed that the cellular effects of compounds **7–9** were not due to their potential decomposition in weak basic medium into compound **10** and salicylaldehyde. Finally, results obtained with compound **9** in the U937 cell line were observed in HL-60, NB4 and THP-1 cell lines. It remains to be seen whether these cells, like U937 cells, respond to the lethal effects of compounds **3–8**.

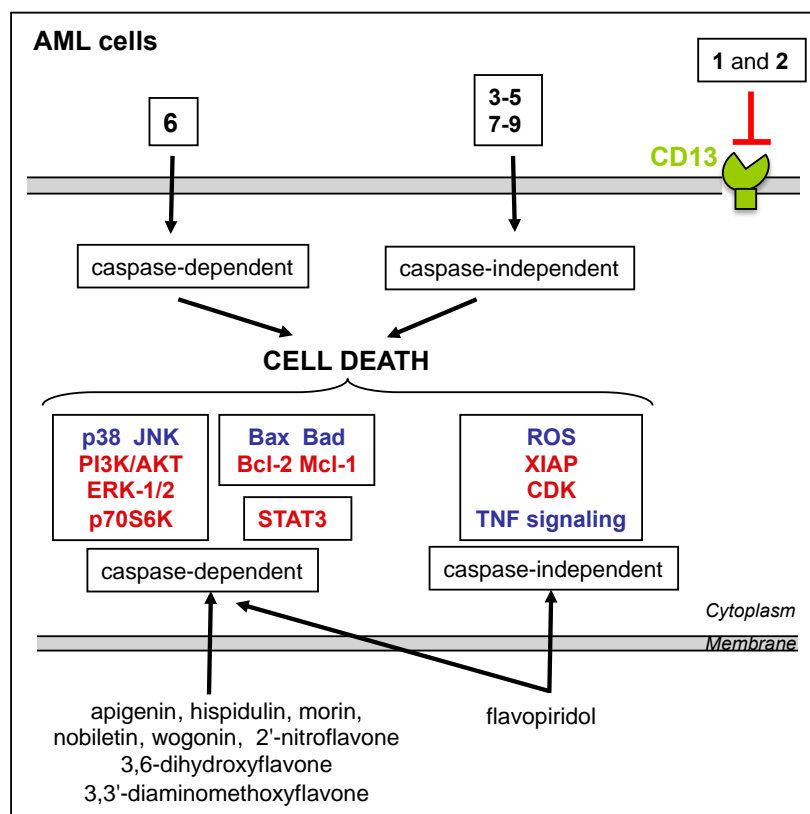


Figure 7. Putative model for the activity profiles of flavonoid derivatives in AML cells.

Compounds **1** and **2** bind to surface APN/CD13, and then inhibit its catalytic activity. Compounds **3–10** induce caspase-dependent (**6**) and caspase-independent (**3–5**, **7–9**) death in AML cells. The molecular mechanisms are not known. The cell signalling pathways associated with the induction of AML cell death by other flavonoids (including apigenin, hispidulin, flavopiridol, nobiletin, quercetin, wogonin, 2'-nitroflavone, 3,6-dihydroxyflavone, 3,3'-diaminomethoxyflavone) are shown. Induction of cell death by (i) activation of pro-apoptotic proteins (Bax and Bad) and repression of anti-apoptotic proteins (Bcl-2 and Mcl-1), (ii) inhibition of the PI3K/AKT, ERK1/2 and STAT3 signaling pathways, (iii) dephosphorylation of p70S6K, and (iv) activation of the p38 and JNK pathways. Red = downregulation; blue = upregulation.

We started to investigate the signalling pathway underlying U937 cell death induced by compounds **3–9**. The broad-spectrum caspase inhibitor Z-VAD-fmk was found to block U937 cell death mediated by compound **6**, thus indicating that this molecule induced a caspase-dependent death response. Caspase-dependent apoptotic processes can be achieved through death receptors (in the extrinsic pathway) or the mitochondria (in the intrinsic pathway) [36,44,45]. In the intrinsic apoptotic pathway, mitochondria may function as an amplifier by activating the downstream caspases-9 and -3 [36,44]. In the extrinsic apoptosis pathway, triggering of death receptors (such as Fas antigen and TNF-Receptor 1) leads to strong caspase-8 activation. This bypasses mitochondria and leads directly to caspase-3 activation and then apoptosis [36,44]. Another mode of cell death, necroptosis, is also defined by caspase-8 activity [46,47]. Conversely, the lack of effect of Z-VAD-fmk on cell death mediated by compounds **3–5** and **7–9** indicates that these molecules do not induce AML cell death through the caspase-dependent pathways. Various types of caspase-independent cell death have been

identified including parthanatos, AIF-mediated programmed necrosis and mitochondrial permeability transition (MPT)-dependent regulated necrosis [45,48-50]. Further research is required to clarify the mechanisms of AML cell death by these compounds **3–9**.

During the process of our study, several papers reported the ability of other flavonoids to induce cell death in AML cell lines through regulation of different signal transduction cascades. A non-exhaustive summary of the mechanisms of action of these flavonoids is added in Figure 7. For example, flavopiridol [11,12], quercetin [11,13,51,52], wogonin [53,54], nobiletin [55], apigenin [56,57], morin [58], hispidulin [59], 2'-nitroflavone [60] and 3,6-dihydroxyflavone [61] were shown to induce caspase-dependent AML cell death associated (i) with the inhibition of the PI3K/AKT and ERK1/2 signaling pathways (apigenin, hispidulin, flavopiridol, nobiletin, quercetin, wogonin, 2'-nitroflavone), (ii) the inhibition of the STAT3 signaling pathway (hispidulin), (iii) the activation of the p38 and/or JNK pathways (apigenin, flavopiridol, nobiletin, 2'-nitroflavone, 3,6-dihydroxyflavone), and (iv) the change of expression of pro-apoptotic (Bad, Bax) and/or anti-apoptotic (Bcl-2, Mcl-1) members of the Bcl-2 family (apigenin, flavopiridol, quercetin, wogonin, morin, 2'-nitroflavone). Our laboratory previously showed the concerted effects of 3,3'-diamino-4'-methoxyflavone on proteasome inhibition and activation of caspase-dependent cascades (with Bax activation and P70S6K degradation) in the induction of apoptosis in AML cells [20]. Whether the apoptotic effect of compound **6** in AML cells could be mediated at least by one of these molecular mechanisms remains to be determined. In addition, flavopiridol has been shown to induce caspase-independent cell death by proceeding through multiple targets (including cyclin-dependent kinase inhibition, downregulation of XIAP, and interference with the TNF signaling pathway) [12-14,62]. Outside of the hematopoietic system, flavonoids including quercetin and (-)-epigallocatechin gallate (EGCG) have been reported to induce cell death through a caspase-independent pathway [63-67]. Further investigation will be essential to determine the molecular mechanisms related to caspase-independent AML cell death mediated by compounds **3–5** and **7–9**.

Flavone acetic acid was the first synthetic flavonoid shown to exhibit anti-angiogenic properties in animal studies [68]. Several studies have highlighted the anti-angiogenic effects of flavonoids in human cancer treatment by modulating signalling pathways and inhibiting the activity and/or expression of metalloproteinases (MMPs) [69-72]. For example, EGCG inhibits MMPs -2, -3 and -9 through direct binding to the catalytic site and gene expression repression [73]. Most MMPs are secreted as proforms [74]. There is now growing evidence pointing the ability of proMMP-2 and proMMP-9 to directly activate classical signalling pathways involved in cell growth, survival, migration and angiogenesis [74]. AML is associated with an increased angiogenesis which disappears if complete hematological remission is achieved [75,76]. Secreted proMMP-2 and proMMP-9 are abnormally expressed in human AML [77-79]. By ELISA analysis, we showed that unstimulated U937 cells secreted very low levels of proMMP-2 (142 ± 8 pg/mL/ 10^5 cells) and proMMP-9 (50 ± 8 pg/mL/ 10^5 cells) which were not altered by compounds **1–9**. Taken as a whole, our data suggest that these compounds are not good candidates as anti-angiogenic inhibitors in AML. We previously showed that compound **1** did not alter the gelatinolytic activity of MMP-2/-9 [18]. It remains to examine the effects of other compounds on MMP-2/-9 activities.

In conclusion, this work provides first evidence that a novel series of benzopyran derivatives (compounds **3–9**) may exhibit antitumor activity against AML cell lines by inducing cell death. These molecules may therefore represent valuable tools for investigating mechanisms involved in these

processes. Further studies are required to validate our observations with these compounds in AML patients' cells.

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Conflicts of interest

The authors declare no conflicts of interest.

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