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New Synthesized Derivatives from *N*-Substituted-4-Oxo-[1] Benzopyrano [4,3-c] Pyrazole Influenced Proliferation, Viability, Spreading and Invasion of Human Liver Tumor Cells

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Abstract

Background/Aim: There is an unsatisfied clinical demand to develop new anticancer agents. The aim of the current study was to synthesize new coumarin derivatives using two different synthetic methodologies and to evaluate their anticancer activity. Materials and methods: Four coumarin derivatives were synthesized and evaluated for their anticancer activities. The structures of all compounds were confirmed by infrared (IR), UV-vis, Nuclear magnetic resonance (NMR) ^{13}C NMR, ^{1}H NMR, and high-resolution mass spectrometry (HRMS) analysis.

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All the synthesized compounds (**4, 5**, **8 and 9**) were analyzed for their anti-proliferative (MTT and LDH assays and cell cycle studied with flow cytometry) and anti-invasive activity (spreading and invasion tests) on human hepatoma cell lines Huh-7 in vitro. Doxorubicin was used as control in order to compare their anti-tumoral effects. Results. All the synthesized compounds are potential inhibitors of proliferation, viability, spreading and invasion of human liver tumor cells with a 50% inhibitory Concentration range, IC50=10.37 μM to 12.94 μM. Conclusion. This study could lead to the identification of a new target therapy for human *Hepatocellular carcinoma* (*HCC*) or other cancers.

Keywords: Fraud pentagon; fraudulent financial reporting; non-financial company.

1. Introduction

Coumarins, natural or synthetic, have become an interesting subject of investigation for many researchers due to their biological properties and characteristics and conjugated molecular architecture. Coumarins have proven to have an interesting role in anticancer drug development since many of their derivatives have shown an antitumor activity on various cell lines [1]. Research conducted on these coumarins and their metabolites showed promising activity against several types of cancer such as lung, malignant melanoma, prostate, breast, leukemia, and hepatocellular carcinoma. Anticancer activity of coumarin derivatives has been previously shown [1, 2, 3, 4, 5, 6, 7] and it has been shown that coumarins can act on various cancer cells by different mechanisms; they inhibit the protein kinase telomerase enzyme activity, down regulate oncogene expression or induce the caspase-9-mediated apoptosis, suppress tumor cell proliferation by arresting cell cycle in G0/G1 or G2/M phase in cancer cells [8, 9]. Some researches proved that 7-hydroxycoumarin can inhibit growth of human cancer cell lines [10], such as A549 (lung), ACHN (renal), H727 (lung), MCF7 (breast) and HL-60 (leukaemia) and in some clinical trials they exhibited anti-proliferative activity in prostate cancer [11], renal carcinoma [12] and malignant melanoma [13].

The invasion is one of the most important prerequisite for cancer progression and metastasis because the growth of the primary tumor is dependent on its invasive potential within the host tissue. For this reason the development of new coumarin derivatives able to inhibit tumor invasion is a logical continuation of the research of new anticancer products.

Knowing that cancer cells, including Human hepatoma cells Huh7, are capable of degrading the constituents of the extracellular matrix (ECM), and the basement membrane which is a highly cross-linked network composed mainly of type IV collagen, laminin and heparan sulfate-based proteoglycans [14].

This proteolysis involves enzymes secreted by the cancer cells and by stromal cells (fibroblasts) stimulated by soluble factors secreted by the cancer cells. These enzymes are in particular matrix metalloproteases. In addition, the process also involves a break in the equilibrium between these enzymes and their inhibitors and the chemiokines, which are involved in cancer progression. Chemokines, chemotactic cytokines, like Regulated upon Activation, Normal T cell Expressed, and Secreted (RANTES/CCL5) and monocyte chemoattractant protein-1 (MCP-1)/CCL2, play an important role in tumor biology because they may influence cell growth,

angiogenesis, invasion and metastasis [15, 16, 17].

It has been previously shown that 4-hydroxycoumarin follow different pathways such as aromatase and sulphatase inhibition, cell cycle arrest and cell apoptosis induction [14]. However, coumarin itself also exhibited a cytotoxic effect against human epithelial cells which showed some typical characteristics of apoptosis with loss of membrane microvilli and nuclear DNA fragmentation. These anticancer properties cited above encouraged us to synthesize a novel series of 4-substituted-4-hydroxycoumarin derivatives (HCD) obtained with two different synthesis processes from 4-hydroxycoumarin.

Each process was performed in three steps and led to two regioisomers. The first process has been already reported in the literature for the preparation of 1-phenyl [1]benzopyrano[4,3-c]pyrazol-4(2*H*)-one (4) and 1-Benzyl [1]benzopyrano[4,3-c]pyrazol-4(*2H*)-one (5) [18], while the second one was realized for the first time in our team and led to the 2-phenyl[1]benzopyrano[4,3-c]pyrazol-4(*2H*)-one (8) and the 2-Benzyl[1]benzopyrano[4,3-c]pyrazol-4(*2H*)-one (9). These processes were chosen for their selective properties leading to the targeted regioisomers. The aim of our work was to synthesize the new 4-hydroxycoumarin derivatives, determine whether these new coumarin derivatives have anticancer effect and are involved in inhibition of hepatocellular carcinoma cells Huh7 proliferation, spreading, cell cycle and invasion.

2. Materials and methods

2.1. Reagents and materials

The starting materials 6 and 7 were prepared according to the literature [19, 20]. All reactions were followed by TLC using aluminum sheets of Merck silica gel 60 F254, 0.2 mm and plates were revealed using a UV 254 light. Melting points were taken on a Buchi-510 capillary apparatus and are uncorrected.

^{1}H, ^{13}C and two-dimensional NMR spectra were recorded with AC-400 Bruker spectrometer at room temperature (rt) in $CDCl_3$or DMSO d-6 at 400 MHz and at 100 MHz, using residual non deuterated solvent peaks as internal reference. Coupling constants are given in Hz. IR spectra were recorded on a Perkin Elmer Spectrum two FT-IR instrument with the Universal ATR Sampling Accessory. The HRMS spectra were acquired with an electrospray- time-of-flight (ESI-TOF, LCT Premier XE, and Waters) mass spectrometer in the positive ion mode.

2.2. General procedure for the preparation of 4-chloro-2-oxo-2H chromene-3-carbaldehyde (2)

To a stirred mixture of 4-hydroxycoumarin (2.2 g) in anhydrous DMF (15 ml) were added drop wise $POCl_3$ (15 ml) at -10 to -5°C. The reaction mixture was then stirred for 1 h at room temperature, heated, and stirred for 12 h at 60°C. After the reaction was completed, the mixture was poured onto crushed ice (100 g) under vigorous stirring. After storing the mixture overnight at 0°C, the pale yellow solid was collected by filtration and washed successively with aqueous Na_2CO_3 (5%) and water, and then was air-dried.

Recrystallization from acetone gave 1.7 g (65%) of **2** as a pale yellow powder with mp 115–120 °C. IR (ATR, $\nu_{max}cm^{-1}$): 2920, 2874, 1720, 1702, 1603, 1587, 1541 cm¬¹; ^{1H} NMR (400 MHz, $CDCl_3$) δH : 10.39 (1H, s,

CH=O), 8.19–7.40 (4H, m, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$) δC : 186.81, 158.44, 153.28, 153.27,135.68, 127.65, 125.56, 118.39, 118.22, 117.20.

2.3. Synthesis of (E)-4-chloro-3-(2-phenylhydrazono)-2H-chromen-2-one (3a)

A mixture of phenylhydrazine hydrochloride (0.160 g,1.1 mmol) and triethylamine (2.2 mmol) in(10 ml)of ethanol was slowly added drop wise to a stirred solution of 4-chloro-2-oxo-2H-chromene-3-carbaldehyde (0.208 g,1.0 mmol) in (10 ml) ethanol at 60°C and the reaction was continued for another 15 min at same temperature. The reaction was monitored by TLC and an orange-red precipitate formation was observed. The reaction mixture was cooled to room temperature; precipitate was rapidly filtered off and then washed with cold water to afford (E)-4-chloro-3-((2-phenylhydrazono) methyl)-2H-chromen-2-one **(3a)** (0.275 g) as orange red color solid in 92% yield. M.p: 181-183°C;IR (ATR, $\nu_{max}cm^{-1}$): 3284, 2921,2851, 1696, 1596, 1521, 1488, 1447, 1280, 1253, 1084 cm¬¹;^{1}H NMR (400 MHz, DMSO-d6)δ_H: 11.11 (bs, 1H, NH), 8.16 (s, 1H, $=CH_{imine}$), 8.04 (d, 1H,*J* = 8 Hz, aromatic), 7.75 (t, 1H, *J* = 8 Hz, aromatic), 7.56 (t, 2H, *J* = 4 Hz,aromatic), 7.36 (t, 2H, *J* = 8 Hz, aromatic), 7.20 (d, 2H, *J* = 8 Hz, aromatic), 6.93 (t, 1H, *J* = 4 Hz,aromatic).^{13}C NMR (100 MHz, DMSO-d6) δ_C: 158.7, 150.7, 144.8, 139.5, 132.6, 129.9, 129.7, 125.7, 125.6, 120.5, 120.2, 119.4, 116.8, 112.9. HRMS (ESI-TOF) for $C_{16}H_{11}ClN_2O_2$ $[M + H]^+$: calcd, 299.0511 found 299.3011.

2.4. Synthesis of (E)-4-chloro-3-(2-benzylhydrazono)-2H-chromen-2-one (3b)

Benzylhydrazine dihydrochloride (0.422 g, 2.2 mmol) in mixture of H_2O and AcOH was added to a stirred solution of 4-chloro-2-oxo-2H-chromene-3-carbaldehyde **2** (0.416 g, 2.0 mmol) in methanol at 60 °C and the reaction was continued for another 30 min at same temperature.

The reaction was monitored by TLC and an orange precipitate formation was observed. After completion of the reaction, the reaction mixture was cooled to room temperature, precipitate was filtered off, and the resulted precipitate was dissolved in ethyl acetate and washed with cold water to remove acetic acid. The organic layer was separated and dried over Na_2SO_4, solvent was removed under reduced pressure afforded (E)-4-chloro-3-((2-benzylhydrazono) methyl)-*2H*-chromen-2-one **(3b)** (0.522 g) as orange color solid in 85% yield. M.p: 195-198°C;^{1}H NMR (400 MHz, $CDCl_3$)δ_H: 11.11 (bs, 1H, NH), 8.16 (s, 1H, $=CH_{imine}$), 8.04 (t, 1H,*J* = 4 Hz, aromatic), 7.33-7.38 (m, 4H, aromatic), 7.21-7.25 (m, 2H, aromatic), 4.19 (s, 2H, CH2).^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 156.3, 151.8, 139.8, 138.0, 133.9, 130.0, 128.2, 127.2, 125.1, 123.4, 121.5, 117.3, 110.4, 107.7, 54.7.HRMS (ESI-TOF) for $C_{17}H_{13}ClN_2O_2$ $[M + H]^+$: calcd, 313.0666 found 313.0671.

2.5. General procedure of the preparation of 1-phenylchromeno[4, 3-c]pyrazol-4(1H)-one (4) and 1-benzylchromeno[4,3-c]pyrazol-4(1H)-one (5)

Catalytic amount of piperidine was added to a stirred solution of4-chloro-3-(N-substituted hydrazino)-2*H*-chromen-2-ones (1mmol) in pyridine (5mL), and contents were refluxed for 2 h. After completion of the reaction indicated by TLC and the formation of a dark red color, reaction mixture was cooled to room temperature and diluted with water (3 mL).

Dilute chlorhydric acid was added to the crude mixture, to quench the excess of pyridine. The brown precipitate so-formed was filtered, washed with water and purified over silica gel (60-120) afforded compounds **4-5** as pale yellow color solids in 78-85% yield.

2.6. Preparation of 1-phenylchromeno [4, 3-c]pyrazol-4(1H)-one (4)

Yield: 78%,yellow color solid, mp = 192°C; IR(ATR, $\nu_{max}cm^{-1}$): 1753, 1535, 981cm⁻¹; ^{1}H NMR (400 MHz, DMSO-d6)δ_H: 8.52 (s, 1H, $H_{olefinic}$), 7.65-7.71 (m,5H, aromatic), 7.53-7.71 (m, 2H, aromatic), 7.20 (t, 1H,*J* = 8 Hz, aromatic), 7.00 (d, 1H,*J* = 8 Hz, aromatic).^{13}C NMR (100 MHz, DMSO-d6) δ_C:156.9; 152.9, 141.3, 139.9, 139.5, 132.1, 131.1, 130.5, 127.5, 124.8, 122.5, 118.3, 111.6, 108.3.HRMS (ESI-TOF) for $C_{16}H_{10}N_2O_2$ $[M + H]^+$: calcd, 263.0741found 263.0741.

2.7. Preparation of 1-benzylchromeno [4, 3-c] pyrazol-4(1H)-one (5)

yield:85% ,Yellow color solid, mp = 123°C; IR(ATR, $\nu_{max}cm^{-1}$):1727,1535, 1022 cm⁻¹; ^{1}H NMR (400 MHz, DMSO-d6)δ_H: 8.27 (s, 1H, $H_{olefinic}$), 7.7 (d, 1H,*J* = 8 Hz, aromatic),7.49 (t, 1H, *J* = 8 Hz, aromatic),7.44 (t, 1H,*J* = 8 Hz, aromatic), 7.29-7.36 (m, 3H, aromatic),7.20 (t,1H,*J* = 8 Hz, aromatic),7.13 (d, 2H,*J* = 8 Hz, aromatic), 5.86 (s, 2H, CH_2).^{13}C NMR(400 MHz, DMSO-d6)δ_C: 157.3, 152.8, 140.8, 139.0, 134.9, 131.0, 129.2, 128.3, 126.1, 124.5, 122.5, 118.3, 111.3, 108.7, 55.8.HRMS (ESI-TOF) for $C_{17}H_{12}N_2O_2$ $[M + H]^+$: calcd, 277.0898 found 277.0905.

2.8. General procedure of the preparation of 2-phenylchromeno[4,3-c] pyrazol-4(1H)-one (8) and 2-benzylchromeno[4,3-c]pyrazol-4(1H)-one (9)

The starting material **1**was prepared according to the literature **[21]** using the two-step formylation/cyclisation reaction between 4-(2-hydroxyphenyl)-1, 5-benzodiazepin-2-one and excess of DMFDMA. For the synthesis of chromeno-pyrazolone derivatives: (10 mmol) of compound **1** and an excess of phenylhydrazine chlorhydrate or benzylhydrazine chlorhydrate (50 mmol) were dissolved in (5 ml) of glacial acetic acid, and refluxed for 2h. The reaction mixture was poured into ice and the precipitate filtered then washed successively with water then recrystallized from ethanol.

2.9. Preparation of 2-phenylchromeno [4, 3-c] pyrazol-4(1H)-one (8)

Yield: 70%, pale yellow color solid, mp = 225°C; IR(ATR, νmaxcm-1): 1733 (C=O),1600(C=N) 1461(C=C arom), 1111(C-O-C arom) cm⁻¹; ^{1}H NMR (400 MHz, DMSO-d6)δ_H: 9.61 (s, 1H, $H_{olefinic}$), 8.07–8.12 (m, 3H, aromatic),7.58–7.63 (m, 3H, aromatic), 7.40–7.50 (m, 3H, aromatic), ^{13}C NMR (100 MHz, DMSO-d6)δ_C: 157.2, 153.0, 149.2, 139.2, 132.0, 131.5, 130.2, 128.8, 125.3, 123.0, 120.4, 117.8, 114.5, 109.2 .HRMS (ESI-TOF) for $C_{16}H_{10}N_2O_2$ [M + H]+: calcd, 263.0742; found 263.0750.

2.10. Preparation of 2-benzylchromeno [4, 3-c] pyrazol-4(1H)-one (9)

Yield: 55%, White cottony, mp =169°C. IR(ATR, νmaxcm-1): 1744 (C=O),1622(C=N) 1464(C=C arom),

1089(C-O-C arom) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d6)δ_H: 9.01 (s, 1H, $H_{olefenic}$), 7.96 (d, 1H,*J* = 8 Hz, aromatic), 7.54 (t, 1H,*J* = 8 Hz, aromatic), 7.33–7.44 (m, 7H, aromatic), 5.59(s, 2H, CH_2).^{13}C NMR (400 MHz, DMSO-d6)δ_C: 157.3, 152.8, 148.8, 136.5, 134.5, 130.9; 129.2; 128.6, 128.4, 125.1, 122.8, 117.7, 114.8, 107.3, 56.4.HRMS (ESI-TOF) for $C_{17}H_{12}N_2O_2$ [M + H] +: calcd, 277.0899 ; found 277.0951.

2.11. Cell culture and reagents

Human hepatoma *Huh7* cells were obtained from ATCC (LGC Standards, Molsheim, France). These Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal bovine serum (FBS), 2 mM l-glutamine, 1 mM sodium pyruvate, 50 U/ml streptomycin (all obtained from Life Technologies Inc.), at 37°C in a 5% CO2 humidified atmosphere. Cells were subcultivated every 7 days. The medium was changed twice a week. All in vitro cell experiments (viability, spreading and invasion assays) were carried out at 37°C in a 5% CO_2 incubator [22].

2.12. Cell Viability Assay

The viability of the cancer cells line Huh7 after treatment by our particles was demonstrated using MTT (3-[4.5-Dimethylthiazol-2-yl]-2.5-dephenyltetrazolium bromide) assay (Thiazolyl Blue Tetrazolium Bromide, Sigma, St. Louis, MO) [23]. 5000 cells/well were seeded in a 96-wells plate for 24, 48 and 72 h with or without the different particles at increasing concentration (0.01 µM, 0.1 µM, 1 µM, 10 µM and 100 µM). MTT solution at 1 mg/mL was added to the medium for 2 h and coloration was revealed in DMSO. The metabolic activity was correlated by the absorbance at 570 nm (Bio-rad© Microplate reader, Model 680). Experiments were performed in quadruplicate. Dose–response curves were obtained allowing the determination of IC50 values, which refer to the concentration inducing a response halfway between the baseline and the maximum plateau reached [24].

2.13. Measurement of lactate dehydrogenase (LDH) release

1. Cellinjurywasalsoquantitatively
2. assessed by measurement of released lactate dehydrogenase (LDH)
3. from damaged cells. The LDH assay was performed using the
4. CytoTOX96 Non-Radioactive CytotoxicityAssay Kit (Promega,
5. Madison, WI, USA), according to the manufacturer's protocol.
6. Glutamate-induced LDH release was expressed as a percentage of
7. experimental LDH release/maximal LDH release which was
8. induced by treatment of 0.9% Triton X-100. One-way
9. ANOVA
10. was
11. used for the statistical analysis, and signi®cant differences in cell
12. viability were determined by post hoc comparisons of means using
13. Bonferroni test
14. Cellinjurywasalsoquantitatively

assessed by measurement of released lactate dehydrogenase (LDH) from damaged cells. The LDH assay was performed using the CytoTOX96 Non-Radioactive CytotoxicityAssay Kit (Promega, Madison, WI, USA), according to the manufacturer's protocol. Glutamate-induced LDH release was expressed as a percentage of experimental LDH release/maximal LDH release which was induced by treatment of 0.9% Triton X-100. One-way ANOVA was used for the statistical analysis, and signi®cant differences in cell viability were determined by post hoc comparisons of means using Bonferroni test

Cell injury was also quantitatively assessed by measurement of released lactate dehydrogenase (LDH)from damaged cells.[29]

The LDH assay was performed using the CytoTOX96 Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI, USA), in accordance with the instructions of the manufacturer's protocol. Briefly, after treating Huh7 at 90% confluence in 24-well plates with different prodrugs (0.01–100µM) for 24 h, culture medium was collected. The induced LDH release was measured as the absorbance at 490 nm using a standard 96-well plate reader. Cytotoxicity was expressed relative to basal LDH release in untreated control cells. One-way ANOVA was used for the statistical analysis and significant differences in cell viability were determined by post comparisons of means [25].

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2.14. Cell Spreading assay

Quantitative cell spreading assays were carried out in respective supplemented basal cell culture media. 6500 cells/well were seeded on the fibronectin (100 µg/mL; Corning)-coated LabTek chambers for 3h at 37C°, 5% CO_2. Then, the samples were rinsed with PBS then fixed with 150µl of PFA 1% for 20 min at 4C°, and followed with 150 µl of Triton 0.05% for 5 min at ambient temperature. Cells were stained subsequently with Alexa Fluor 546 phalloidin (dilution 1/100, Invitrogen, France) and DAPI (dilution 1/1000, Invitrogen, France) for 1h30 min in order to visualize filamentous actin and observed with a fluorescence microscope (Zeiss Axiophot, Carl Zeiss France). Cells were photographed using digital camera fixed on top of the microscope (Nikon COOL PIX 8400, Japan) and ten fields of stained cells were photographed for each treatment. Cell areas expressed in square inches were evaluated on 30 individual cells, measured by Scion Image software (Scion Corporation) **[26].** Histograms represent mean of cell areas ± SD of three different experiments.

2.15. Cell Invasion assay

Huh7 cell invasion was investigated using Boyden invasion chambers (Becton Dickinson) with 8 mm pore size filters coated with Matrigel membrane matrix (100 µg/ml; BD Bioscience PharMingen). 2.5 $\times 10^5$ Huh7 cells with (0.01 µM, 0.1 µM, 1 µM, 10 µM and 100 µM) of each compound (4, 5, 8 and 9) were seeded in the upper well of the Boyden chamber. Hepatocyte growth factor (HGF; 20 ng/mL) was used as a positive control [27]. After 24 h, non-migrated cells in the upper chamber were wiped with a cotton swab and the cells were fixed in methanol and stained with Mayer's hemalum, and counted. Cells invading the lower surface of the filter were counted in 10- fields using a Zeiss microscope. Results were expressed as a percentage relative to controls and therefore normalized to 100%. Experiments were performed in triplicate [28].

2.16. Flow cytometry analysis

To analyze the cell cycle of Huh7, 10^5 cells were seeded into 12- well plates and serum deprived during one night. Then, Huh7 were treated or not with the coumarinic derivatives products at 1 or 10 µM during 24h. Supernatant and trypsinated cells were then centrifuged during 5 minutes at 1500 rpm. Samples were fixed with pre-cooled 75% ethanol for 1h at 4°C. Finally, cells were incubated 15 minutes at room temperature with 10 µg/mL of propidium iodide (PI)-PBS for flow cytometry analysis. Data were analyzed using FACScalibur (Becton Dickinson, CA, USA).

2.17. Statistical Analysis

For the determination of statistical significance, an ANOVA test was performed with the Stat view software (Stat View 4.5 Abacus Concepts, Berkeley, USA). A p value of <0.05 was used as the criterion for statistical significance.

3. Results

3.1. Chemistry

The coumarin derivatives 4, 5, 8 and 9 were prepared using two general synthetic methods. The regioisomere 4 and 5 were prepared using the methodology outlined in Figure 3 (Pathway I). 4-Hydroxycoumarin (1) was treated with $POCl_3$ and DMF, similarly to standard Vilsmeier-Haack conditions (at -10 to -5°C) [29]. After hydrolysis, 4-chloro-3-formylcoumarin (2) was generated. Condensation of 2 with hydrazine hydrochlorides derivatives in acidic or basic medium provided the corresponding (E)-4-chloro-3-((2-phenylhydrazono)-*2H*-chromen-2-one (3a) and (E)-4-chloro-3-((2-benzylhydrazono)-*2H*-chromen-2-one (3b) [30].

The compounds 4 and 5 were obtained by the regioselective cyclisation of their corresponding 2H-chromenohydrazones 3a and 3b at 100°C with a catalytic amount of piperidine in pyridine in high yields, .

As shown in Scheme 1(Pathway II), analogues 8 and 9 were prepared using the second methodology. The condensation of one equivalent of [1] benzopyrano[4,3-c][1,5]benzodiazepin-7(*8H*)-one (7) [31] with an excess of hydrazine hydrochloride derivatives in boiling acetic acid has given the corresponding products. The starting material 7 was prepared according to the literature [32] using the two-step formylation/cyclisation reaction between 4-(2-hydroxyphenyl)-1, 5-benzodiazepin-2-one (6) and an excess of dimethylformamide dimethylacetal

(DMF-DMA).

The IR spectra of compound 4 chosen as an illustrative example, has shown absorption bands at 1753, , 1618, 1535 and 981 cm^{-1} which corresponded respectively to the stretching vibrations of the (lactone >C=O), (pyrazol (C=N), aromatic (C=C), and (C-O-C) bonds ^{1}H NMR spectra recorded at 400 MHz in DMSO-d_6, displayed characteristic signals which can be, according to their multiplicity, readily assigned to olefinic proton (singlet at 8.53 ppm) and to aromatic protons (multiplets at 7.68 ppm and 7.58ppm, triplet at 7.2 ppm and doublet at 7 ppm). Compound 4 was further confirmed by ^{13}C NMR and two-dimensional heteronuclear ^{1}H-^{13}C HSQC and HMBC spectroscopy. The signal at d 156.9 ppm corresponded to coumarin carbonyl carbon C-2 and three separate signals at δ 141.3, 139.9 and 111.6 ppm corresponded in turn respectively to C-9b, C-3 and C-3a (Figure.2).

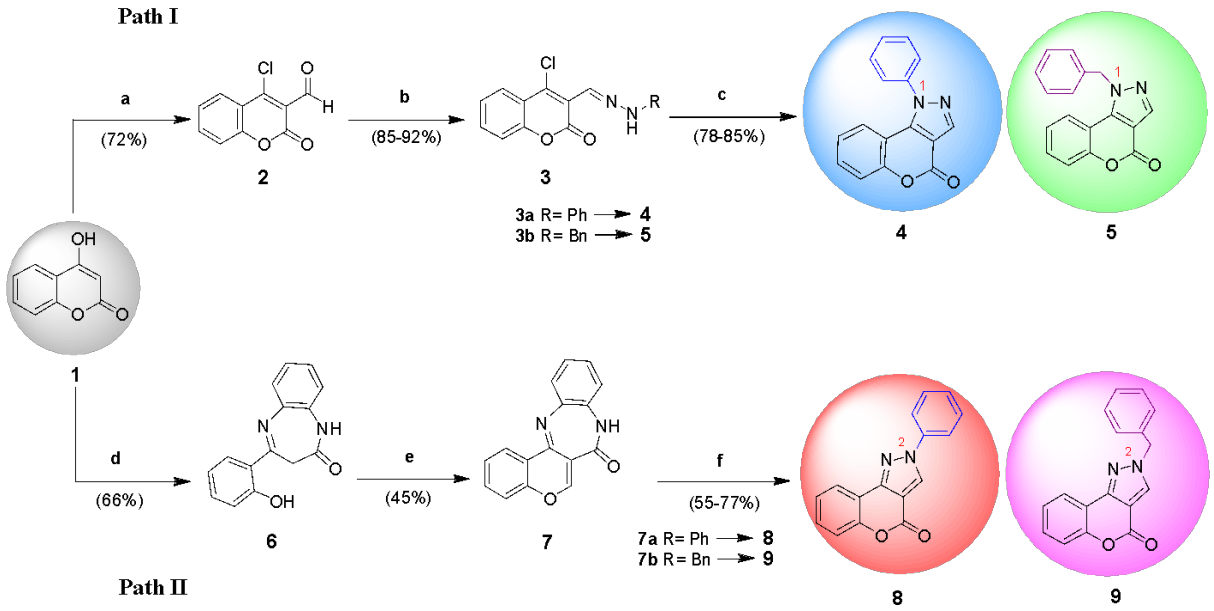

Figure 3: Synthesis of compounds 4-5 and 8-9 (a) Vilsmeier-Haack conditions: (DMF+$POCl_3$),-10 to -5°C. (b)R-NH-NH_2.HCl/Et_3N, EtOH, 60°C. (c)Piperidine (cat) Pyridine (solvent) 100°C. (d) 1, 2-diaminobenzene, Xylene reflux. (e)DMF-DMA, Toluene 100°C. (f) R-NH-NH_2.HCl, AcOH reflux. Synthesized compounds are:

***4**: 1-phenylchromeno [4, 3-c] pyrazol-4(1H)-one*

***5**: 1-benzylchromeno [4, 3-c] pyrazol-4(1H)-one*

***8**: 2-phenylchromeno [4, 3-c] pyrazol-4(1H)-one*

***9**: 2-benzylchromeno [4, 3-c] pyrazol-4(1H)-one*

3.2. Biological assays

3.2.1. Anti-proliferative activity analyzed by MTT assay

In order to analyze anti-proliferative properties of all the synthesized compounds, we evaluated their cytotoxicity with MTT assay. The incubation of Human hepatoma Huh7 cell line with different concentrations (0.01µM-100µM) of all coumarinic derivatives was performed up to 48h. Doxorubicin was used as the reference drug. Our results showed that all compounds 4, 5, 8 and 9 displayed good cytotoxic effects on Huh7 with half maximal inhibitory concentrations (IC_{50}) between 10.37 ± 0.05 µM and 12.94 ± 0.04 µM. These data are shown in Table 1. It may be noted that the substituted benzyl ring compounds 5 showed a higher anti-proliferative activity (IC_{50}=10.37 ± 0.05 µM) than the other compounds 9 (IC_{50} = 12.94 ± 0.04 µM), 4 (IC_{50} = 10.84 ± 0.04 µM) and 8 (IC_{50} = 11.02 ± 0.04 µM).

In addition, the lipophilicity (*log P*) of the compound may contribute to optimum anti-proliferative activity in as much as the higher partition coefficient means that the compound is more likely to penetrate into the membrane and bind with intracellular receptor [33]. Actually, according to Table 2, the higher *Log P* the more the compounds were active.

Table 1 : Anti-proliferative values IC_{50} (µM) of the 4-Hydroxycoumarin derivatives. Huh7 were treated or not with our different compounds at concentrations from 0.01 to 100 µM for 48h. Cell viability was determined by a MTT assay and then the IC_{50} was calculated. The table presents the IC_{50} of the different compound ± SEM.

Compound	IC_{50} (µM)
Dox	5.84±0.06
4	10.84±0.04
5	10.37±0.05
8	11.02±0.04
9	12.94±0.04

Table 2: Log P (lipo-hydro partition coefficient) properties of anti-proliferative agents.

Compound	*Log Pa*
4	2.67
5	3.47
8	2.67
9	3.47

[a]The *log P* values were predicted using ChemBioDraw.

3.2.2. Structural integrity of cell membrane analyzed by LDH assay

The breakdown of membrane integrity is one of the characteristic features of the process of apoptosis and cell

death. There by the cytotoxicity results in a phase of massive cell lysis. To monitor the evolution of the integrity of the cells we measured the release of the enzyme LDH in the supernatant of the cells treated or not by all our compounds after 24h, 48h and 72h of incubation (**Figure 1).**

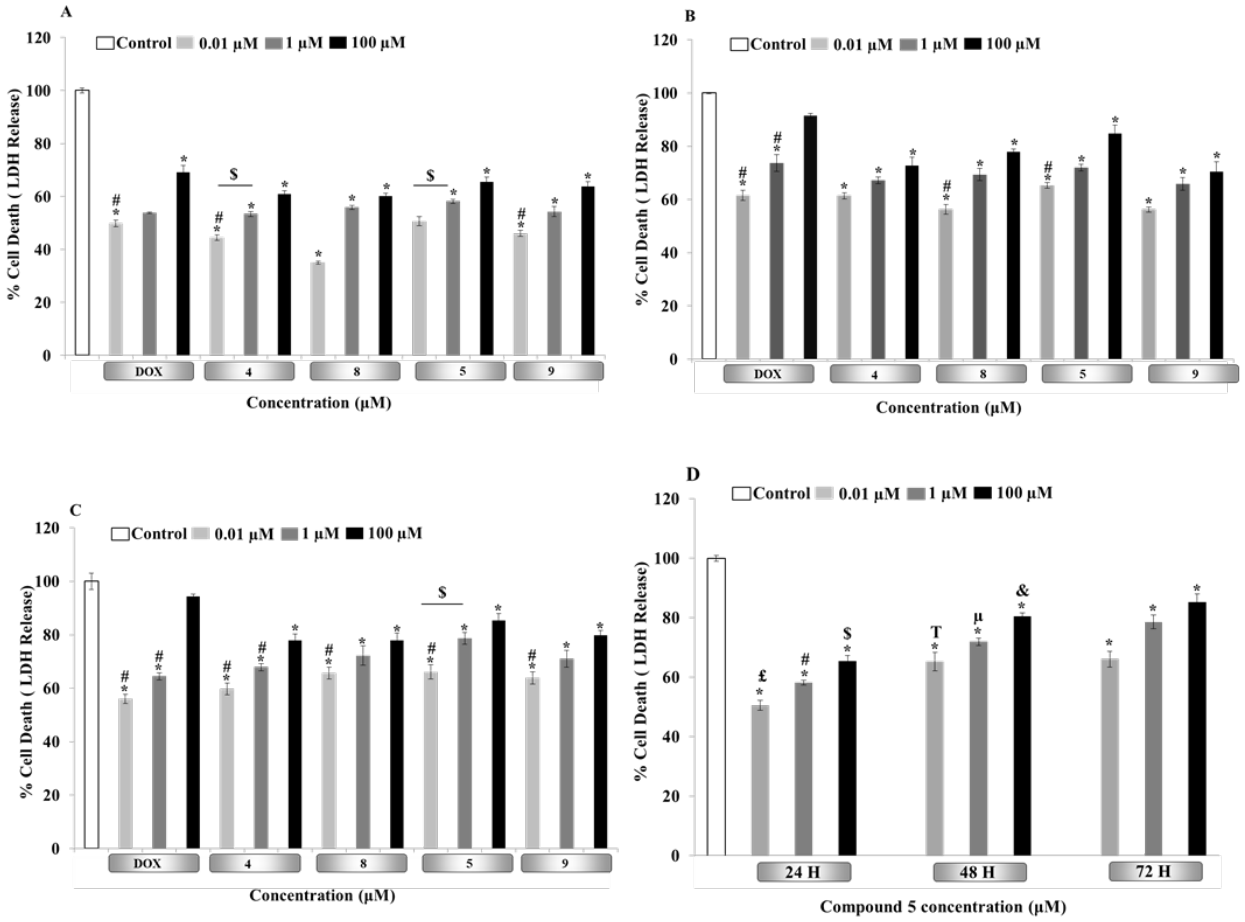

Figure 1: Effect of compounds **4**, **5**, **8** and **9** on LDH release induced by Huh7 cells (A, B, C). The addition of 100 µM, 1 µM and 0.01 µM was during 24h (A), 48h (B) and 72h (C).Results are represented as percent Huh7 death.

(**A**) *****P<0.05 versus compounds Dox, **4**, **5**, **8** and **9**, **$** P <0.05 versus the concentration of 1µM of all compounds, #P <0.05 versus the concentration of 100 µM of all compounds

(**B**) *P<0.05 versus Dox, **4**, **5**, **8** and **9** except Dox 100 µM, #P <0.05 versus the concentration 100 µM of all compounds

(**C**)*****P<0.05 versus Dox, **4**, **5**, **8** and **9** except Dox 100 µM, **$**P<0.001 versus 0.01 µM

#P<0.05 versus the concentration 100 µM of all compounds.

(**D**): Effect of compound **5** only on LDH release for 24h, 48h and 72h.*****P<0.001 versus control, **£** P<0.05 versus 0.01µM 48h and 0.01µM 72h,# P<0.001 versus 1µM 48 h and 1µM 72h, **T** P<0.05 versus 0.01µM 72h,

µP<0.05 versus 1µM 72h, **&** P<0.05 versus 100µM 72h.

The results showed that the LDH activity of each compound was statistically different from that of the control group. Thus, we note a significant liberation of LDH into the cell supernatant for the first 24h **(Figure 1a)** which increased significantly with the extension of time, indicating that the different derivatives had both time dependent effects which cause cell membrane damage (**Figure 1 a-c).** The maximum release of LDH was thus obtained after 72h of incubation with cytotoxicity higher than 65% for all the compounds. Under basal conditions, the LDH is already present in the cytoplasm.

However, if the cell membrane is damaged, it will leak out of the cell. Therefore, the amount of LDH measured in a conditioned media can indirectly give an idea of the degree of damage of the cell membrane integrity. Furthermore, the compound **5** remains the most effective with a percentage of cytotoxicity of 50.64% (24h), 65. 26% (48h), 66.14% (72h) at the concentration of 0.01 µM whereas for that of 1µM the percentages are 58.17% (24h), 71.94% (48h),78.63% (72h) and finally the percentages are 65.53% (24h), 84.85% (48h), 85.32% (72h) for the concentration of 100µM.

3.2.3. Cell spreading assay

Cell spreading take part in cell movement and progression processes since before migration the cell must adhere well and spread to the surface [19, 20], During spreading, the cells undergo morphological changes involving the extension of lamellipodia along the extracellular matrix, formation of focal adhesion complexes, translocation of the cell body, and posterior detachment from the extracellular matrix.

Meanwhile, the cells drastically reorganize their cytoskeleton during this extremely dynamic process. The main visible feature is the increase of the surface area of contact between the cells and the extracellular matrix, which is accompanied by a significant flattening of the cell body and an increase in the total cell area [26].

Thus, it was interesting to measure the effectiveness of synthesized HCDs on the spreading of Huh7 liver cancer cells, which is a relevant step in tumor progression. Cell morphology was studied after 3h-incubation of Huh7 cells in the presence or absence of HCD by analysis of red labeling actin, a major cytoskeletal protein.

After 3h of cell culture with compounds 4, 5, 8 and 9. The results showed that the morphology of the cells cultured in the presence of HCD did not have the same morphology as those cultured in its absence and this depends on the concentration of HCD (0.01µM, 1µM and 100µM).

The results obtained using fluorescent microscopy showed that the red labeled cytoskeleton in the cells pass from a spherical to a star-shaped form in the presence of all derivatives. The qualitative analysis of the morphology of the cells carried out by fluorescence microscopy was confirmed quantitatively by image analysis with Scion Image software and expressed in square inch.

The cell areas were calculated in absence and in the presence of HCD at increasing concentrations (0.01 µM, 1 µM or 100 µM). We consider a cell spread out as round and with a higher contrast. The areas representing

aggregates or clusters of cells were excluded from the data set based on the evaluation of nuclear DAPI fluorescence, so that all tagged data were derived from individual cells. The percentage of inhibition of cell spreading was determined relative to the control group. All results are presented in the Figure 2.

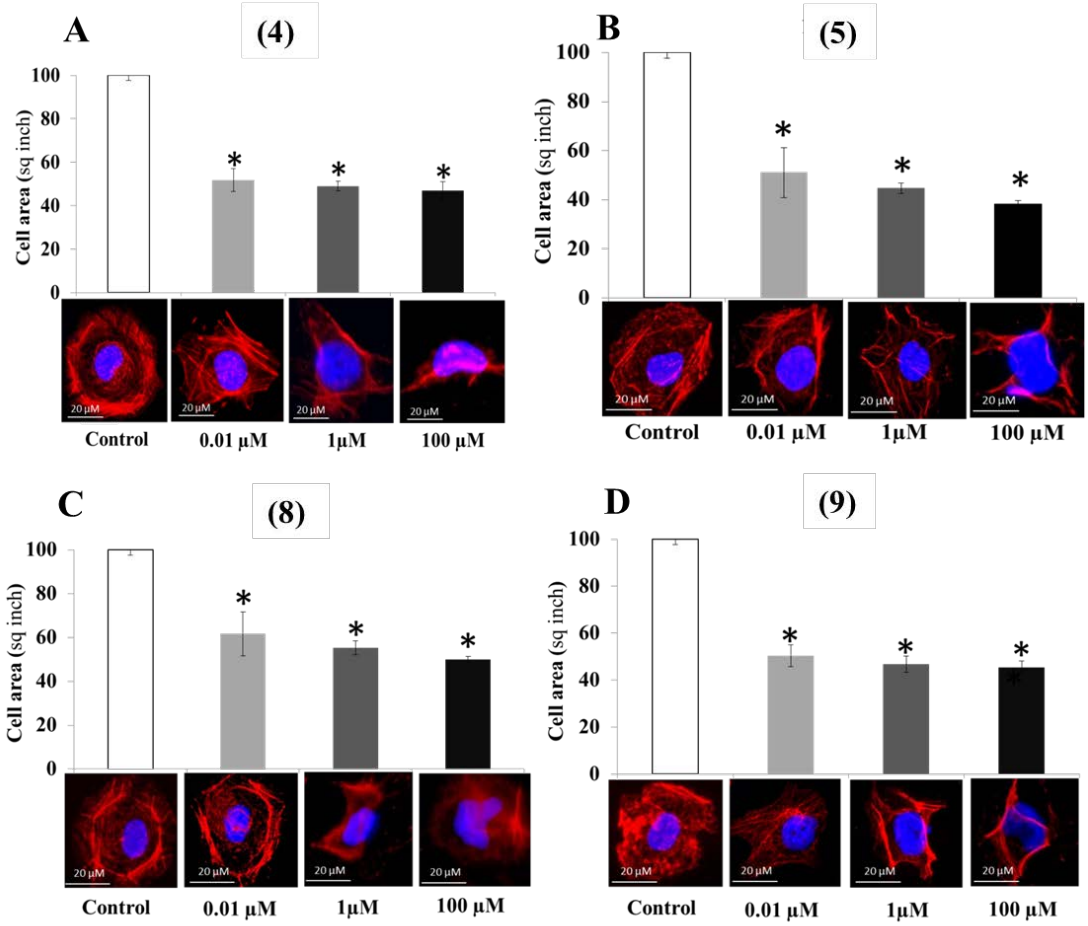

Figure 2: HCD inhibition of Huh7 cell spreading. In adequate time (3h) and for adequate concentration (0.01µM, 1µM and 100µM) 3000 cells/well were plated in glass LabTek, washed with PBS and fixed with 1% PFA. Huh7 were then stained with Phalloidin (F-actin: red) and DAPI (Nucleus: blue), then were imaged with fluorescence microscope. Cell spreading was quantified using Scion image software. High resolution zoom images of F-actin distribution in Huh7 cells. White scale bar = 20 μm. * P<0.0001 as compared to untreated control cells.

In the absence of HCD, the cells spread out and the large spreading areas were observed, whereas in their presence the cell spreading areas were significantly decreased. As shown in **Figure 2-B**, Huh7 spreading was significantly decreased by 55% when the cells were incubated with 1 µM of compound **5** as compared with untreated cells (P<0.005, n=3). Meanwhile, the other compounds **4**, **8** and **9** had less decreasing effect on Huh7 spreading by 53%, 51 % and 45 %, respectively, in the same experimental conditions (**Figure 2A, C and D).**

3.2.4. Cell invasion assay

The four compounds (**4, 5, 8 and 9**) were studied in an *in vitro* model of tumor invasion in modified Boyden chambers. This model makes it possible to evaluate the capacity of Huh7 cancer cells to pass through a filter covered with Matrigel, a biological element of extracellular matrix (ECM), in the presence of a chemotactic cytokine Regulated upon Activation, Normal T cell Expressed, and Secreted (RANTES/CCL5). The results showed that all coumarin derivatives inhibited to some extent the basal invasion of Huh7 cells through the Matrigel. Only for Doxorubicin there was a significant and high inhibition of Huh7 invasion by 60±3 % in basal condition. RANTES/CCL5 was then used to stimulate chemotactic Huh7 invasion. The results indicated a significant increase in cell invasion by 41±2% in control condition. Interestingly, there was a significant decrease of RANTES/CCL5-induced invasion after Doxorubicin, **5** and **8** treatments, by 30%, 34% and 24%, respectively.

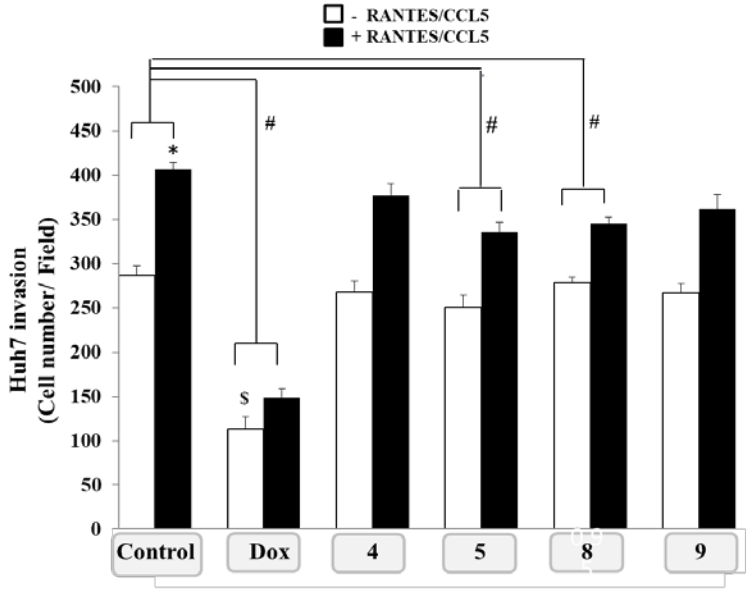

Figure 3: Invasiveness of Huh7 in presence of 4-hydroxycoumarin derivatives. Huh7 cell invasion was performed for 24h in presence of 1 µM of compounds Dox, 4, 5, 8 and 9 and was chemoattracted by RANTES/CCL5 (+RANTES/CCL5) or not (- RANTES/CCL5). Results are indicated as cell number/field. *P < 0.05 versus control – RANTES/CCL5, $P < 0.05 versus control - RANTES/CCL5, # P < 0.05 versus induction by RANTES/CCL5 for control, n=3.

3.2.5. Cell cycle analyzed by flow cytometry

The cytostatic effect of the HCD compounds on the *Huh7* was evaluated in order to confirm the inhibition of cell growth. Huh7 cells were treated with different HCDs at a concentration of 1 µM or 10 µM, then the cell were stained with propidium iodide and were analyzed by flow cytometer which detects early changes in apoptosis and provides a lucid picture on the nature of cell cycle progression. The cell cycle distribution was examined at various times and doses. The results indicate the percentage of living cells in sub-G1, G0/G1 and S/G2/M at a concentration of 1 or 10µM. Our results showed that there was a significant increase of Huh7 cells at the sub-G1 position (Table 3) after compounds 5 (54.64±17.29%) and 9 (54.41±10.35%) treatments at 10 µM,

as compared to control untreated cells (27.92±11.38%). In addition, standard Doxorubicin (Dox) treatment led to significant increase of Huh7 cells at the sub-G1 position at 1 µM (67.42±6.21) and at 10 µM (59.42±7.46). In parallel we observe a significant decrease of cells in phase S and G2/M only for the Doxorubicin treatment for 1µM (11.13±4.42%) and for 10 µM (15.63±3.91%) when compared to control untreated cells.

Table 3: Effect of coumarinic derivatives products on Huh7 cell cycle progression. Huh7 were serum deprived for one night and treated or not with coumarinic derivatives or Doxorubicin for 24h at 1 or 10µM. Then, cells were stained with propidium iodide-PBS and analysis by flow cytometry. Cell histogram on FL2 was divided into three parts according to the cell cycle phases, sub-G_1, G_0-G_1 and S+G_2-M. Data are presented with the mean ± SEM of three independent experiments. *p<0.05 versus untreated cells.

	% of Huh7 in cell cycle phases (mean ± SEM)		
Treatments	**Sub-G1**	**G0-G1**	**S+G2/M**
Untreated cells	27,92 % ± 11,38 %	37,50 % ± 4,08 %	34,59 % ± 9,73 %
Dox (1µM)	**67,42 % ± 6,21 % ***	21,45 % ± 10,57%	**11,13 % ± 4,42 % ***
Dox (10µM)	**59,42 % ± 7,46 % ***	24,96 % ± 5,52 %	**15,63 % ± 3,91 % ***
4 (1µM)	42.87 % ± 11,93 %	31,46 % ± 4,26 %	25,67 % ± 8,40 %
4 (10µM)	35,31 % ± 7,55 %	35,63 % ± 1,58 %	29,06 % ± 7,89 %
5 (1µM)	34,72 % ± 3,49 %	35,91 % ± 4,40 %	29,37 % ± 3,50 %
5 (10µM)	**54,64 % ± 17,29 % ***	22,11 % ± 5,08 %	21,58 % ± 10,6 %
8 (1µM)	37,40 % ± 7,77 %	34,05 % ± 1,27 %	28,55 % ± 6,70 %
8 (10µM)	41,44 % ± 5,70 %	33,76 % ± 2,70 %	24,80 % ± 8,01 %
9 (1µM)	34,00 % ± 3,41 %	35,06 % ± 2,88 %	30,94 % ± 1,34 %
9 (10µM)	**54,41 % ± 10,35 % ***	27,01 % ± 5,33 %	18,59 % ± 5,12 %

4. Discussion

A new versatile synthetic route to a new derivatives from *N*-substituted-4-Oxo-[1] benzopyrano [4, 3-c] pyrazole (4, 5, 8 and 9) by the treatment of 4-hydroxycoumarin with different reagents is described; the method is rapid and produced all compounds in good yields. The newly synthesized compounds were evaluated for their antitumoral activity against Huh7 cells. This series of derivatives were widely active in almost all bioassays. With regard to the Anti-proliferative activity analyzed by MTT assay all the title compounds are potent

inhibitors and the compound 5 exhibited the most potent inhibitory activity (IC_{50}=10.37μM) against Huh7 cells. The cytotoxicity effect was dose-dependent and time-dependent. Indeed all compounds did affect significantly Huh7 spreading as compared to untreated control cells even at low concentrations (0.01 μM to 100 μM). The ability of derivatives (4, 5, 8 and 9) to inhibit cell spreading was concentration dependent. These results showed that the presence of each compound decrease cellular spreading and thus inhibited adhesion of Huh7 cancer cells *in vitro* and that their action was dependent on their concentration, confirming to some extent the results obtained by LDH assays. The mechanism of inhibition of cell spreading remains unknown, but we can suppose that down-regulation of cell spreading by our compounds may involve effects on cytoskeletal processes required for cell spreading in as much as the observed impairment of the actin network forming the peripheral cytoskeleton of the cell is involved in both membrane integrity and integrin-mediated cell spreading. All of the derivatives (4, 5, 8 and 9) used in this study may thus function as tumor suppressors by causing the embrittlment of the membrane and by negatively regulating cell interactions with the extracellular matrix. For example, the compound 5 prevented cells from spreading at normal rates, and it reduced to about 38% the number of spread cells, at a concentration of 100 μM, with altered morphology and impaired cytoskeletal formation. Also the cell viability of Huh7 cells treated with these derivatives and especially with the compound 9 was potentially reduced by 81.41 % in the G2 / M phase. So, the presence of apoptotic cells results in an increasing percentage at the sub-G1 position [22]. These results suggest that the cell cycle is stopped after treatment, and it may also confirm the induction of apoptosis in Huh7 cells by compounds 4, 5, 8 and 9 in a dose dependent manner.

On the other hand, all derivatives inhibited to some extent the basal invasion of Huh7 cells through the Matrigel .This effect may involve actually either inhibition of the spreading of Huh7 cells to the Matrigel and therefore their interaction with the components of the MEC.

Together, these results of the current study should be further confirmed by evaluating anti-cancer activity *in vivo* and may open new avenues for cancer treatment, leading to possible clinical applications.

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