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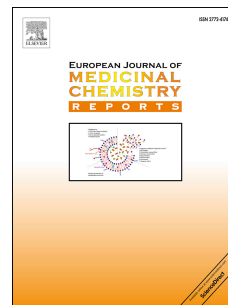


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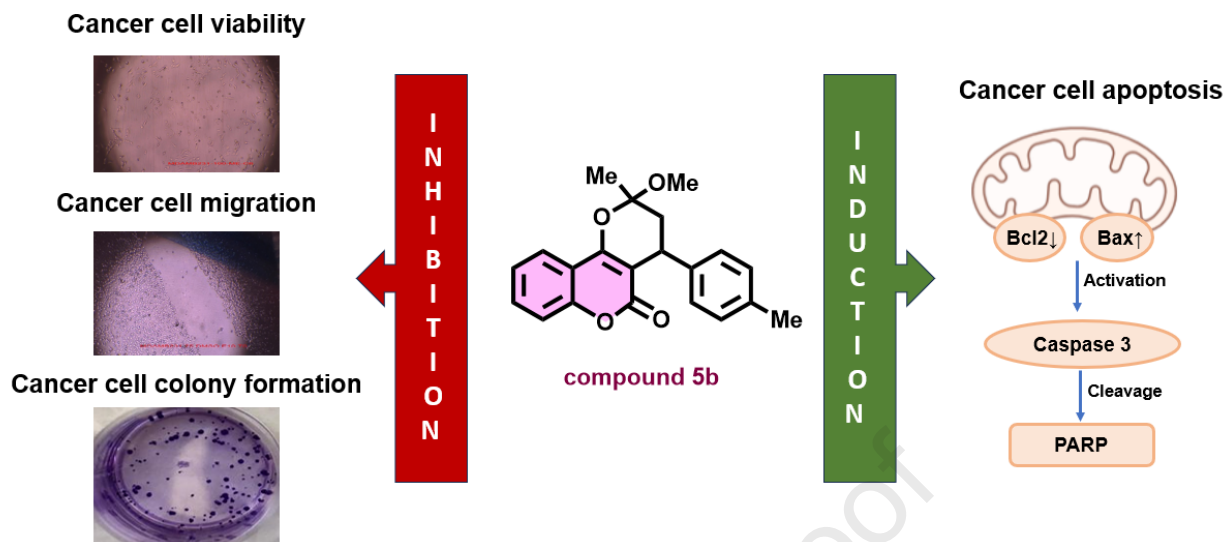
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Anticancer effect of new cyclocoumarol derivatives

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33 **1. Abstract:**

34 Coumarins have demonstrated a broad spectrum of pharmacological activities, including
35 significant anticancer properties. Recently, a series of cyclocoumarol derivatives, a
36 pyranocoumarin known for its anticoagulant and anti-inflammatory effects, have been
37 synthesized and shown to inhibit cyclooxygenase-2 (COX-2) activity selectively. In this study,
38 we evaluated the anticancer effects of these cyclocoumarol derivatives for the first time. We
39 tested their antiproliferative effects on several human cancer cell lines, including MDAMB231,
40 A549, MCF7, SF268, HCT116, HeLa, and Jurkat. The MTT assay revealed that the methylated
41 cyclocoumarol derivative, 2-methoxy-2-methyl-(1-(p-tolyl))-3,4-dihydropyrano [3,2-c]
42 chromen-5(2H)-one, **5b** exhibited the most potent antiproliferative activity, particularly against
43 MDAMB231 cells. Further investigations demonstrated that **5b** induced apoptosis in
44 MDAMB231 cells via the mitochondrial pathway, as indicated by increased Bax and decreased
45 Bcl2 levels, along with caspase-3 activation and PARP cleavage. Additionally, compound **5b**
46 significantly inhibited the clonogenic potential of MCF7 cells and reduced the migration
47 capacity of MDAMB231, SF268, and A549 cells. These findings suggest that **5b** is a promising
48 lead compound for developing new anticancer agents, with the potential for chemical
49 optimization to enhance its efficacy.

50

51

52 2. Keywords

53 *Coumarins, cyclocoumarol, anticancer effect, apoptosis, migration*

54

55 3. Abbreviations

56 Akt, protein kinase B (PKB)

57 Bax, Bcl-2-associated X-protein

58 Bcl2, B-cell lymphoma 2

59 COX-2, cyclooxygenase-2

60 DMEM, Dulbecco's Modified Eagle Medium

61 DMSO, dimethylsulfoxide

62 ELISA, enzyme-linked immunosorbent assay

63 FBS, fetal bovine serum

64 IC₅₀, inhibitory concentration 50

65 mTOR, mammalian target of rapamycin

66 MTT, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide

67 NMR, nuclear magnetic resonance

68 NQO1, NAD(P)H Quinone Dehydrogenase 1,

69 OD, optical density

70 PARP, poly ADP-ribose polymerase

71 PBS, Phosphate-buffered saline

72 PI3K, Phosphoinositide 3-kinase

73 RIPA, Radio-Immunoprecipitation Assay

74 RPMI, Roswell Park Memorial Institute

75 SD, standard deviation

76

77 4. Introduction:

78

79 Cancer is a leading cause of death worldwide. According to GLOBOCAN estimates, nearly 10
80 million deaths in 2020 were attributed to cancer (1). The global cancer burden is rising with
81 time because of increased incidence and rapid development of cancer resistance to current
82 therapies (2) making the search for new and efficient treatments a continuous need.

83 Coumarins are oxygenated polycyclic and polyphenolic compounds that are widely distributed
84 in nature. Synthetic and natural Coumarins have been extensively studied and were shown to
85 display several pharmacological activities such as anti-infectious, anticancer, antioxidant, anti-
86 inflammatory, anticoagulant, and neuroprotective activities (3). The anticancer effects of
87 coumarins have been largely reviewed (4,5), and several anticancer mechanisms were
88 identified including, induction of cell cycle arrest and apoptosis, induction of oxidative stress,
89 inhibition of carbonic anhydrase, inhibition of topoisomerase, inhibition of microtubule
90 polymerization, inhibition of PI3K/AKT/mTOR signaling pathway and inhibition of
91 angiogenesis (6–8). Moreover, coumarin compounds were found very promising in drug-
92 resistant cancers as they can inhibit the activity of multidrug resistant proteins (9).

93 Cyclocoumarol is a pyranocoumarin with anticoagulant and anti-inflammatory activity. We
94 previously designed, synthesized and characterized a series of six cyclocoumarol derivatives
95 and demonstrated *in vitro* their selective inhibitory activity on COX-2. These compounds were
96 prepared by cyclisation of corresponding functionalized warfarins in methanolic HCl 4% under
97 reflux. After chromatographic purification major and minor diastereomers were isolated and
98 their structures were confirmed by ¹H NMR and X-ray crystallographic. Moreover, we showed
99 *in silico* the binding mode of these derivatives to the COX-2 binding site. Interestingly, the
100 major diastereomers displayed higher COX-2 inhibitory activity than the minor ones (10).

101 In an effort to continue the research on cyclocoumarol derivatives, we assessed for the first
102 time, in this work, the anticancer effect of the major diastereomers of these cyclocoumarol
103 derivatives and characterized the anticancer mechanism of the most promising cyclocoumarol
104 derivative, 2-methoxy-2-methyl-(1-(p-tolyl))-3,4-dihydropyrano [3,2-c]chromen-5(2H)-one,
105 or compound **5b** bearing a methyl group.

106

107 5. Materials and Methods

108

109 5.1. Cell culture

110 Human triple negative breast cancer MDAMB231, Human non-small cell lung carcinoma
111 (NSCLC) A549, human receptor positive breast cancer MCF7, human glioblastoma SF268,
112 human colorectal cancer HCT116, human cervical cancer HeLa, and human T-cell leukemia
113 Jurkat cell lines were purchased from the American Type Culture Collection (ATCC). Solid
114 cancer cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma life
115 science, D0819) supplemented with 10% fetal bovine serum (FBS) (Sigma life science F9665),
116 1% of a mixture of penicillin and streptomycin (Biowest, L0022-100) and 1% L-Glutamine
117 (Sigma life science, G7513) and Jurkat cells were cultured in RPMI-1640 (Sigma life science,
118 R8758) supplemented with 10% FBS and 1% of penicillin/streptomycin mixture. Cultures were
119 maintained at a humid atmosphere with 5% CO₂ at 37°C.

120

121 **5.2. Coumarins solution preparation**

122 Coumarin derivatives were solubilized using a mixture of equal volumes of dimethylsulfoxide
123 (DMSO) and acetone. These concentrated stock solutions (20 mM) were prepared
124 extemporaneously before each experiment and diluted to the final concentrations using the
125 complete cell medium. The control groups consisted of the same mixture used as solvent for
126 the coumarins.

127

128 **5.3. Tetrazolium bromide salt (MTT) test**

129 Cancer cells were seeded in 96-well plates (3000 cells/well) and allowed to adhere for 24 h.
130 The next day, cells were treated with cyclocoumarol derivatives that were serially diluted to
131 reach final concentrations ranging from 10 µM to 150 µM. Control groups were treated with a
132 1V/1V mixture of DMSO/acetone. After 48 hours of incubation, cell media were aspirated and
133 replaced with 100 µL of DMEM mixed with Tetrazolium bromide salt (MTT, Sigma M5655)
134 at a final concentration of 0.5 mg/mL. Cells were incubated in MTT solution at 37 °C for 3
135 hours to allow the conversion of MTT to formazan by the mitochondrial enzymes. MTT
136 solution was then removed, and the formed formazan product was solubilized with 100 µL of
137 lysis buffer (SDS 20%, DMF 33%, acetic acid 2%, HCl 0.005 N, pH7). The optical density
138 (OD) of the formazan was determined at 570 nm in an enzyme-linked immunosorbent assay
139 (ELISA) reader (Biotek synerg 2, 266278). This experiment was repeated three times and
140 wells were prepared in quadruplicate for each condition. The percentage of viability was
141 calculated according to the following equation:

$$142 \frac{\text{mean OD of treated cells} - \text{mean OD of blank}}{\text{mean OD of control cells} - \text{mean of blank}} * 100$$

143 where the blank was the lysis buffer.

144

145 **5.4. Scratch-wound assay**

146 Cancer cells were seeded in 96-well plates in complete culture medium and maintained until
147 confluence. When confluent, the cell monolayer was scratched with the end of a sterile tip of
148 10 μ L; then the culture medium was replaced by a medium containing the compound **5b** at
149 different concentrations (0 - 150 μ M). Images were taken using an inverted microscope
150 immediately after wound creation (T_0) and 24 hours after treatment (T_{24}). Cell migration was
151 evaluated by measuring the size of the cell-free surface between the two sides of the scratch at
152 T_0 and T_{24} . Results are expressed as the percentage of wound closure in each condition.

153

154 **5.5. Clonogenic assay**

155 MCF7 cancer cells were seeded in 6 well plates (400 cells per well) and allowed to adhere
156 overnight. After 24 hours, cells were treated with 10, 25, 50, 75, 100 or 150 μ M of compound
157 **5b** or control and incubated for 10 days to allow colonies formation. Cells were washed with
158 PBS, fixed with methanol, and stained with 0.5 % crystal violet for 30 minutes. Finally, cells
159 were washed with distilled water and were left to dry overnight at room temperature. Colonies
160 were counted, and results were expressed as plating efficiency (PE).

$$161 \quad PE = \text{Number of colonies counted} \div \text{Number of cells seeded} \times 100$$

162

163 **5.6. Protein extraction and western blot**

164 MDAMB231 cells were seeded in 6-well plates (300,000 cells/well) and treated, the following
165 day, with compound **5b** then incubated for 48 hours. Cells were washed twice with cold PBS
166 solution and lysed in RIPA buffer (Sigma, R0278). Then, cell lysates were transferred to 1.5
167 mL tubes and centrifuged at 12000 rpm for 10 minutes at 4 $^{\circ}$ C. The supernatant containing the
168 proteins was separated from the pellet, and the samples were frozen in aliquots at -80 $^{\circ}$ C until
169 the day of the experiment. The protein concentration of the lysates was determined using the
170 Bradford method.

171 Fifty μ g of proteins were separated on SDS-PAGE (SDS polyacrylamide gel electrophoresis)
172 and transferred onto a nitrocellulose membrane. The membranes were blocked with 5%
173 skimmed milk for 1 h, and then incubated overnight with primary antibodies (abcam, 1:1000)
174 at 4 $^{\circ}$ C. After three washes with PBST, the membranes were incubated with peroxidase-
175 coupled anti-rabbit antibody (abcam, 1: 10000) for 2 h at room temperature. After a final wash,
176 peroxidase activity was revealed using Clarity ECL reagent (Bio-Rad). ImageJ was used to
177 quantify protein bands relatively.

178

179 5.7. Statistical analysis

180 Data were analyzed using Microsoft Excel (Microsoft Office Professional Plus 2019 version).
181 The unpaired Student's t-test was performed to compare the means of the control group to those
182 of each coumarin derivative concentration. All experiments were repeated independently at
183 least twice. A $p < 0.05$ was considered statistically significant.

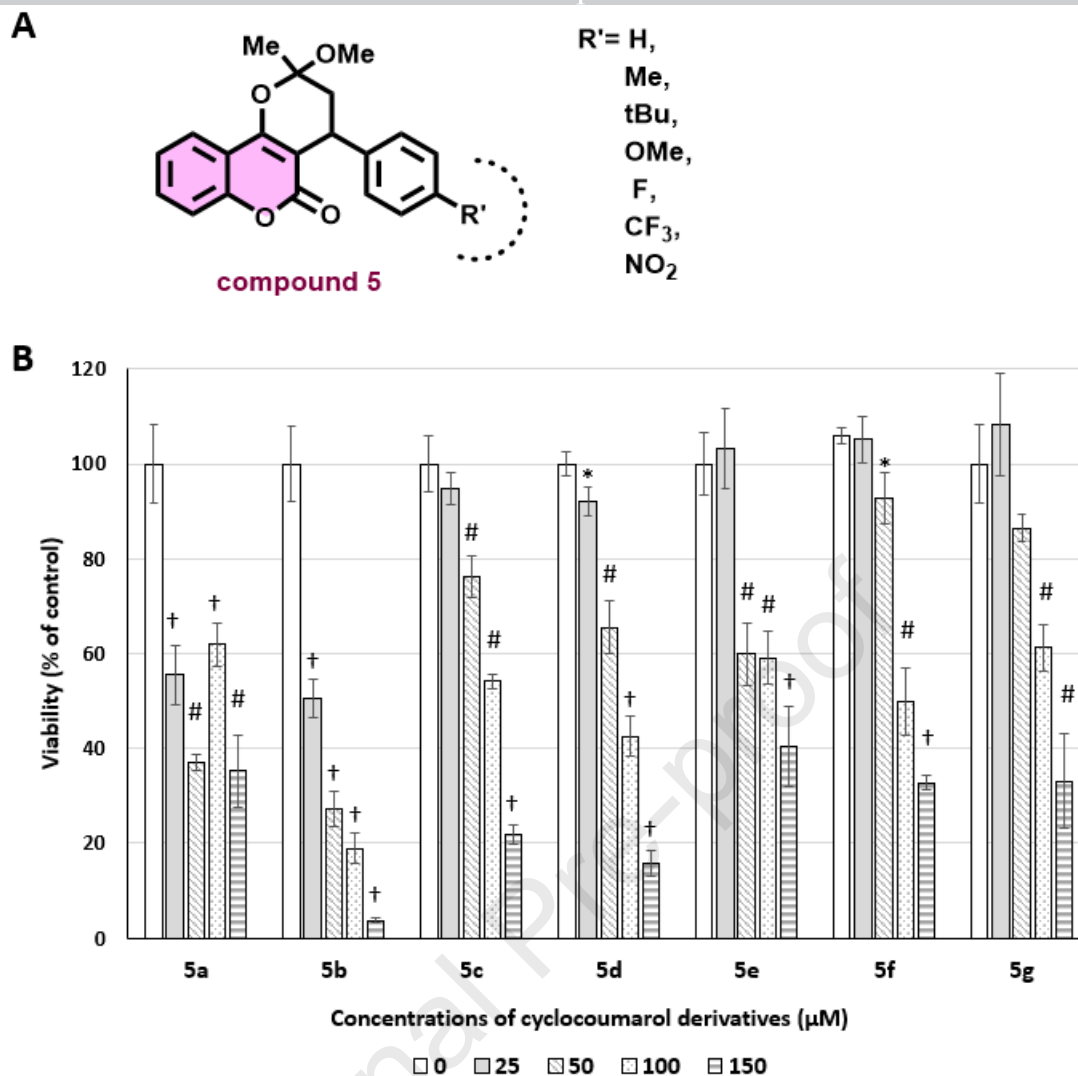
184 6. Results and discussion

185 6.1. Cyclocoumarol derivatives show various antiproliferative effects.

186
187 We began by evaluating the effect of all the cyclocoumarol derivatives on the viability of the
188 MDAMB231 cell line using the MTT test. It is noteworthy that crystallization occurred in the
189 wells containing 100 and 150 μM of compounds **5a** (cyclocoumarol), the trifluoromethylated
190 compound (**5f**) and the compound bearing a *t*-Bu (**5c**). Thus, we could not determine their
191 effects as, firstly, the concentration cannot be well calculated, and, secondly, there is a
192 possibility that the observed effect on the viability could be related to the toxicity of the
193 precipitated crystals. As shown in figure 1, the other cyclocoumarol derivatives showed various
194 antiproliferative effects where the highest concentration (150 μM) of the compounds **5b**, **5d**,
195 **5g**, and **5e** (bearing the groups Me, OMe, F, and NO_2 respectively) decreased cell viability by
196 96, 84, 66 and 59% respectively. Interestingly, the compounds **5b** and **5d** that showed the
197 highest antiproliferative effect on the MDAMB231 cell line are the same that exhibited
198 previously the highest percentage of PGE2 synthesis inhibition (10).

199
200 As the compound **5b** bearing the methyl group showed the most potent antiproliferative effect,
201 we chose this compound for further studies.

202
203



204

205 Figure 1. Structure of the major diastereomers of cyclocoumarol (**5a**) and its derivatives (**5b-**
 206 **g**) and their antiproliferative effect on MDAMB231 cell line. A. chemical structure of
 207 cyclocoumarol and its derivatives. B. MDAMB231 cells were treated with the indicated
 208 concentrations of the coumarin derivatives. Cell viability was assessed after 48 hours using the
 209 MTT assay. Data is represented as average \pm SD. The significance of the difference between
 210 the control group and different concentrations of coumarin derivatives was calculated by
 211 Student's t-test (* $p < 0.05$, # $p < 0.01$, † $p < 0.001$).

212

213 **6.2.** The compound **5b** inhibits the proliferation of several cancer cell lines.

214 We then conducted a screening of the antiproliferative effect of the compound **5b** using
 215 different cancer cell lines. Cells were treated with ascending concentrations of **5b** for 48 hours,
 216 then viability was assessed using MTT assay, and inhibitory concentrations 50 (IC₅₀) were
 217 calculated (table 1). Our results show that the compound **5b** decreased the viability of all cancer
 218 cell lines. The triple negative breast cancer MDAMB231, the glioblastoma SF268, and the

219 NSCLC A549 cells lines were associated with IC₅₀ below 100 μM whereas the hormone
 220 positive breast cancer MCF7, the cervical cancer HeLa, the colorectal cancer HCT116 and the
 221 Leukemia Jurkat cell lines had IC₅₀ equal or above 100 μM. Moreover, the most sensitive cell
 222 line in our cell screening was MDAMB231 with an IC₅₀ of 25 μM. A possible explanation for
 223 the difference in cell line sensitivity to **5b** could be the level of expression of COX-2. Indeed,
 224 COX-2 is known to promote oncogenesis, in particular for breast cancer (11,12). Several
 225 studies showed that MDAMB231 cells express higher levels of the COX-2 protein than MCF7
 226 cells (13–16) and that suppressing COX-2 expression or activity decreases the viability,
 227 motility, and invasiveness of breast cancer cell lines. Therefore, the higher COX-2 expression
 228 level of MDAMB231 can account at least in part for the higher sensitivity to the compound **5b**
 229 compared to MCF7 cell lines. However, other mechanisms of action should also be involved
 230 as other cells also known to express high levels of COX-2 (HeLa and A549 (17)) showed high
 231 IC₅₀ when treated with the compound **5b**.

232

233 Table 1. IC₅₀ values of the compound **5b** after 48 hours of treatment of cancer cell lines.

	MDAMB231	MCF7	SF268	A549	HCT116	Jurkat	Hela
IC ₅₀ (μM)	25	113	49	96	124	137	100

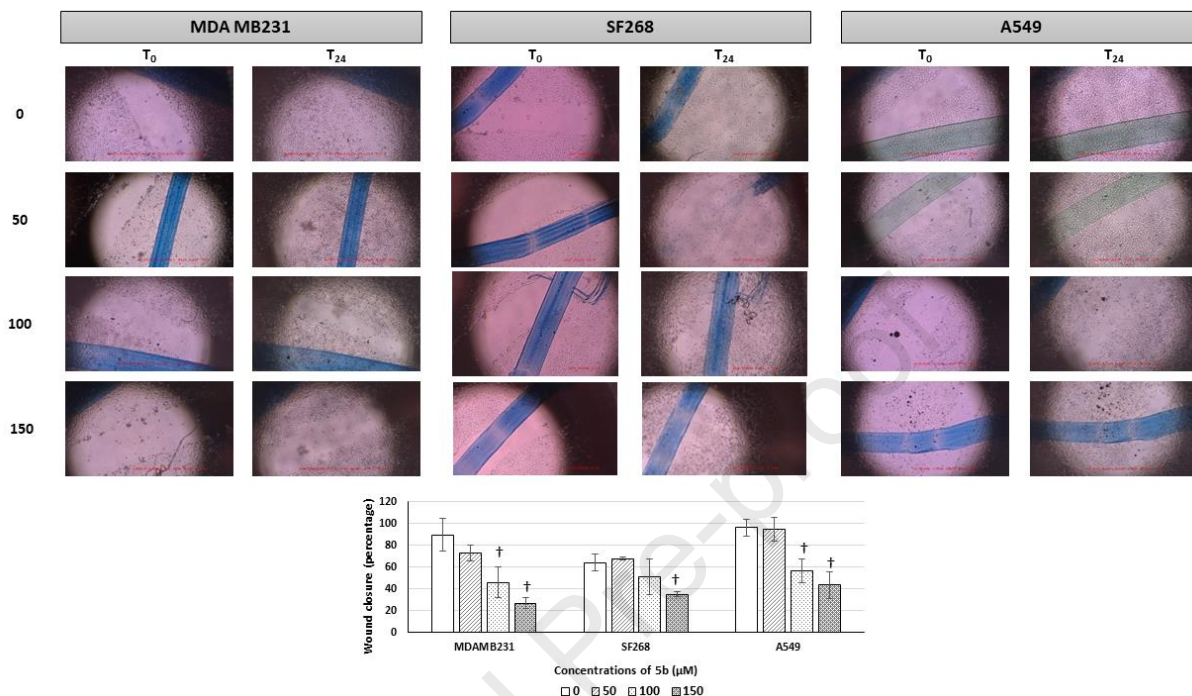
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235

236 6.3. The compound **5b** inhibits migration of cancer cell lines

237 Migration and invasion constitute an important hallmark of cancer cells and allow these to form
 238 metastasis. We chose the three cancer cell lines displaying the lowest IC₅₀ at 48 hours (table 1)
 239 and assessed the effect of compound **5b** on their migration potential using the scratch assay.
 240 As seen in figure 2, within 24 hours, the wound closed almost completely (95%) for control
 241 A549 cells, while it closed by 90% and 67% for control MDAMB231 and SF268 cells,
 242 respectively. The compound **5b** decreased the percentage of wound closure dose-dependently
 243 in the three cell lines. Again, the highest activity was observed in MDAMB231 cells where
 244 150 μM of compound **5b** blocked wound closure by 70% compared to control cells. The anti-
 245 migratory activity of the compound **5b** was lower for A549 and SF268 cells with an inhibition
 246 of wound closure by 54 % and 45 %, respectively, at the concentration 150 μM compared to
 247 control cells. Several coumarins were reported to block migration of cancer cells and some
 248 mechanisms of these effects were identified. For example, osthole was found to block the
 249 migration and invasion of MDAMB231 and MCF7 cells by downregulating integrins α3 and
 250 β5 (18); esculetin could inhibit the migration of prostate cancer PC3 cells, this effect could be
 251 related to its ability to modulate PI3K/Akt/mTOR axis (19); scopoletin inhibited the migration

252 of the cholangiocarcinoma KKKU-100 cells by decreasing the MMP9/TIMP1 ratio and
 253 inhibiting NQO1 (20); auraptene reduced migration and invasion of melanoma B16F10 cells
 254 through suppressing the activity of MMP2 and MMP9 and downregulating mesenchymal
 255 markers as fibronectin and *N*-cadherin (21).
 256



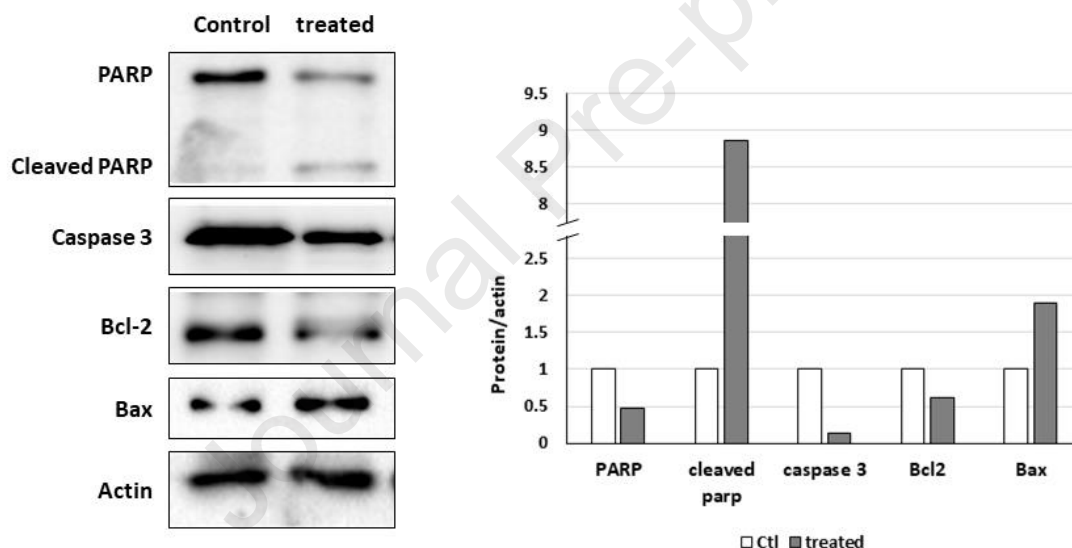
257
 258 Figure 2. Compound **5b** decreases the migration potential of cancer cell lines. Confluent cancer
 259 cells were scratched using a pipette tip and then treated with the indicated concentrations of
 260 compound **5b** for 24 hours. A. Representative images (x100) of MDAMB231, SF268 and A549
 261 cells at times 0 (T₀) and 24 hours (T₂₄) of treatment. B. Percentage of wound closure for each
 262 condition. Data is represented as average ± SD. The significance of the difference between the
 263 control group and different concentrations of compound **5b** was calculated by Student's t-test
 264 (†p<0.001).

265

266 **6.4. The compound 5b induces apoptosis of MDAMB231 cells.**

267 In order to reveal the mechanism underlying the antiproliferative effect of compound **5b**, we
 268 investigated its effect on several key proteins in the apoptosis machinery. The intrinsic pathway
 269 of apoptosis, also called the mitochondrial pathway, is regulated by proteins from the Bcl2
 270 family. Cancer cells resist apoptosis by upregulating the anti-apoptotic Bcl2 protein and
 271 downregulating proapoptotic proteins as Bax (22). Caspases are proteases that are responsible
 272 for the initiation and execution of the apoptotic cascade. Caspase 3 is an executioner caspase
 273 that inactivates, through cleavage, several substrates, particularly poly ADP-ribose polymerase

274 (PARP), a key enzyme in the DNA repair mechanism (23). We treated MDAMB231 cells with
 275 100 μ M of compound **5b** for 24 hours, then assessed several apoptosis-related proteins by
 276 western blotting. Our results show that compound **5b** induces cell apoptosis through the
 277 mitochondrial pathway as expression levels of the pro-apoptotic protein Bax increased and
 278 those of the anti-apoptotic protein Bcl2 decreased (Figure 3). Furthermore, levels of procaspase
 279 3 were decreased, and the activation of the caspase 3 was confirmed by demonstrating the
 280 cleavage of its substrate PARP. Similar modulation of these apoptosis-related proteins was
 281 demonstrated for several coumarin derivatives, e.g., decursin and decursinol angelate in breast
 282 cancer MDAMB231 and MCF7 cells (24); ferulin C in breast cancer MDAMB231 and MCF7
 283 cells (25), pulchrin A in ovarian cancer CAOV-3 and SKOV-3 cells (26), coumarin and 7-
 284 hydroxycoumarin in human lung cancer cell lines, A427 and Calu-1 (27),
 285 thiazolylpyrazolyl coumarin derivatives in breast cancer MCF7 cells (28) and auraptene in
 286 prostate cancer PC3 and DU145 cells (29).



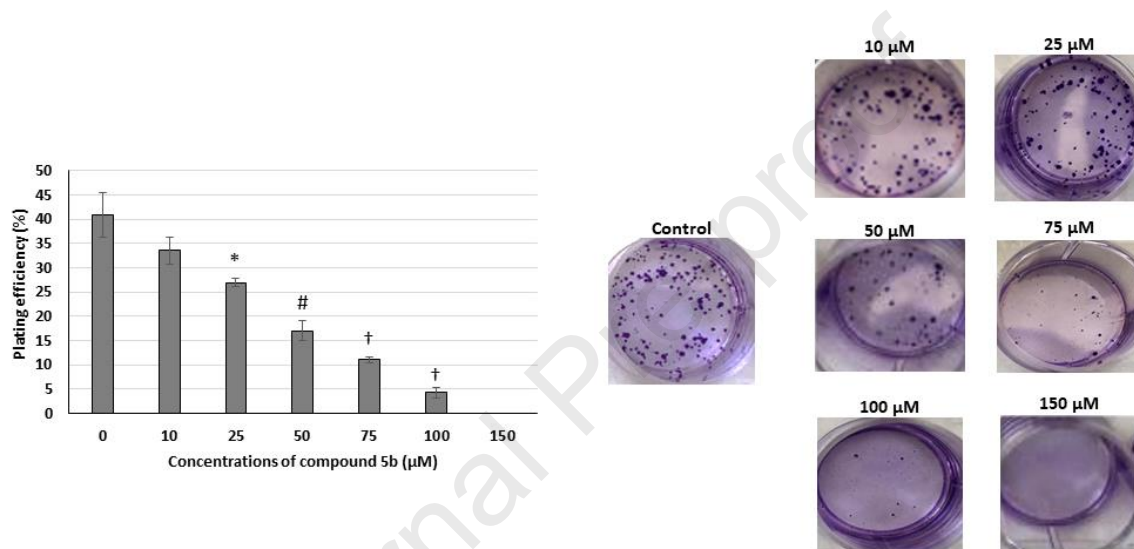
287
 288 Figure 3. Compound **5b** induces apoptosis of MDAMB231 cells. Cells were treated with 100
 289 μ M of compound **5b** for 24 hours, then lysed, and proteins were analyzed by western blotting
 290 using several antibodies. β -actin served as a loading control. The right panel shows band
 291 intensity quantification represented as fold change to the control.

292

293 **6.5. The compound **5b** inhibits clonogenic potential of MCF7.**

294 The clonogenic assay is based on the ability of a single cell to develop into a colony that
 295 consists of at least 50 cells (30). It is considered as a gold standard test that allows the
 296 evaluation of the reproductive capacity of a cell *in vitro*. We wanted to assess the long-term

297 effect of compound **5b** on MCF7 cells; we chose this cell line because it was not very sensitive
 298 to the short-term effect of this compound with an IC_{50} at 48 hours above 100 μM . Interestingly,
 299 a continuous treatment of 10 days of MCF7 cells inhibited the clonogenic potential of these
 300 cells in a dose-dependent and very efficient way. Indeed, a concentration of 50 μM (almost half
 301 of the short-term IC_{50} of 113 μM) decreased the number of colonies by 60% compared to the
 302 control (Figure 4). Moreover, the concentration of 100 μM (very close to the short-term IC_{50})
 303 substantially reduced the colonies by 90% compared to the control, and, interestingly, no
 304 colony was formed in the wells treated by the concentration 150 μM . This result suggests that
 305 the effect of the compound **5b** becomes more important with time.
 306



307
 308 Figure 4. Compound **5b** decreases the clonogenicity of MCF7 cells. Cells were seeded at very
 309 low density and treated with indicated concentrations of compound **5b** for 10 days. Then,
 310 colonies were fixed and stained with crystal violet and counted, and plating efficiency (PE)
 311 was calculated. Data are expressed as averages \pm SD (* $p < 0.05$, # $p < 0.01$, † $p < 0.001$)
 312

313 Conclusion

314 We report hereby, for the first time, the anticancer activity of cyclocoumarol derivatives. Our
 315 study identified the methylated cyclocoumarol (compound **5b**) as the most promising molecule
 316 as it was the most potent derivative regarding antiproliferative activity on cancer cells in
 317 particular MDAMB231, it blocked the clonogenic potential of MCF7, reduced the migration
 318 capacity of MDAMB231, SF268 and A549 cells and induced apoptosis of MDAMB231 cells
 319 through the intrinsic pathway. Therefore, we used the compound **5b** as a lead molecule for new
 320 chemical optimizations and generated a new series of coumarin derivatives. Biological studies

321 of these compounds are currently underway with the hope to reach higher potency and get good
322 candidates for the development of new anticancer agents.

323

324 **Author Contributions:**

325 Racha Karaky: Conceptualization, Data curation, Formal analysis, Investigation, Methodology,
326 Project administration, Resources, Supervision, Validation, Visualization, Writing - original
327 draft, Writing - review & editing

328 Joumana Al-Akhdar: Data curation, Formal analysis, Investigation, Visualization

329 Fatima Saab: Data curation, Formal analysis, Investigation, Visualization

330 Batoul Rostom: Data curation, Formal analysis, Investigation, Visualization

331 Wassim Shebaby: Conceptualization, Data curation, Formal analysis, Investigation,
332 Methodology, Resources, Validation, Visualization, Writing - review & editing

333 Mohamed Mroueh: Conceptualization, Funding acquisition, Methodology, Resources, Writing
334 - review & editing

335 Mona Diab-Assaf: Conceptualization, Funding acquisition, Resources, Writing - review &
336 editing

337 Issam Kassab: Conceptualization, Methodology, Supervision, Writing - review & editing

338 Maité Sylla-Iyarreta Veitía: Conceptualization, Funding acquisition, Methodology, Project
339 administration, Resources, Supervision, Validation, Visualization, Writing - review & editing

340

341 **Conflict of interest statement**

342 The authors have no conflicts of interest to declare.

343

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Highlights

- The anticancer effect of the major synthetic diastereomers of cyclocoumarol and its derivatives was assessed.
- Cyclocoumarol derivatives decreased viability of MDAMB231 breast cancer cells
- Compound **5b**, 2-methoxy-2-methyl-(1-(p-tolyl))-3,4-dihydropyrano [3,2-c] chromen-5(2H)-one, was the most potent with an IC₅₀ on MDAMB231 of 25 μM
- Compound **5b** reduced cancer cell line migration and clonogenic potential
- Compound **5b** induced apoptosis of MDAMB231 cells

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The author is an Editorial Board Member/Editor-in-Chief/Associate Editor/Guest Editor for *[Journal name]* and was not involved in the editorial review or the decision to publish this article.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: