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Coumarin derivatives exert anti-lung cancer activity by inhibition of epithelial-mesenchymal transition and migration in A549 cells

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ABSTRACT: A series of coumarin derivatives and isosteres were synthesized from the reaction of triflic intermediates with phenylboronic acids, terminal alkynes and organozinc compounds, through palladium-catalyzed cross-coupling reactions. The *in vitro* cytotoxic effect of the compounds was evaluated against two non-small cell lung carcinoma (NSCLC) cell lines (A-549 and H2170 (human lung adenocarcinoma cells)) and normal cell line (fibroblast healthy NIH-3T3) using cisplatin (2.6 μ M) as reference drug. Additionally, the effects of the most promising coumarin derivative (**9f**) in reversing the epithelial-to-mesenchymal transition (EMT) in IL-1 β -stimulated A549 cells, and in inhibiting the EMT-associated migratory ability in A549 cells were also evaluated. Compound **9f** had the greatest cytotoxic effect ($CC_{50} = 7.1 \pm 0.8$ and 3.3 ± 0.5 μ M, respectively against A549 and H2170 cells) and CC_{50} value of 25.8 μ M for NIH-3T3 cells. Compound **9f** inhibited the IL-1 β -induced EMT in epithelial cells by inhibiting the F-actin reorganization, attenuating changes in the actin cytoskeleton reorganization and by downregulating of vimentin in A549 cells stimulated by IL-1 β . Treatment of A549 cells with **9f** at 7 μ M for 24 h significantly reduced migration of IL-1 β -stimulated cells, phenomenon confirmed by qualitative assessment of the wound closure. Taken together, our finding suggest that coumarin derivatives, especially compound **9f** may become a promising candidate for lung cancer therapy, especially in lung cancer promoted by non-small cell lung carcinoma (NSCLC) cell lines.

Keywords: anticancer activity, lung cancer, non-small-cell lung cancer, epithelial-mesenchymal transition, methastasis, coumarin derivative.

These authors contributed equally to this work.

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39 1. Introduction

40 Cancer is characterized by cells with uncontrolled division, genome heterogeneity, and
41 invasiveness to other tissues via blood or lymph nodes. According to the World Health Organization
42 (WHO) reports, almost 9 million cancer-related deaths annually occur [1]. Among cancers, the lung
43 cancer is one of the most common type, with a mortality rate of around 18.4% according to
44 GLOBOCAN report [2].

45 Cancer metastasis is defined as the formation of new tumors in tissues away from the primary
46 site, account for a vast majority of morbidity and mortality of patients and is associated with about
47 90% of all cancer-associated deaths [3,4]. In the past decade, an increasing number of studies have
48 provided strong evidence to proposed that epithelial-mesenchymal transition (EMT) — a known
49 cellular program allowing polarized cells to shift to a mesenchymal phenotype with increased cellular
50 motility [5] — has a central role in cancer progression and metastatic dissemination [6-8]. For this
51 reason, the EMT has become as a target of interest for anticancer therapy [9, 10].

52 Furthermore, cancer therapy is complex due mainly to drug-resistance, which leads to less
53 effectiveness of the anticancer agents. Therefore, the discovery and development of new
54 chemotherapeutic agents with greater efficacy is very urgent need.

55 Coumarins are a class of secondary metabolites chemically characterized by the fusion of a
56 benzene with an α -pirone ring [11]. Their pharmacological applications are widely described [12-17],
57 highlighting its applications in the treatment of several human cancer and in the inhibition of cell
58 growth of several cancer cell lines [18-27], including lung cancer [21, 28-37]. Its low toxicities [38,
59 39], associated with its potential to inhibit several proteins associated with lung cancer (tyrosine
60 kinase, telomerase, NF- κ B, ERK1/2, EGFR, STAT proteins, HSP 90, PI3K, Bax, among others) [24,
61 26, 28], makes them as promising prototypes for the development of new anti-lung cancer drugs.

62 In view of the above, the aim of this study was to synthesize a series of coumarins derivatives
63 obtained through palladium-catalyzed cross-coupling reactions (PCCCR), and evaluate their cytotoxic
64 effects *in vitro* in two non-small two cell lung carcinoma (NSCLC) cell lines (A549 and H2170).
65 Additionally, were investigated the potential of the most promising coumarin derivative (**6f**) in
66 reversing the epithelial-to-mesenchymal transition (EMT) in IL-1 β -stimulated A549 cells, and in
67 inhibiting the EMT-associated migratory ability in A549 cells.

68 2. Materials and Methods

69 2.1. Compounds (synthetic coumarins)

70 Compounds **1d** [40], **3** [41], **5b** [42], **6** [43], **8a** [44], **8d** [45], **9a** [44], **9b** and **9c** [46], **9d** [44],
71 **9g** [47], **10** [43], **12a** [48], **13a** [49] and **17** [50] were synthesized and structure of these compounds
72 has been confirmed by comparison with NMR spectral data from the literature.

73 All other coumarin derivatives: triflic intermediates (**4**, **5a**, **5b**, **6**); Suzuki-Miyaura adducts (**7**,
74 **8a-g**, **9a-f**); Sonogashira adducts **11**, **12a-c**, **13 a-c**); Negishi adducts (**14** and **15**); and alkyl coumarin
75 derivatives obtained by catalytic hydrogenation (**16** and **17**) were prepared according to the synthetic
76 procedures described in the supplementary material.

77 2.2. Biological Assays

78 2.2.1. Cell line and cell culture

79 A549, H2170 and normal mouse fibroblast (NIH-3T3) cell lines were obtained from the Rio de
80 Janeiro Cell Bank (BCRJ). A549 and NIH-3T3 cells were maintained in Dulbecco's Modified Eagle
81 Medium (DMEM), while H2170 cell line was maintained in Roswell Park Memorial Institute (RPMI)-
82 1640. The culture media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine,
83 40 $\mu\text{g/mL}$ gentamicin. All cells were cultured in a humidified atmosphere contained 5% CO₂ incubator

84 at 37 °C. For experiments, cells were grown to 90% confluence. All experiments were conducted using
85 cells with passage numbers less than 10.

86 **2.2.2. Cell viability assay and treatment**

87 The effect of coumarin derivatives on cell viability was evaluated by the MTT assay at a single
88 dose according to NCI testing protocol or at different concentrations for IC₅₀ determination [51].
89 Coumarin derivatives were dissolved in dimethyl sulfoxide (DMSO) and then diluted with DMEM.
90 Briefly, cells were plated in 96-well plates (2×10⁴/well) and each coumarin derivatives at 12 μM were
91 added to the culture medium and the cell cultures were continued for 24 h. Cisplatin (2.6 μM) was
92 used as a reference drug. Thereafter, the medium was replaced with fresh DMEM containing 5 mg/mL
93 MTT. Following an incubation period (4 h) in a humidified CO₂ incubator at 37 °C and 5% CO₂, the
94 supernatant was removed and dimethyl sulfoxide solution (DMSO, 150 mL/well) was added to each
95 cultured plate. After incubation at room temperature for 15 min, the absorbance of the solubilized MTT
96 formazan product was spectrophotometrically measured at 540 nm. Three individual wells were
97 assayed for each treatment and the percentage viability relative to the control sample was determined
98 as (absorbance of treated cells/absorbance of untreated cells)×100%. Only the compound which
99 reduced the viability by half the value of control cells progressed to the full 5-dose assay with the
100 H2170 cell lines. The concentration of **9f** compound that reduced the viable cell number by 50% (CC₅₀)
101 was determined using a non-linear regression approach and the mean value of CC₅₀ for each cell type
102 was calculated from triplicate.

103 **2.2.3. Epithelial-to-mesenchymal transition (EMT) induction and coumarin derivatives** 104 **treatment**

105 For induction of EMT process, A549 cells (1×10⁵ per well) were seeded in 24-well culture
106 plates and treated with 1 ng/ml IL-1β (Peprotech, NJ, USA) for 24 h. In the unstimulated cells DMEM
107 medium was added. Then, the morphological alteration of cells was observed under a microscope. This

108 protocol for EMT induction is as reported in previous literature [52]. To evaluate the effects of
109 coumarin derivative with respect to EMT induced by IL-1 β , cells were pretreated with compound **9f**
110 at 7 μ M, being this treatment also maintained during stimulation with IL-1 β for 24 h.

111 **2.2.4. Immunofluorescence staining**

112 After 24 h, cells were fixed for 15 minutes at 4°C with 4% paraformaldehyde in PBS. Cells
113 were permeabilized with 0.1% Triton X-100, washed with PBS. Next, cells were incubated with FITC-
114 conjugated phalloidin (1:100) for 2 h at room temperature, and then rinsed several times with PBS.
115 Following an additional wash step with PBS, cells were stained with 10 μ g/ml DAPI at room
116 temperature for 10 min for the visualization of cell nuclei. Cell morphology was determined using an
117 inverted epifluorescence microscope (Nikon Eclipse 50i).

118 In another set of experiment, the analysis for vimentin, a well-recognized marker for its
119 selective expression and specific role in mesenchymal state, was performed. After treatment, cells were
120 fixed, permeabilized and washed as described above. Next, the slides were incubated with an anti-
121 vimentin antibody (1:100) at 4 °C overnight. The next day, the slides were incubated with secondary
122 antibody goat anti-rabbit-FITC (1:100) dilutions at room temperature for 1 h. Lastly, cells were stained
123 with DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) and washed with PBS. Stained cells were
124 analyzed by a flow cytometer (FACSCanto II, Becton Dickinson, San Jose, CA) accompanied with
125 the BD FACSDIVA™ software for data analysis. The cell-associated fluorescence of 5,000 cells per
126 sample was measured as mean fluorescence intensity (MFI) in the FL1 channel. The MFI values were
127 corrected for unspecific staining by subtracting the fluorescence of cells unstained (negative control).

128 **2.2.5. *In vitro* scratch wound healing assay**

129 To evaluate the effect of **9f** on epithelial motility, we performed the scratch assay as described
130 by Cardoso and coworkers [53]. Cells were maintained in 24 well plates until they reached 90%

131 confluency. Thereafter, a vertical stripe on the cell monolayer was made using a sterile pipette (200
132 μ l) tip. The wells were washed with PBS to remove dead cells and debris, and then **9f** was added at
133 concentration of 7 μ M. As a control, the cells were treated with cell culture medium. Photographs were
134 captured by a digital camera connected to inverted microscope (Olympus IX70) at 0 and 24 h after
135 scratch. The migration gap area of the cells was measured by ImageJ software
136 (<https://imagej.nih.gov/ij/>; Center for Information Technology, National Institute of Health, Bethesda,
137 MA, USA). Each measurement was repeated three times.

138 **2.2.6. Statistical analysis**

139 Data were expressed as mean \pm standard deviation (S.D.). The statistical analysis involving two
140 groups was done using Student's t-test. Analysis of variance followed by the Tukey's test were used to
141 compare three or more groups. Values of $p < 0.05$ were considered as indicative of significance.

142 **3. Results and Discussion**

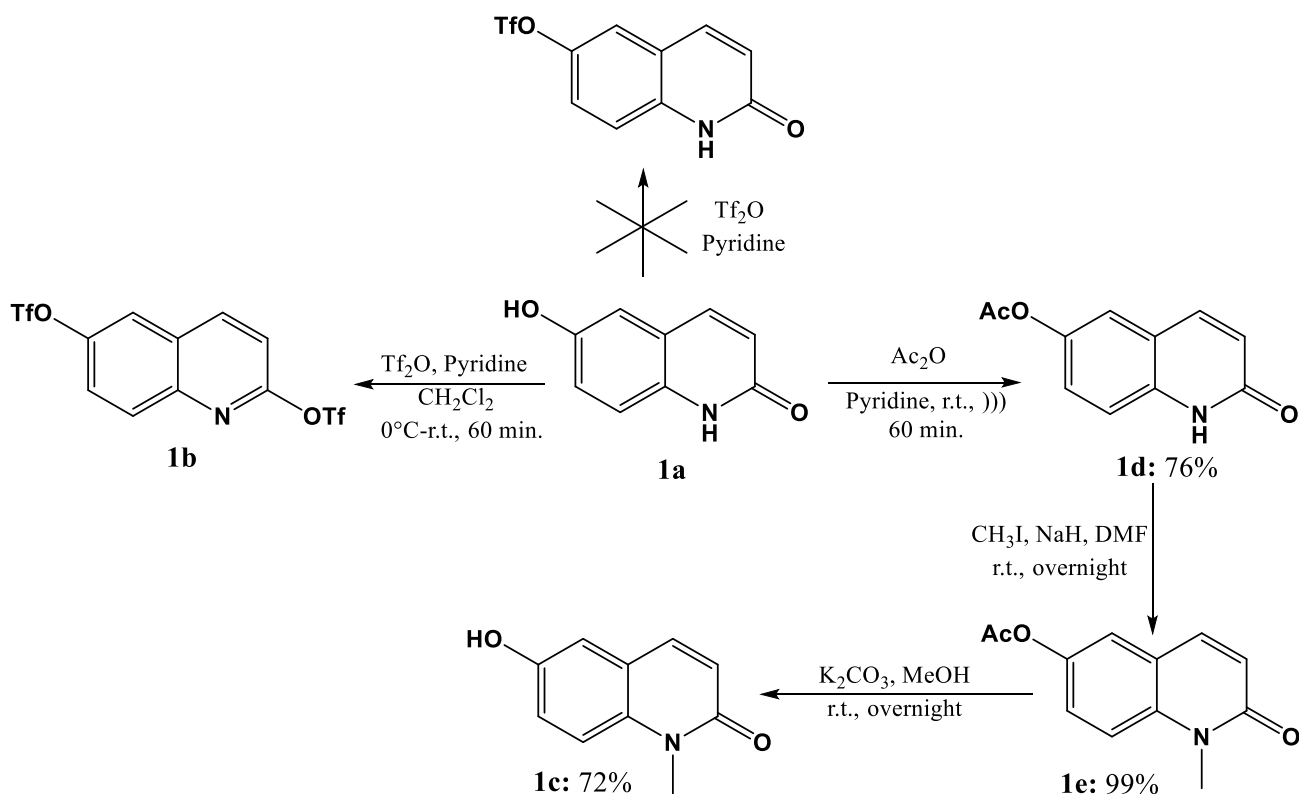
143 **3.1. Chemistry**

144 The synthesis of the coumarin core can be performed by different synthetic methodologies,
145 among which the most common are Pechmann, Wittig, Knoevenagel and Perkin reactions [11]. Cross-
146 coupling reactions catalyzed by transition metals, like the Suzuki-Miyaura, Negishi [54] and
147 Sonogashira [55] reactions, have become powerful alternatives to the formation of carbon-carbon
148 bonds [56, 57] and allowed the introduction of various substituents in all positions of the basic nucleus,
149 leading to analogous, homologous or libraries of compounds [58].

150 The preparation of target compounds (coumarins, quinolones and chromen-4-ones) involved
151 the formation of triflic methanesulfonate derivatives as key intermediates **4**, **5a-b** and **6**, thanks to the
152 cross coupling reactions. 6- and 7-hydroxycoumarin **2a-b** and 6-hydroxyquinolone **1a** are
153 commercially available, and 3-hydroxy-chromen-4-one **3** was synthesized following a reported

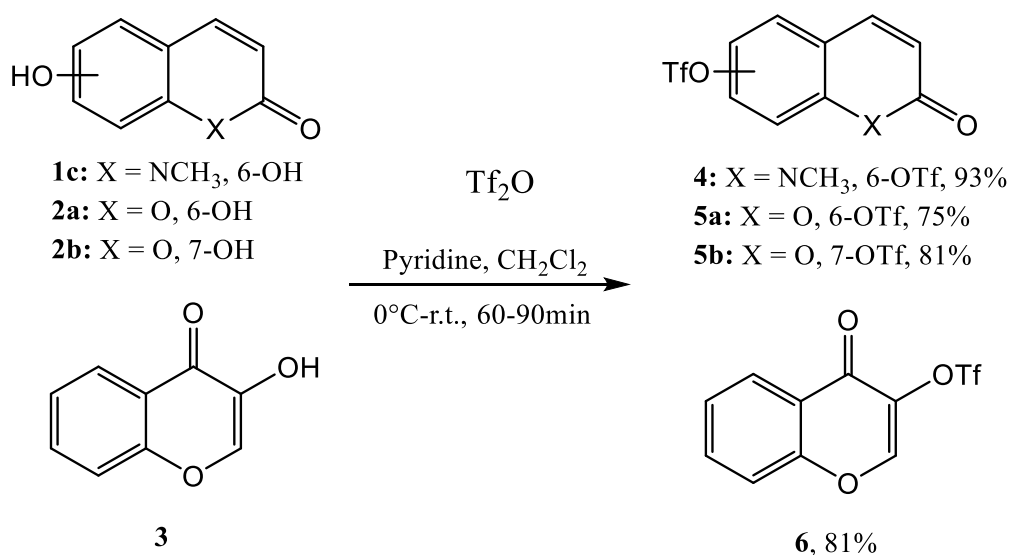
154 preparation [41]. Attempts to prepare 6-OTf quinolone from **1a** and triflic anhydride, resulted
155 exclusively in the formation of the 2,6 di-triflic adduct **1b**. Therefore, our efforts focused on the
156 preparation of N-Me quinolone **1c**. However, N-alkylation of quinolone **1a** needed a first transient
157 protection of the phenol by an acetyl group (compounds **1d-e**) according to scheme 1.

158 **Scheme 1.** Synthesis of 1-methyl-6-hydroxy-quinol-2-one (**2c**).



160 Reaction of the respective hydroxyl cores (cpds **1c**, **2a-b** and **3**) with triflic anhydride in
161 presence of pyridine afforded the corresponding triflic intermediates **4**, **5a-b** and **6** in high yields (\geq
162 75%), as illustrated in scheme 2.

163 **Scheme 2.** Synthesis of the triflic intermediates **4**, **5a-b**, **6**.



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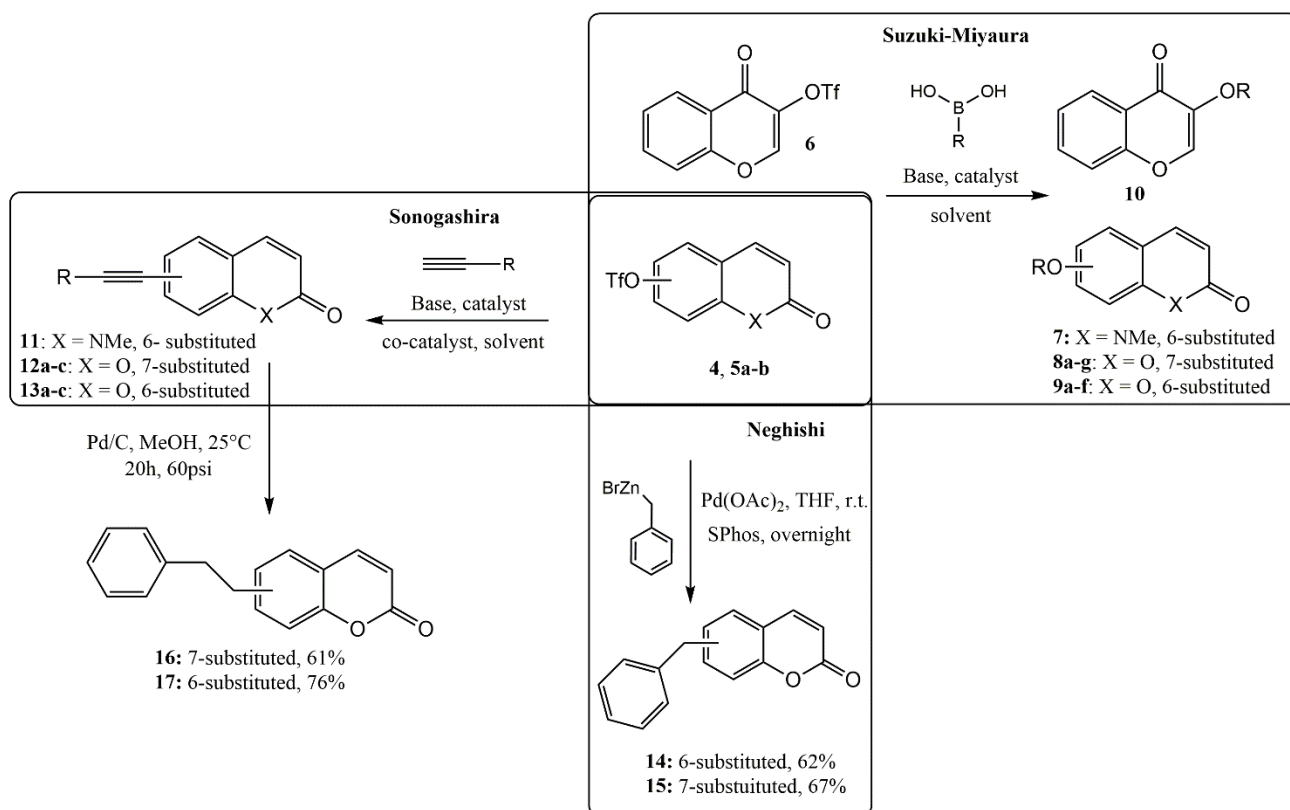
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With triflic intermediates **4**, **5a-b** and **6** in hand, our attention next turn to the Suzuki-Miyaura cross coupling reaction. Reaction with various boronic acids enable preparation of a small library of 6- and 7-substituted coumarins (cpds **8a-f**, **9a-g**). The use of a catalytic amount of tetrakis(triphenylphosphine) palladium(0) (5.0 mol%) in presence of NaHCO₃ as base, led efficiently to the target compounds (see table 1). However, for the introduction of a pyridin moiety in the coumarin structure, K₃PO₄ was preferred over NaHCO₃ (table 1, cpds **8d** and **9g**). For these 2 cpds, the reaction was performed in Toluene/EtOH/H₂O and yielded the expected compounds **8d** and **9g** in 74 % and 82 % yields respectively. Starting from the OTf-flavone derivative **6**, the use of Pd(OAc)₂ (5.0 mol%) in presence of KF furnished **10** in moderate yield (50%) (Scheme 3).

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Scheme 3. Palladium-catalyzed cross-coupling reactions to synthesized coumarin, quinolones and chromen-4-one derivatives.



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177

Table 1. Preparation of compounds 7, 8a-g, 9a-f and 10 through Suzuki-Miyaura conditions.

Cpd	X	R	Base	Catalyst. (5.0 mol%)	Solvent	Yield (%)
8a	O	7-(4-OCH ₃)-Ph	NaHCO ₃	Pd(PPh ₃) ₄	MeOH	71
8b	O	7-(2-OCH ₃)-Ph	NaHCO ₃	Pd(PPh ₃) ₄	MeOH	84
8c	O	7-(2-Cl)-Ph	NaHCO ₃	Pd(PPh ₃) ₄	MeOH	71
8d	O	7-(Pyridin-4-yl)	K ₃ PO ₄	Pd(PPh ₃) ₄	Toluene/EtOH/H ₂ O (4:1:1)	74
8e	O	7-(4-CF ₃)-Ph	NaHCO ₃	Pd(PPh ₃) ₄	MeOH	78
8f	O	7-(3,4-Cl)-Ph	NaHCO ₃	Pd(PPh ₃) ₄	MeOH	59
9a	O	6-(4-OCH ₃)-Ph	NaHCO ₃	Pd(PPh ₃) ₄	MeOH	73
9b	O	6-(3-OCH ₃)-Ph	NaHCO ₃	Pd(PPh ₃) ₄	MeOH	71
9c	O	6-(2-OCH ₃)-Ph	NaHCO ₃	Pd(PPh ₃) ₄	MeOH	76

9d	O	6-(4-Cl)-Ph	NaHCO ₃	Pd(PPh ₃) ₄	MeOH	45
9e	O	6-(2-Cl)-Ph	NaHCO ₃	Pd(PPh ₃) ₄	MeOH	71
9f	O	6-(3,4-Cl)-Ph	NaHCO ₃	Pd(PPh ₃) ₄	MeOH	68
9g	O	6-(Pyridin-4-yl)	K ₃ PO ₄	Pd(PPh ₃) ₄	Toluene/EtOH/H ₂ O (4:1:1)	82
7	NCH ₃	6-(4-OCH ₃)-Ph	NaHCO ₃	Pd(PPh ₃) ₄	MeOH	81
10	-	3-(4-OMe)-Ph	KF	Pd(OAc) ₂	MeOH	50

178

179

Sonogashira reaction with different terminal alkynes resulted in 7 compounds (Table 2).

180

181

Table 2. Preparation of compounds **11**, **12a-c**, **13a-c** through Sonogashira cross-coupling reaction in CH₃CN.

Cpd	X	R	Base	Ligand/Catalyst	Additive	Yield %
12a	O	Ph	Et ₃ N	Pd(PPh ₃) ₂ Cl ₂	CuI	75
12b	O	CH ₂ OCH ₂ Ph	Et ₃ N	Pd(PPh ₃) ₂ Cl ₂	CuI	38
12c	O	CH ₂ OH	K ₂ CO ₃	S-Phos/Pd(OAc) ₂	TBAI	75
13a	O	Ph	K ₂ CO ₃	S-Phos/Pd(OAc) ₂	TBAI	78
13b	O	(CH ₂) ₃ Ph	K ₂ CO ₃	S-Phos/Pd(OAc) ₂	TBAI	76
13c	O	CH ₂ OH	K ₂ CO ₃	S-Phos/Pd(OAc) ₂	TBAI	72
11	NCH ₃	Ph	K ₂ CO ₃	S-Phos/Pd(OAc) ₂	TBAI	78

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Palladium-catalyzed Sonogashira cross-coupling is a widely used method to synthesize functional molecules containing an alkyne unit. Traditional Sonogashira coupling with Pd(PPh₃)₂Cl₂ (3.0 mol %) and Et₃N typically requires the use of a Cu(I) halide salt as a cocatalyst to have high reaction productivity. So, starting from coumarin **5b** and under these conditions, the phenyl acetylene moiety was introduced under microwave irradiation in 75% yield (cpd **12a**). However, with OBn

188 propargylalcohol, same conditons yielded **12b** in only 38% yield. Recently, Chorley et al. highlighted
189 the efficacy of Pd(OAc)₂ and 2-dicyclohexylphosphino-2',6'-dimethoxybiphenyl (SPhos) as effective
190 catalytic system for the Sonogashira cross coupling reaction [59]. In addition, the presence of
191 tetrabutylammonium iodide (TBAI) as additive increased the yield of the reaction [59]. Under these
192 conditions and without protection of propargylic alcohol, we isolated the target alkyne derivative **12c**
193 in 75% yield. These last conditions applied to OTf intermediates **4** and **5b** in presence of various
194 terminal alkynes, yielded target compounds **11** and **13a-c** in satisfactory yields (> 70 %, see table 2)
195 (Scheme 3).

196 Negishi cross coupling reactions represent an extremely versatile tool for the introduction of
197 alkyl substituents. As reported by Knochel et al. [60] and in presence of SPhos (10.0 mol %) and
198 Pd(OAc)₂ (5.0 mol %) it was possible to perform at room temperature, efficient cross coupling reaction
199 between OTf coumarin **5a-b** and benzyl zinc reagent (see scheme 3, cpds **14** and **15**).

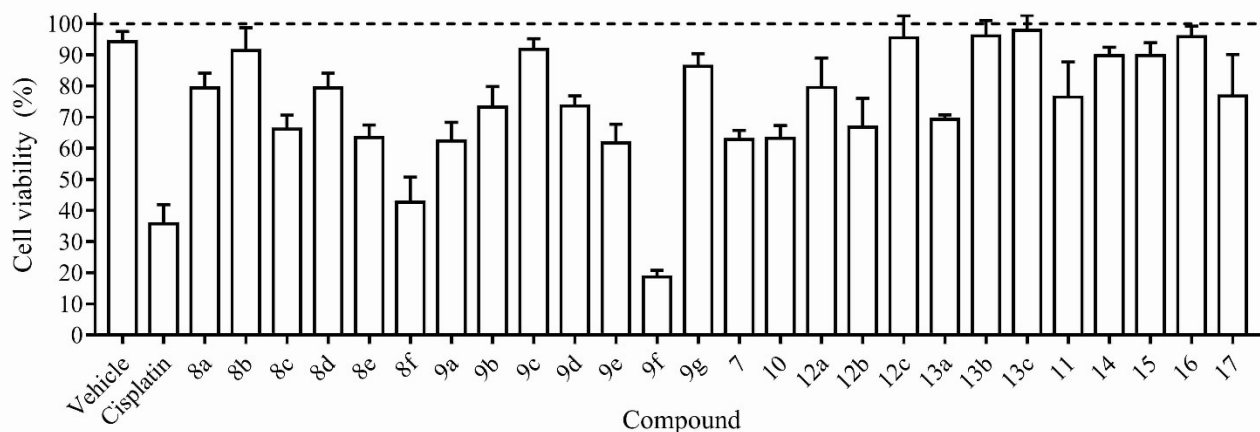
200 Lastly, subsequent reduction of alkynes **12a** and **13a** was performed by catalytic
201 hydrogenation, leading respectively to cpds **16** and **17** as depicted in scheme 3.

202 All synthesized compounds had their chemical structures confirmed by ¹H and ¹³C NMR and
203 mass spectrometry, and all spectra data are available in supplementary material.

204 **3.2. Biological evaluation**

205 **3.2.1. Effects of coumarin derivatives on cell viability**

206 All the synthetic coumarins (**7**, **8a-f**, **9a-g**, **10**, **11**, **12 a-c**, **13a-c**, **14-17**) were first screened for
207 their *in vitro* cytotoxic activity at a single concentration (12 μM) against NSCLC cell line (human lung
208 adenocarcinoma A549) for 24 h, using the well-established 3-(4,5-dimethylthiazol-2-yl)-2,5-
209 diphenyltetrazolium bromide (MTT) assay. The results of A549 cells viability are reported in Figure
210 1.



211

212 **Figure 1.** Effect of synthetic coumarin derivatives on cell viability. The viability of A549 cells was
 213 determined using the MTT assay after exposure at a single concentration (12 μ M) of the compounds
 214 or cisplatin (2.6 μ M) for 24 h. The percentage of inhibition was calculated considering the cells treated
 215 with medium (DMEM), considered as 100% of cell viability (dotted line). Bars represent the mean \pm
 216 S.D. from three independent experiments.

217

218 The results showed that compounds **8b**, **9c**, **9g**, **12c**, **13b**, **13c**, **14**, **15**, and **16** showed little or
 219 no cytotoxicity. Compounds **7**, **8a**, **8c**, **8d**, **8e**, **9a**, **9b**, **9d**, **9e**, **10**, **11**, **12a-b**, **13a** and **17** showed a
 220 moderate cytotoxicity by inducing a reduction in cell viability within 80%–60%. And compounds **8f**
 221 and **9f**, which have in common the presence of a 3,4-dichloro-phenyl group ((3,4-Cl)-Ph), induced a
 222 strong cytotoxicity by decreasing the A549 cells viability to values below 50%.

223

224 Among these two compounds, **9f** was the most promising, reducing the A549 cells viability to
 225 less than 20%. Thus, this compound was selected and had its concentration that reduces the viable cell
 226 number by 50% (CC_{50}) determined against two cancer cell lines (human lung adenocarcinoma A549
 227 and H2170 cell lines) and one non-cancer cell line (NIH-3T3), and showed CC_{50} values (mean \pm S.D.)
 228 of $7.1 \pm 0.8 \mu$ M, $3.3 \pm 0.5 \mu$ M and $25.8 \pm 1.7 \mu$ M against A549, H2170 and NIH-3T3 cells,
 229 respectively. Facing these results, we can notate that **9f** showed to be the most potent against cancer
 229 cells (A549 and H2170 cell lines) than against healthy cell (NIH-3T3 cell line).

230 These cytotoxicity results are better than those observed for the most cytotoxic coumarins from
231 other studies against the A549 cells, such as: Umbelliprenin ($IC_{50} = 52 \pm 1.97 \mu M$) [33]; 3-
232 arylcoumarin derivative (8-(acetyloxy)-3-(4-methanesulfonyl phenyl)-2-oxo-2*H*-chromen-7-yl
233 acetate) with $IC_{50} = 24.2 \mu M$ [31]; and iodinated-4-aryloxymethylcoumarins (6-chloro- and 7-chloro-
234 4-(4-iodo-phenoxy-methyl)-chromen-2-one) with $IC_{50} = 7.57 \mu M$ [35]; these latter bearing chlorine
235 atoms in their structures, as observed in the most active compounds of the present study (coumarins **8f**
236 and **9f**).

237 On the basis of cytotoxic effect, the concentration of $7 \mu M$ of **9f** was chosen for further to
238 characterize the antitumor activity, by investigating their effects on the process of inhibition of the
239 EMT-associated migratory ability and epithelial-to-mesenchymal transition (EMT) in IL-1 β -
240 stimulated A549 cells.

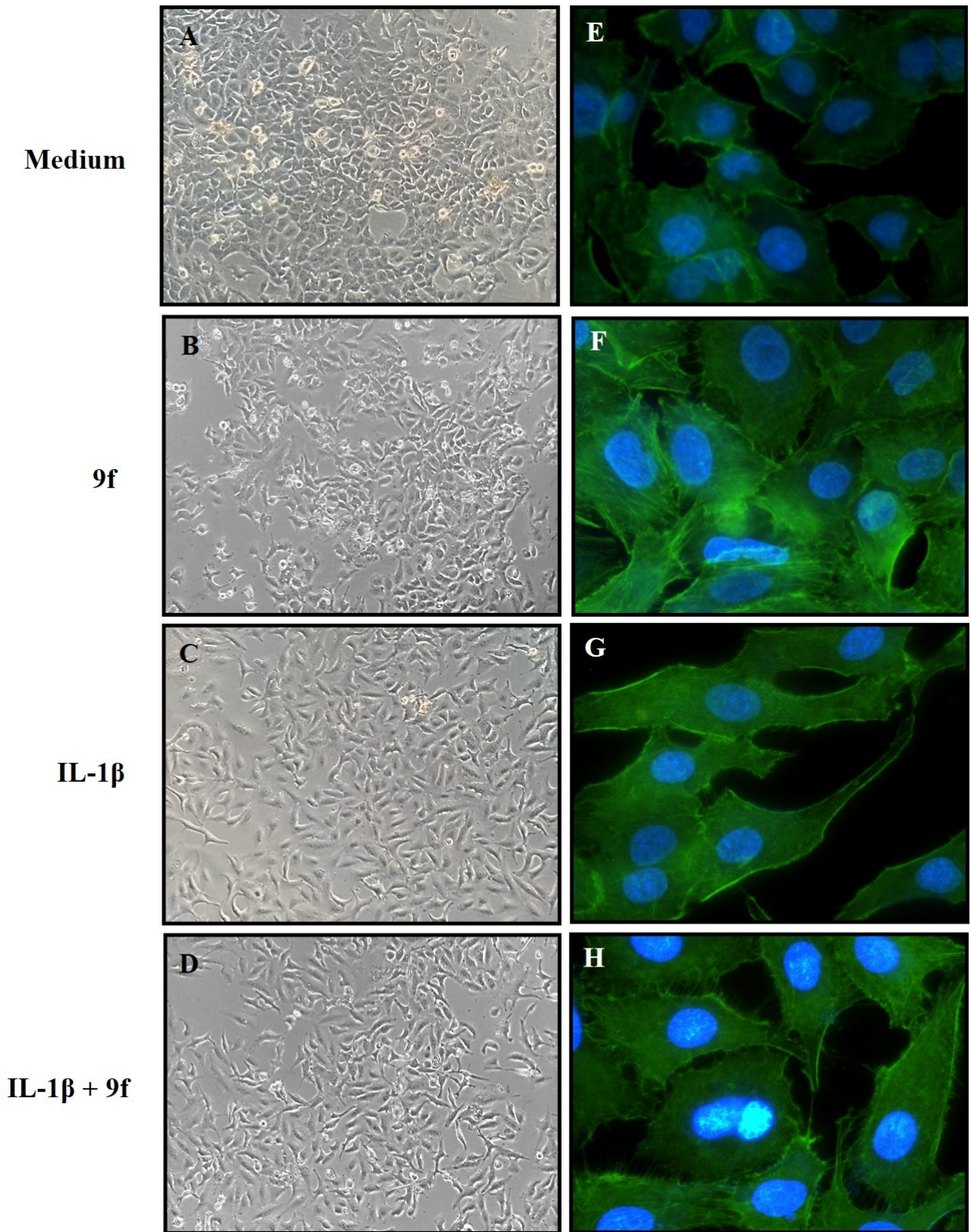
241 3.2.2. Effect of **9f** on IL-1 β -induced EMT in A549 cells

242 The EMT process is characterized by the phenotypic conversion of epithelial into mesenchymal
243 cells that occurs with great frequency in fibrotic tissues, embryonic cells, and cancer. This transition
244 increases the invasion capacity and the migratory potential of cells, which are characteristic of
245 metastatic cancer, contributing additionally to the development of drug-resistance in cancer [61-66].

246 To determine whether compound **9f** acts as an inhibitory compound of EMT in epithelial cells,
247 the morphological changes induced by IL1- β on A549 cells was observed. As shown in Figure 2A and
248 2B, the A549 cells maintained to culture medium (DMEM) or treated with compound **9f** exhibited, in
249 a confluent monolayer, a cobblestone-like cell morphology, which is characteristic of epithelial cells.
250 Cells treated with 1 ng/mL IL-1 β exhibited an evident morphological change and acquired a spindle-
251 like morphology with loss of cell-cell interactions that is characteristic features of mesenchymal cells
252 (Figure 2C). A549 cells treated with **9f** exhibited an impairment in changes in its mesenchymal

253 characteristics induced by IL-1 β (Figure 2D), suggesting that **9f** possess inhibitory effects on IL-1 β -
254 induced F-actin reorganization.

255 To evaluate the effect of compound **9f** on actin cytoskeleton organization, A549 cells were IL-
256 1 β -stimulated and evaluated by staining with FITC-labeled phalloidin. As presented in the Figure 2E
257 and 2F, the A549 cells maintained to culture medium (DMEM) or treated with **9f** exhibited an abundant
258 deposition of actin filament in the cortical region, which determines a cellular cobblestone-like
259 morphology, typical of epithelial cells. Stimulation with IL-1 β induced a cytoskeleton reorganization,
260 leading to the activation of actin polymerization and the morphologic cell reorganization, which
261 indicate a differentiation from the epithelial to mesenchymal phenotype (Figure 2G). Treatment with
262 **9f** attenuated the changes in the actin cytoskeleton reorganization in A549 cells stimulated by IL-1 β
263 (Figure 2H).



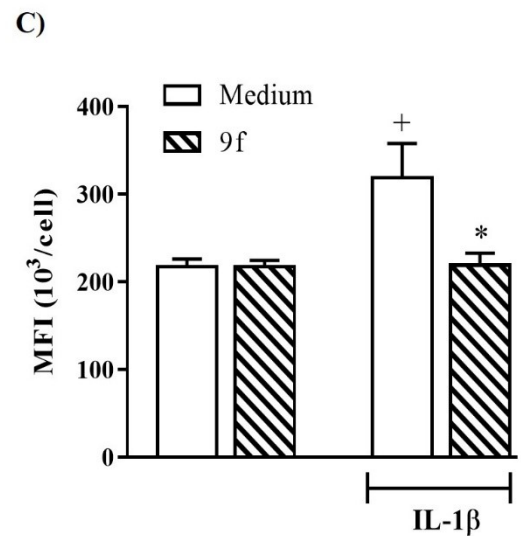
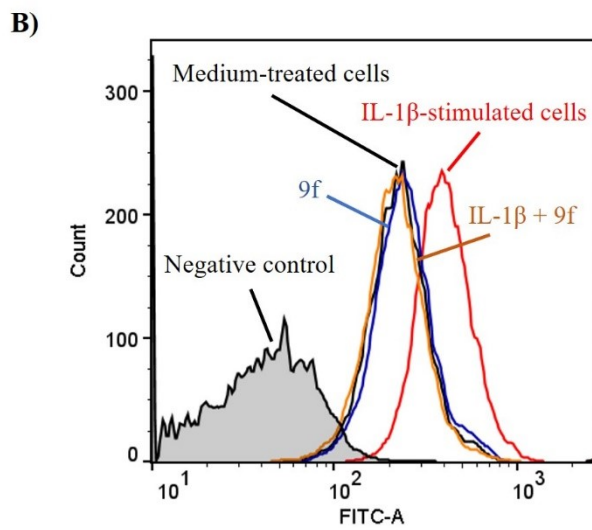
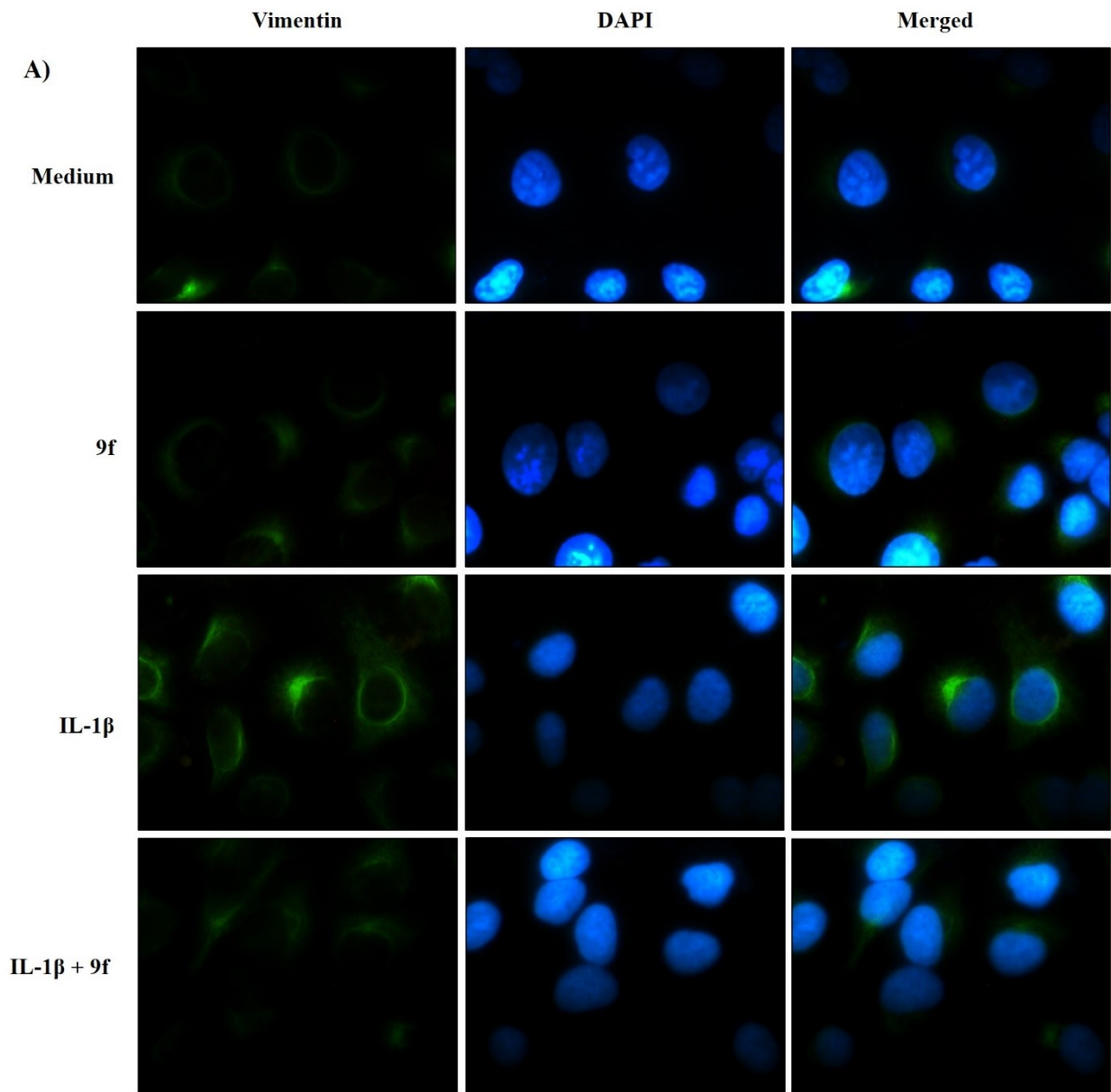
264

265 **Figure 2.** Compound **9f** inhibited IL-1 β -induced EMT *in vitro*. A549 cells were treated with **9f** (7 μ M)
 266 in the presence or absence of 1 ng/mL IL-1 β . Cells were photographed using phase-contrast
 267 microscopy (A, B, C and D) or fluorescence microscopy (E, F, G e H). Cells were exposure to
 268 treatment with DMEM-medium (A and E), **9f** (B and F), IL-1 β (C and G) or **9f** + IL-1 β (D and H) for

269 24 h. Actin (green) was detected via immunofluorescence in formaldehyde-fixed cells with FITC-
270 conjugated phalloidin (1:100). Nuclei were counterstained with DAPI. Magnification $\times 100$ for phase-
271 contrast microscope and $\times 400$ for fluorescence microscope.

272 To corroborate whether this morphological transformation represents EMT,
273 immunofluorescent staining was used to quantify the vimentin, a mesenchymal marker most
274 commonly associated with EMT and involved in cancer progression [67].

275 As shown in Figure 3A, 24 h incubation with 1 ng/mL IL-1 β increased significantly the
276 expression of vimentin in A549 cells compared with those maintained DMEM medium (control). We
277 found that treatment of cells with **9f** (7 μ M) significantly diminished the expression of mesenchymal
278 marker vimentin in IL-1 β -stimulated A549 cells (Figure 3A), phenomenon confirmed by quantitative
279 assessment by flow cytometer (Figure 3B-C). Treatment of cells with **9f** did not change levels of
280 vimentin expression in unstimulated cells with IL-1 β (Figure 3A-C). This result showed that **9f**
281 treatment suppresses IL-1 β -induced EMT in A549 cells through downregulating vimentin.

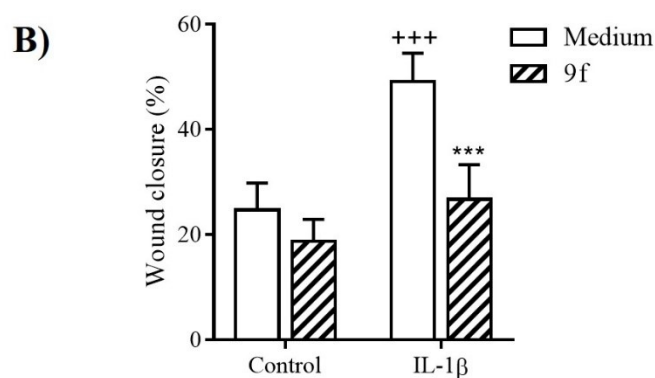
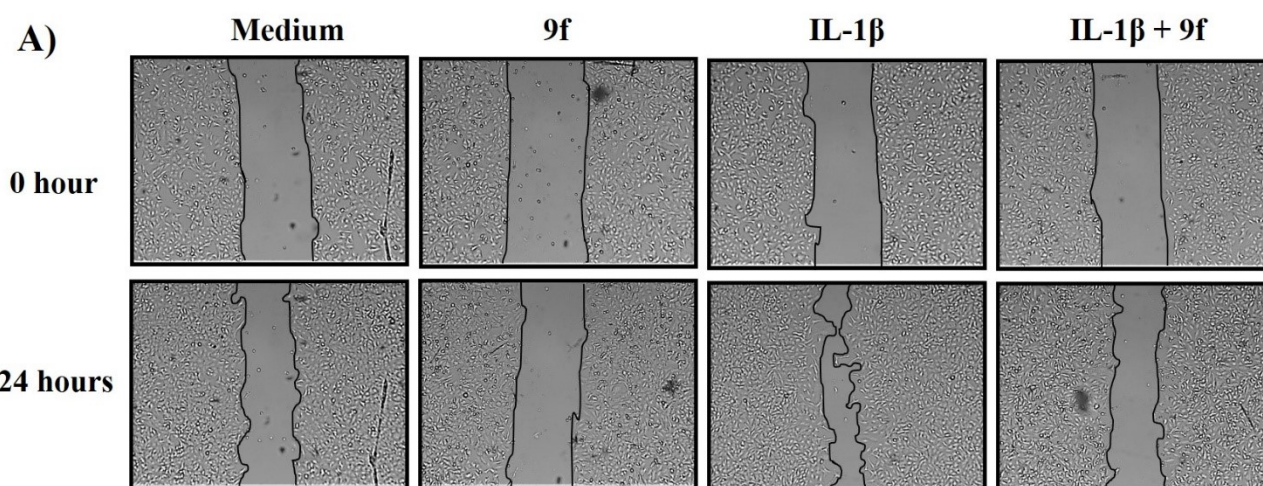


283 **Figure 3.** Compound **9f** downregulates the expression of mesenchymal cell marker vimentin in IL-1 β -
284 induced EMT. A549 cells were treated with compound **9f** (7 μ M) in the presence or absence of 1 ng/mL
285 IL-1 β . The mesenchymal markers vimentin was detected by immunofluorescence. (A) Cells were
286 exposure to treatment with DMEM-medium, **9f**, IL-1 β or IL-1 β +6f for 24 h. Vimentin (green) was
287 detected via immunofluorescence in formaldehyde-fixed cells with FITC-conjugated antibody. Nuclei
288 were counterstained with DAPI. Magnification \times 400 for fluorescence microscope. (B) and (C) Flow
289 cytometry analysis showing the reduced expression of vimentin in IL-1 β -induced EMT under **9f**
290 treatment. In graph in (C), bars represent mean \pm S.D. from three independent experiments. (+) P <
291 0.01 compared with respective DMEM-treated cells and (*) P < 0.01 compared with IL-1 β -stimulated
292 cell medium-treated cells.

293 **3.2.3. Effects of 9f on cells motility**

294 Given the good results of **9f** in inhibiting the IL-1 β -induced EMT in epithelial cells, was
295 investigated whether **9f** could affected the EMT-associated migratory ability in A549 cells. For this,
296 *in vitro* wound-healing assay was performed to evaluate as **9f** acts as anti-metastatic agent in A549
297 cells.

298 As shown in Figure 4A-B, IL-1 β -treated cells exhibited an increased in wound closure within
299 24 h compared with those not treated with IL-1 β (control). Treatment of cells with **9f** at 7 μ M for 24
300 h significantly reduced migration of IL-1 β -stimulated cells, phenomenon confirmed by qualitative
301 assessment of the wound closure (Figure 4A-B).



302

303 **Figure 4.** The effect of **9f** on the migration of A549 cells assayed by the wound healing assay. Cells
 304 were treated with **9f** at 7 μ M, and images were captured to calculate the scratch closure. In A,
 305 representative photomicrography images showing the cell migration towards the cell-free area after
 306 treatment with DMEM (control) or **9f** and the after 24 h. In B, graph shows percentage of scratch
 307 covered was measured by quantifying the total distance the cells moved from the edge of the scratch
 308 towards the center of the scratch, using ImageJ software, followed by conversion to a percentage of
 309 the wound covered. Values represent mean \pm S.D. from three independent experiments. (+++) $P <$
 310 0.001 compared with respective DMEM-treated cells and (***) $P <$ 0.001 compared with IL-1 β -
 311 stimulated cell vehicle-treated cells.

312 In conclusion, twenty-six coumarin derivatives were synthesized through PCCCR and were
 313 evaluated for their anti-lung cancer properties against two non-small cell lung carcinoma (NSCLC)
 314 cell lines. Coumarins **8f** and **9f**, presenting a 3,4-dichloro-phenyl radical, inhibited *in vitro* the growth
 315 of both human lung adenocarcinoma cells in low micromolar concentration. Derivative **9f** regulate the
 316 epithelial-to-mesenchymal transition (EMT) suppressing the mesenchymal marker vimentin and

317 cancer cell migration in IL-1 β -stimulated A549 cells. Taken together, our finding suggest that
318 coumarin derivatives, especially compound **9f** may become a promising *hit* in the process of lung
319 cancer drug discovery, especially in lung cancer promoted by non-small cell lung carcinoma (NSCLC)
320 cell lines.

321 **Declaration of competing interest**

322 All the authors declare they have no conflict of interests for this work.

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Highlights

- Coumarin derivatives were synthesized through palladium-catalyzed cross-coupling reactions.
- Compound **9f** is high cytotoxicity against NSCLC cell lines (A549 and H2170).
- **9f** reverses EMT by attenuation of changes in the actin cytoskeleton reorganization.
- **9f** suppresses IL-1 β -induced EMT in A549 cells through downregulating vimentin.
- Wound-healing assay confirmed that **9f** reduces cancer cell migration of IL-1 β -stimulated cells.