ANTICANCER RESEARCH *37*: 5225-5233 (2017)
doi:10.21873/anticanres.11946

Anti-proliferative, Cytotoxic and NF-κB Inhibitory Properties of Spiro(Lactone-Cyclohexanone) Compounds in Human Leukemia

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Abstract. *Background/Aim: NF-κB affects most aspects of cellular physiology. Deregulation of NF-κB signaling is associated with inflammatory diseases and cancer. In this study, we evaluated the cytotoxic and NF-κB inhibition potential of new spiro(lactone-cyclohexanone) compounds in two different human leukemia cell lines (U937 and K562). Materials and Methods: The anti-proliferative effects of the spiro(lactone-cyclohexanone) compounds on human K562 and U937 cell lines was evaluated by trypan blue staining, as well as their involvement in NF-kB regulation were analyzed by luciferase reporter gene assay, Caspase-3/7 activities were evaluated to analyze apoptosis induction. Results: Both spiro(coumarin-cyclohexanone)* ***4*** *and spiro(6- methyllactone-cyclohexanone)* ***9*** *down-regulated cancer cell viability and proliferation. Compound* ***4*** *inhibited TNF-α-induced NF-κB activation in a dose-dependent manner and induced caspase-dependent apoptosis in both leukemia cell lines. Conclusion: Results show that compound* ***4*** *and compound* ***9*** *have potential as anti-cancer agents. In addition, compound* ***4*** *exerted NF-kB inhibition activity in leukemia cancer cells.*

After over half a century of chemotherapy research, cancer remains one of the most difficult diseases to treat; a consequence of factors that include limitations of animal models, tumor diversity, drug resistance and the side effects of therapy (1). Since various side-effects are frequently reported for the anticancer drugs currently used in therapy, there is a growing trend towards discovery of new drug scaffolds from natural origins able to inhibit the key hallmarks of cancer (2-6).

Nuclear factor-κB (NF-κB) is a dimeric transcription factor composed of subunits that belong to the Rel family (p105/50, p100/52, p65 (RelA), RelB, and c-Rel) (7). NF-κB is regulated through an N-terminal dimerization/DNA-binding domain known as the Rel Homology domain (RHD). NF-κB affects most aspects of cellular physiology from immunity and inflammation to apoptosis, cell survival, growth, and proliferation. However, deregulation of the corresponding cell signalling pathway is associated with diseases such as chronic inflammation, immunodeficiency and cancer (8, 9). Consequently, inhibition of NF-κB has been considered a novel strategy to improve cancer chemotherapy (10-13).

Plants have always been an excellent source of biologically active agents used in traditional medicine. Spiro-cyclic compounds, lactones and coumarins represent interesting groups of plant-derived chemotypes with anticancer potential (14, 15). Natural and synthetic coumarins have attracted great attention due to their wide range of biological properties, such as anticancer (16-18), anti-mutagenic (19), anti-inflammatory (20), antibacterial (21) and anti-fungal (22) activities.

Spiro-lactones are scaffolds (Figure 1) encountered in a wide range of natural products from plants, fungi, insect secretions, shellfish toxins and other living organisms (23-27). Many of these compounds display diverse biological properties such as attractant, antimicrobial, cytostatic or cytotoxic activities, and are usually used as antibiotic agents (28-30). Important naturally-occurring templates of spiro-

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Key Words: Spiro(lactone-cyclohexanones), anti-cancer, cytotoxicity, anti-proliferation, nuclear factor-κB, apoptosis.

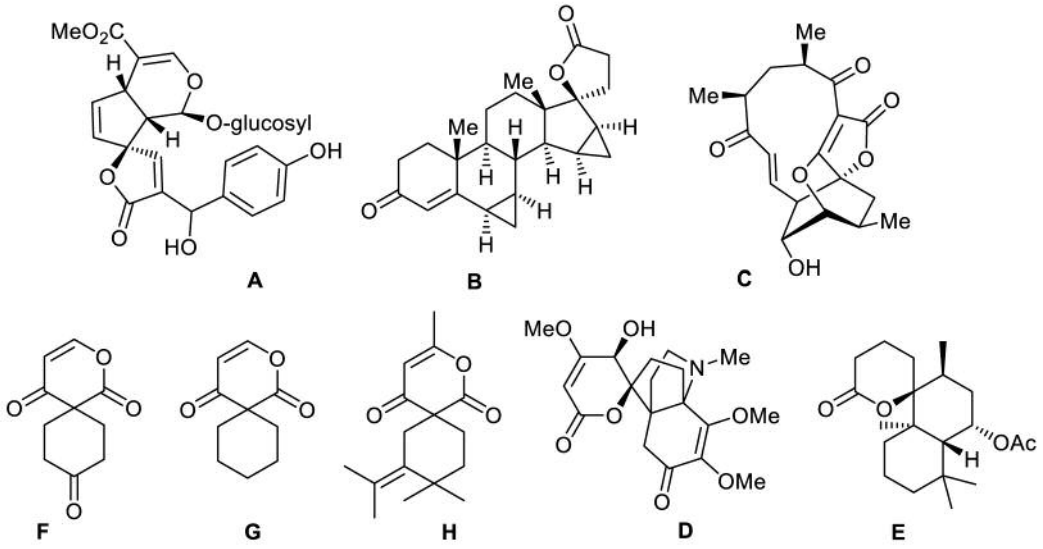

Figure 1. *Natural and synthetic lactone-containing spiro-compounds.*

compounds are reported; for instance gaertneroside (A), an inhibitor of the classical pathway activation of the complement system (31, 32), drospirenone (B) a component of certain birth control formulations (33), and abyssomicin C (C), a potent antibacterial agent used against Gram-positive bacteria (34, 35) (Figure 1). Synthetic derivatives deserved more interest (36, 37), especially the spiro-lactone basic skeletons which serve for the total synthesis of bioactive natural products and pharmaceuticals, including variecolin (38), mevinolin (39), biyouyanagin A (40) and (–)-β-vetivone (41). A large number of natural and synthetic products bearing a spiro-lactone moiety as a key structural element in their molecular scaffold displayed a multitude of biological activities (42-47). Apart from others, this structural moiety is the most important component of the molecular framework of the recently isolated alkaloid hypserpine (D) (48) and of the norlabdane-type diterpene, vitexifolin E (E) (49, 50) (Figure 1).

To the best of our knowledge, there is no natural compound presenting the 2-oxaspiro(5.5)undec-3-ene-1,5,9-trione nucleus (F). However, the 2-oxaspiro(5.5)undec-3-ene-1,5-dione (G) can be found in the commercially available derivative, 3,9,9-trimethyl-8-(propan-2-ylidene)-2-oxaspiro(5.5)undec-3-ene-1,5-dione (H) (Figure 1). In view of our long-standing interest in the chemistry of oxygen heterocyclic spiro-compounds (51, 52), recently we developed a methodology towards the synthesis of spiro(lactone-cyclohexanone) derivatives based on two consecutive 1,4-conjugate (Michael) addition of 1,3-dicarbonyl lactones such as 4-hydroxycoumarin or triacetic lactone on dibenzylideneacetone derivatives (submitted for publication). In this study, we evaluated the cytotoxic and NF-κB inhibition potential of our new spiro(lactone-cyclohexanone) compounds in two different human leukemia cell lines (U937 and K562), hypothesizing that the spiro-lactone moiety may play an important role as a main structural determinant of anti-cancer activity.

Materials and Methods

Compounds. The spiro-compounds **4-9** (Table I) were solubilized in DMSO to obtain a stock concentration of 100 μM. The stock solution was stored at –20˚C and prior to experiments further diluted in DMSO to obtain working aliquots. Control cells were treated with equivalent amounts of DMSO.

Cell culture. Human K562 (chronic myelogenous leukemia) and U937 (acute myelogenous leukemia) cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and a 1% (v/v) antibiotic–antimycotic in a 37˚C, 5% CO_2 humidified atmosphere. The cells were harvested every 3 days. After thawing, the cells were kept in normal culture conditions for 10 days before experiments.

Cell viability assessment. K562 and U937 cells were incubated with different concentrations of spiro-compounds **4-9** for 8, 24, 48 and 72 h periods. K562 and U937 cell viability were assessed by the trypan blue exclusion test, where 20 μl of cell suspension were mixed with an equal volume of 0.4% Trypan blue. The total number of vital cells was then counted using a haemocytometer under light microscopy.

Transient transfection and luciferase reporter gene assay. K562 cells were transiently transfected as described previously (53).

Table I. *Structures of spiro(lactone-cyclohexanones)* ***4-9***.

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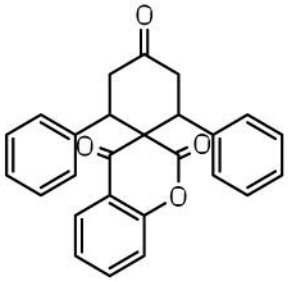

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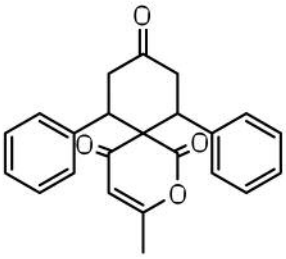

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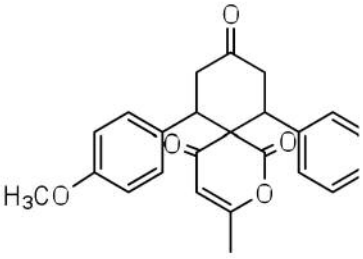

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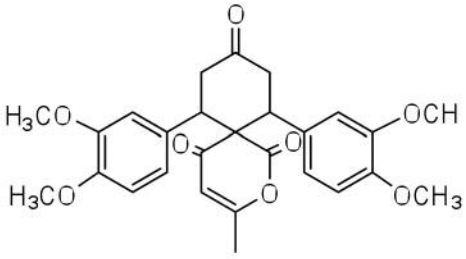

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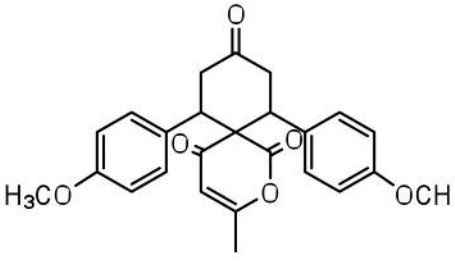

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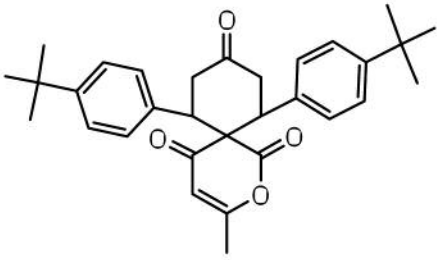

Briefly, for each experiment, 3.75×10^6 cells at a concentration of 1.5×10^7 cells/mL were electroporated at 250 V and 500 μF in the presence of 5 μg of a luciferase reporter gene construct containing five repeats of a consensus nuclear factor-κB (NF-κB) site and 5 μg of *Renilla* luciferase plasmid. 24 h after transfection, cells were re-suspended in a growth medium to a final concentration of 10^6 cells/ml and treated for 2 h with compound **4** or **9** at indicated concentrations followed by 6 h of activation with 20 ng/ml of TNF-α. Goniothalamin was used as a positive inhibitory control at a concentration of 7 μM (corresponding to IC_{50} value) (54). After the treatment, 75 μl of cell culture were incubated with 75 μl Dual-Glo™ Luciferase Reagent (Promega, Seoul, Republic of Korea) for 10 min at 22˚C. After the first measurement, 75 μl Dual-Glo™ Stop&Glo Reagent (Promega, Seoul, Republic of Korea) were added to the cells and incubated for an additional 10 min at 22˚C. Luciferase and *Renilla* activities were measured by an Orion microplate luminometer (Berthold detection systems). Results are expressed as a ratio of arbitrary units of firefly luciferase activity normalized to *Renilla* activity.

Caspase assay. Measurement of caspase 3/7 activities were assessed by homogeneous Caspase-Glo®3/7 luminescent assay (Promega, Seoul, Republic of Korea) following the manufacturer's guidelines. Briefly, 3×10^5 cells/mL were pre-treated or not for 1 h with 50 μM of Caspase Inhibitor I (Z-VAD (OMe)-FMK, Calbiochem), following treatments with compound **4** at 10, 30, 50, 70 and 100 μM for 24 h. After treatments, 75 mL of cell culture were mixed with 75 ml of Caspase- Glo®3/7 reagent and incubated at room temperature for 1 h. Luminescence is proportional to the amount of caspase activity present. The results are expressed as fold change compared to the control.

Statistics. Significant differences were determined using One-way Anova, Dunnett's multiple comparisons test for luciferase and caspase assays. Two-way Anova, Dunnett's multiple comparisons test were done for proliferation and viability assays. Statistical significances were evaluated at *p*-values below 0.05 and represented by the following legend: $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$. IC_{50} calculation was performed with Graphpad Prism software.

Results

Chemistry. We developed a simple and one-pot protocol towards the synthesis of spiro(lactone-cyclohexanone) derivatives **4-9**, which consists in an organobase (4-pyrrolidinopyridine), and catalysed two consecutive 1,4-conjugate addition of 4-hydroxycoumarin **1** or triacetic lactone **2** on dibenzylideneacetones **3a-e** (Table I, Figure 2) (submitted for publication).

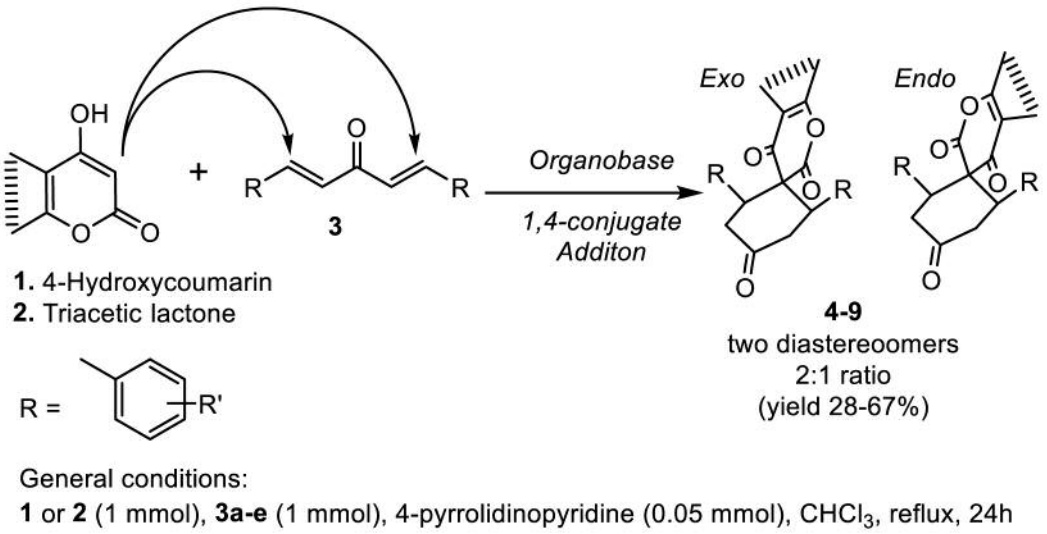

Figure 2. *Reaction pathway for the synthesis of spiro(lactone-cyclohexanone)* ***4-9***.

The NMR spectra of compounds **4-9** confirm the presence of two diastereomers existing in a 1:2 ratio, namely endo/exo respectively, which are associated with the two possible orientations of the oxygen atom in the lactone subunit relative to the cyclohexanone part (Table I). Although several asymmetric carbons can be observed in our spiro(lactone-cyclohexanone) structure, the latter is present in a "meso" form causing a total absence of chirality due to the bilateral symmetry of the whole scaffold. This fact was confirmed by chiral-HPLC analysis, showing therefore the presence of the expected endo/exo diastereomers exactly in the same 1:2 ratio as demonstrated by NMR. In the case of compound **6**, chiral-HPLC analysis revealed a racemic mixture of enantiomers due to the absence of symmetry in such scaffold caused by the difference in substitution of both phenyl groups attached to the cyclohexanone unit (Figure 3).

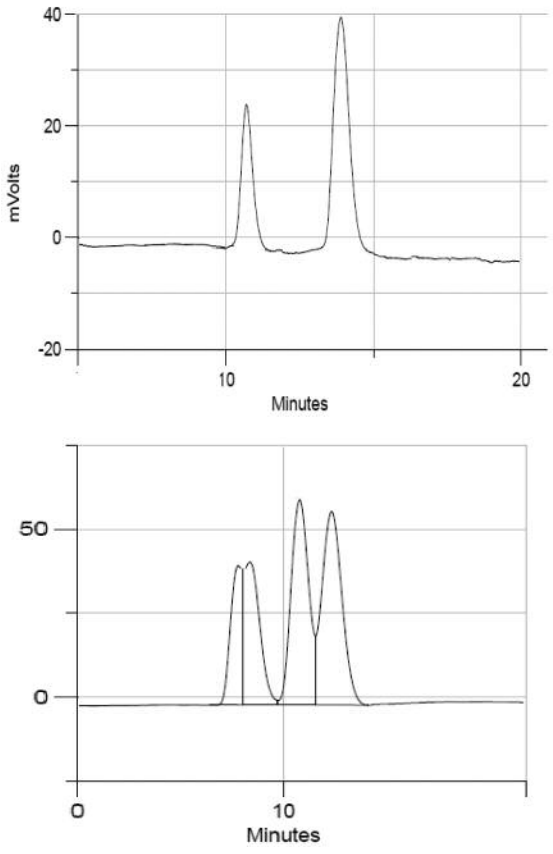

Figure 3. *Chiral-HPLC separation of spiro(lactone-cyclohexanone) endo and exo-diastereomers of the racemates for compound* ***6*** *(right) and meso-compound* ***8*** *(left). Conditions:CHIRALCEL® OD column (particle size 10 μm, 250×4.6 mm ID) at 25°C; mobile phase -hexane/THF (isocratic mode, 80:20 (v/v)) at a flow rate of 1.0 ml/min; UV detector set at 254 nm.*

Biological assays

Compounds ***4*** *and* ***9*** *affect cancer cell proliferation and viability*. The anti-cancer activities of the six spiro-compounds **4-9** were assessed in human chronic myeloid leukemia (K562) and acute myeloid leukemia (U937) cell lines. After an initial viability screening (data not shown), compounds **4** (spiro(pseudocoumarin-cyclohexanone)) and **9** (spiro(6-methyllactone-cyclohexanone)) were selected based on their antiproliferative and cytotoxic characteristics to elucidate underlying mechanisms of action.

Compounds **4** and **9** led to a dose- and time-dependent inhibition of proliferation of K562 and U937 cells from 50 μM (Figure 4), with U937 cells being more sensitive. Decreased proliferation was accompanied by a reduction of viability

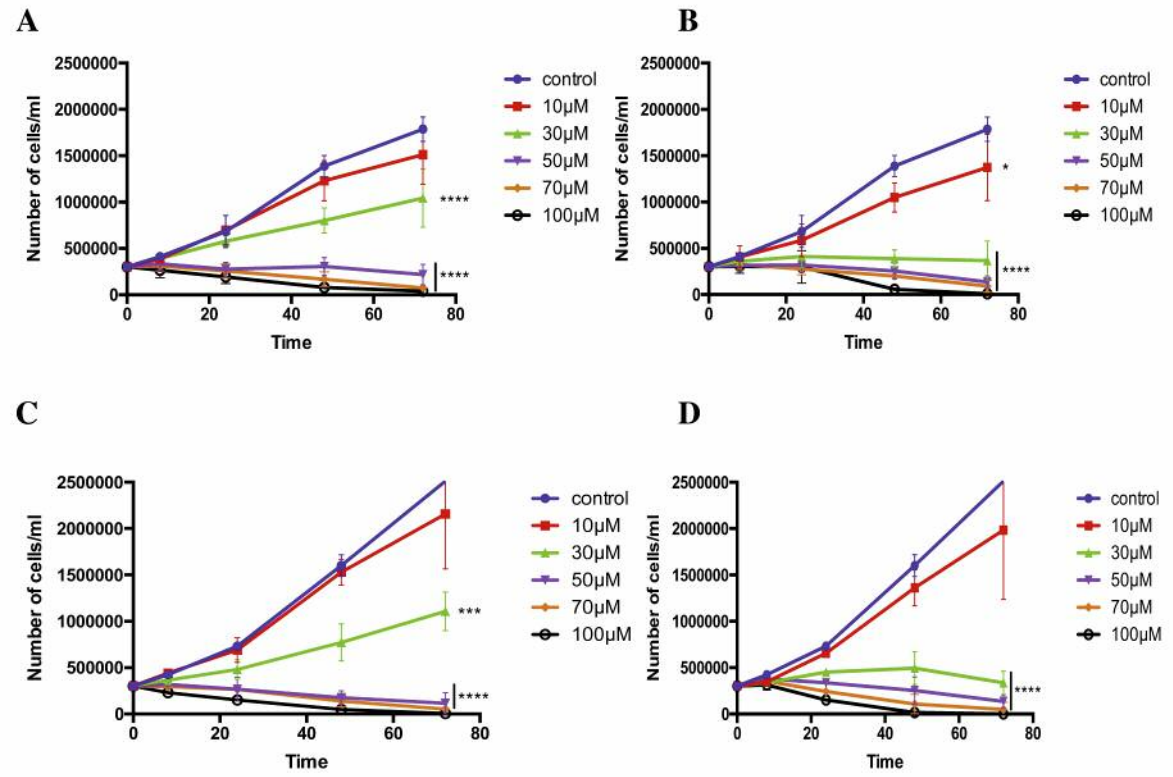

Figure 4. *Effects of compounds **4** and **9** on K562 (A and B) and U937 (C and D) cell proliferation assessed by the trypan blue exclusion test. Control corresponds to solvent-treated cells. Asterisks represent a significant difference between the specific treatment compared to control over time. p-Values below 0.05 were considered significant: ***p<0.001, ****p<0.0001.*

(Figure 5). IC_{50} for compound **4** were 74.02±4.1 μM (K562) and 51.6±4.2 μM (U937) and for compound **9** were 58.6±4.2 μM (K562) and 43.7±1.5 μM (U937) after 72 h, respectively.

*Effects of compounds **4** and **9** on TNFα-activated nuclear factor kappa B (NF-kB) activity in K562 cells*. The nuclear factor kappa B (NF-kB) pathway is constitutively active in many different types of human cancer. This continuous activation of NF-kB contributes to tumor growth and survival by inappropriately activating genes involved in cell cycle regulation, adhesion, and anti-apoptosis (55, 56). To examine the inhibitory potential of compounds **4** and **9** on NF-kB activity we used a reporter gene assay. K562 cells transfected with a luciferase reporter gene were pre-treated with different concentrations of compounds **4** and **9** for 2 h and then treated with TNFα (20 ng/mL) for an additional 6 h.

Our results show that compound **4** reduced TNFα-stimulated NF-κB activation in a dose-dependent manner with an IC_{50} of 15.9±4.0 μM (Figure 6). Compound **9** did not inhibit TNFα-induced NF-κB activity, as the IC_{50} was superior to 100 μM (Figure 6).

*Compound **4** induced caspase-dependent apoptosis in K562 and U937 cells*. As our previous results showed down-regulation of proliferation, viability and NF-κB activity by compound **4** but not compound **9**, and to understand the mechanism of cell death, caspase 3/7 activity was measured in K562 and U937 cell lines treated with compound **4** at various concentrations for 24 h. Compound **4** induced caspase 3/7 activity in a time-dependent manner in both cells lines with a stronger effect in U937 cells (Figure 7). To ascertain the involvement of caspase-dependent apoptotic mechanisms, we pre-treated cells with 50 μM of pan-caspase inhibitor (Z-vad) for 1 h. Under these conditions, induction of caspase 3/7 activity mediated by compound **4** was completely abrogated.

Discussion

There are two major NF-kB signalling pathways, the canonical pathway (or classical) and the non-canonical pathway (or alternative pathway) (57). The non-canonical pathway is activated by LTα/β, CD40 ligand and Blys/BAFF, but not by TNF-α, IL-1 and LPS (58). The canonical pathway is mainly activated by TNF-α, IL-1 and LPS and is

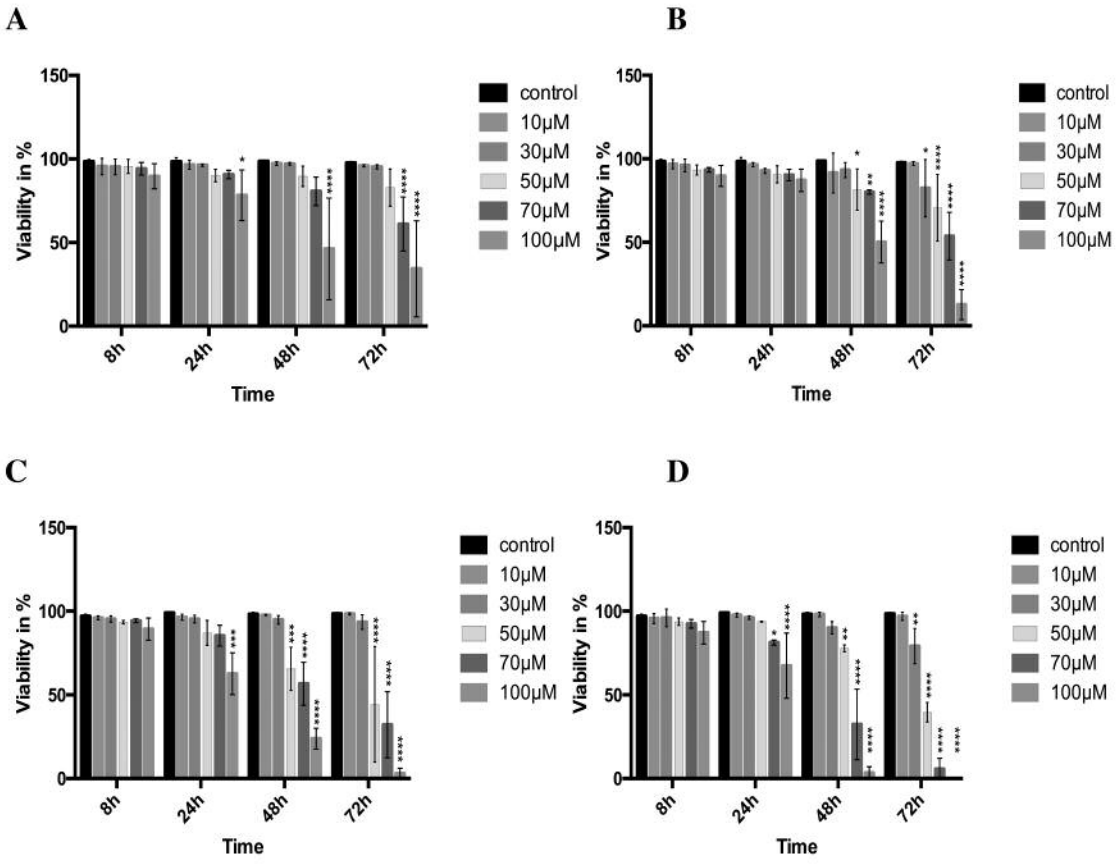

Figure 5. *Effects of compounds **4** and **9** on viability of K562 (A and B) and U937 (C and D) cells assessed by the trypan blue exclusion test. Control corresponds to solvent-treated cells. Asterisks represent a significant difference between the specific treatment compared to control over time. p-Values below 0.05 were considered significant: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

principally related to inflammation, cell proliferation and survival signals. In the present study, we have showed that Compounds **4** and **9** inhibited the canonical NF-kB pathway.

Many studies have revealed numerous anti-proliferative and anti-tumor properties of a variety of coumarin and spiro-compounds and they were reported to inhibit the proliferation of a number of human malignant cell lines *in vitro* (9, 10, 59).

Considering these results, it was not surprising that our compounds showed a significant effect on the down-regulation of cancer cell proliferation and viability in K562 and U937 cells lines. Indeed, we found that compound **4** and compound **9** affected viability with IC_{50}s values of compound **4** at: 74.02±4.1 and 51.6±4.2 μM against K562 and U937 cells, respectively; compound **9** was cytotoxic with IC_{50}s at: 58.6±4.2 and 43.7±1.5 μM against K562 and U937 cells, respectively.

We evaluated the effects of compound **4** and compound **9** on TNF-α inducedNF-kB transactivation. We noted that unlike compound **9**, compound **4** inhibited TNF-α-induced NF-kB activation in a dose-dependent manner with an IC_{50} at 15.9±4.0 μM in K-562 cells (Figure 6). This value is within the range of IC_{50} values previously reported by our laboratory for various compounds against TNF-α-induced NF-kB activation in K562 and U937 cells using the same experimental conditions (16, 60). This activity of compound **4** is not surprising as other coumarin and spiro-compounds (11) were also recently reported to exhibit NF-kB inhibition potential.

Induction of apoptosis occurred in a caspase-dependent manner as revealed by caspase 3/7 assay. Based on previous studies (11, 61), we can hypothesize that the inhibition of the NF-kB pathway by compound **4** may be responsible for the induction of apoptotic cell death in K562 cells, as concentrations necessary for NF-kB inhibition were lower and/or appeared at the shorter time points compared to cytotoxic concentrations. These observations indicate that inhibition of NF-kB activity occurs prior to induction of cell death.

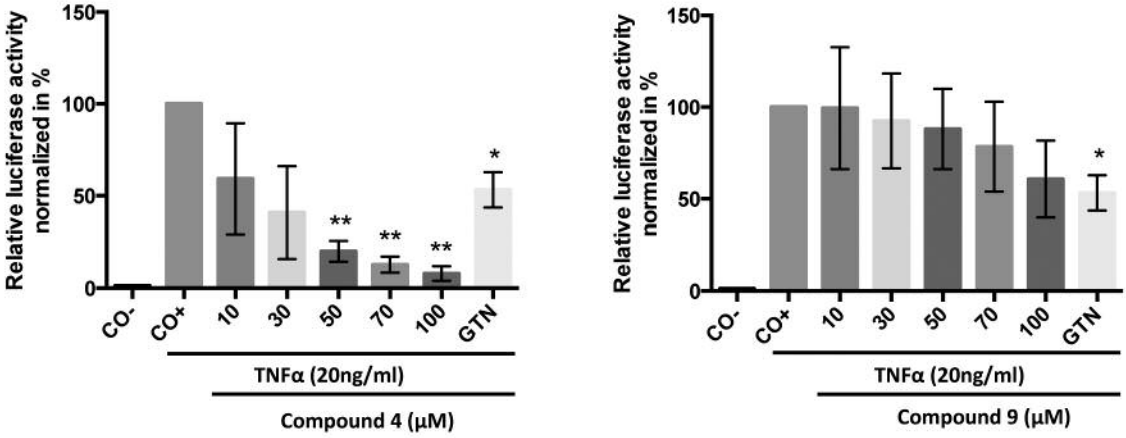

Figure 6. *Compound **4** inhibits TNF-α-induced NF-κB activation. K562 cells were transfected with NF-κB-responsive luciferase reporter plasmid and a Renilla luciferase control reporter plasmid. 24 h after transfection cells were treated with indicated concentrations of compounds **4** and **9** for 2 h followed by 6 h of activation with 20 ng/ml TNFα. Negative control (Co–) corresponds to solvent-treated cells. Positive control (Co+) corresponds to solvent-treated cells activated by TNF-α. Goniothalamin (GNT) at 7 μM was used as a positive inhibitory control. Asterisks represent a significant difference between the specific treatment compared to control over time. p-Values below 0.05 were considered significant: *p<0.05, **p<0.01.*

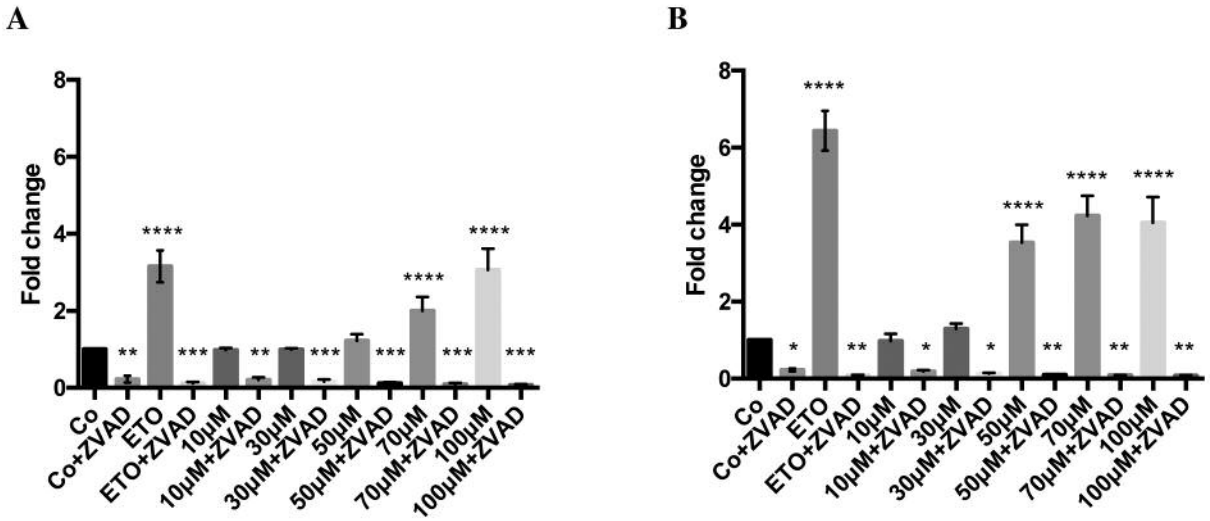

Figure 7. *Induction of caspase 3/7 activity by compound **4** in leukemia cell lines. K562 (A) and U937 (B) cells were pre-treated or not with Z-vad at 50 μM for 1 h, followed by compound **4** treatment at indicated concentrations for 24 h. Induction of caspases-3/7 activity was investigated by substrate cleavage using luminescence based test kit (Promega, Seoul, Republic of Korea). Control (Co) corresponds to solvent-treated cells. Etoposide (ETO) at 100 μM was used as a positive control. Asterisks represent a significant difference between the specific treatment compared to control over time. p-Values below 0.05 were considered significant: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

Conclusion

The anticancer activity of spiro(lactone-cyclohexanone) derivatives **4-9** on leukemia cell lines have been studied. The results reveal that from a set of six tested compounds, two compounds, spiro(pseudocoumarin-cyclohexanone) **4** and spiro(6-methyllactone-cyclohexanone) **9**, showed a significant effect on down-regulation of cancer cell proliferation and viability in K562 and U937 cell lines. Moreover, compound **4** inhibited TNF-α-induced NF-κB activation in a dose-dependent manner with an IC_{50} value of 15.6±4.0 μM, which could be linked to the cell death induction. The results indicate that cell death induction by compound **4** could be mediated through apoptosis as we observed Z-vad–sensitive activation of executioner caspases 3/7.

Acknowledgements

The Authors would like to thank FCT/MEC for their financial support for the project PT-DZ/0005, of the QOPNA research Unit (FCT UID/QUI/00062/2013) through national foundations, and, where applicable, co-financed by the FEDER, within the PT2020 Partnership Agreement, and to the Portuguese NMR Network. The Authors would also like to thank the General Directorate for Scientific Research and Technological Development – DGRSDT of Algeria and Agence Thématique de Recherche en Sciences et Technologie ATRST for approving the co-financed bilateral project PT-DZ/0005. Research at LBMCC is financially supported by the "Fondation de Recherche Cancer et Sang", the "Recherches Scientifiques Luxembourg" Association, the "Een Haerz fir kriibskrank Kanner" Association, the Action Lions "Vaincre le Cancer" Association, the European Union (ITN "RedCat" 215009, Interreg IVA project "Corena") and the Télévie Luxembourg. BO and MD are supported by the NRF by the MEST of Korea for Tumor Microenvironment GCRC 2012-0001184 Grant and by Brain Korea (BK21) PLUS program.

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Received July 3, 2017
Revised July 21, 2017
Accepted July 24, 2017