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Novel coumarin compounds potentiate the effect of cisplatin on lung cancer cells by enhancing pro-apoptotic gene expressions, G2/M cell arrest, oxidative and antiangiogenic effects

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Abstract

Coumarin is a functional compound with a pronounced wide range of biological activities and has recently been shown to have anticancer effects on various human cancer cells. Cisplatin is widely used in the treatment of many cancers but its effectiveness is limited due to acquired resistance and dose-related side effects. This study aimed to reveal the chemosensitizing ability of novel synthesized coumarin-triazole hybrid compounds (**3a-f**) alone or their combination with cisplatin in A549 cells.MTT assay was used for cytotoxic effects. Lactate dehydrogenase (LDH), antioxidant/oxidant status, DNA fragmentation were determined spectrophotometrically by using commercial kits. Muse™ Cell Analyzer was used to assess cell cycle progression. Pro/anti-apoptotic gene expressions were determined by Real-Time qPCR. The antiangiogenic activity was determined by VEGF expression and Hen's chorioallantoic membrane model. Compounds **3c**, **d**, **e**, and **f** potentiated the cisplatin-induced cytotoxicity through the increase of LDH release and DNA fragmentation, induced G2/M cell cycle arrest, overproduction of oxidative stress, and decrease of cellular antioxidant levels. These compounds combined with cisplatin caused upregulation in the pro-apoptotic Bax, Bıd, caspase-3, caspase-8, caspase-9, Fas, and p53 gene expressions while downregulating anti-apoptotic DFFA, NFkB1, and Bcl2 gene expressions. These combinations caused vascular loss and a reduction in VEGF expression. These results suggest that a combinational regimen of coumarin compounds with cisplatin overcome the acquired resistance of cancer cells to cisplatin and, considering compounds have relatively low toxicity in normal cells, decrease the dose requirement of cisplatin in cancer treatments.

Keywords: Angiogenesis, apoptosis, cisplatin, coumarin, cytotoxicity, lung cancer, ROS

Abbreviations

HETCAM.; Hen's Egg Test–Chorioallantoic Membrane, LDH.; Lactate dehydrogenase, ROS.; reactive oxygen species, TAC.; Total antioxidant capacity, TOS.; Total oxidative stress, VEGF.; Vascular endothelial growth factor

Introduction

Cancer is uncontrolled cell proliferation in any part of the body and is characterized by aggressive behavior, high metastasis, and rapid growth. Cancer is the second leading cause of death worldwide accounting for an estimated 19 million new cases and 10 million deaths, in 2020 [1]. Many strategies such as surgery, radiotherapy, and chemotherapy have been developed to prevent or decrease the dramatic outcomes of cancer. Chemotherapeutic drug applications are one of the most important steps for effective treatment. Platinum-based chemotherapeutic drugs are widely used as anticancer agents in different cancer cases. But, the effectiveness of these drugs is limited due to the toxic effects on healthy cells and the acquired resistance of cancer cells to drugs [2].

Natural compounds with less harmfulness and good ability have received great attention in the pharmaceutical industry. Heterocyclic complexes derived from natural compounds can interact more easily with biological targets due to relatively weak binding in their structure compared to the native form [3]. More than half of the approved drugs are derived from synthetic forms of natural compounds [4]. Coumarin is an important secondary metabolite related to defense mechanisms in the plant. Coumarin derivates can readily interact with a variety of cellular components such as proteins, enzymes, and receptors and this feature provide a unique opportunity for new drug designs. Warfarin, novobiocin, scopoletin, esculetin, khellactone, and calanolide are the best-known coumarin derivatives and have been marketed as anticoagulants, antibiotics, antiproliferative, and anti-HIV agent [5,6]. Hybrid structures bearing coumarin have

recently gained great importance to improve the biological activity, increase the specific effect, and overcome the drug resistance by synergistic effect in cancer treatments. Numerous studies have documented that coumarin-based compounds exhibit cytotoxic activity through the different cellular mechanisms on cancer cells [7]. Coumarin molecules bearing organometallic have shown promising results by inhibiting the proliferation of colon, lung, breast, and stomach cancer cells. Previous studies have shown that hybrid formations of coumarin with molecules such as hydrazine, pyrazole, pyridine, thiazole, and chalcone exhibit anticancer effects by inducing cell cycle arrest, pro-apoptotic gene expressions, and DNA damage [4]. Recently, specifically targeted prodrugs bearing coumarin moiety have become an attractive approach to overcome the acquired resistance of cancer cells to cisplatin, particularly through easily interacting with cellular components and promoting cellular signaling pathways [8].

Based on these outcomes, in this study, a novel series of coumarin-triazole derivates (3a, -b, -c, -d, -e, and -f) were synthesized and they were first tested for cytotoxic effects on the human breast (MCF-7), cervix (HeLa), lung (A549) cancer and normal human embryonic kidney cells 293 (HEK293) cell lines. Then, 3-c, -d, -e, and -f compounds that exhibited the highest on cancer cells and lowest on HEK 293 cells effects were chosen to compare with cisplatin treatments on A549 cells. The effectiveness of these compounds alone (treated with IC50 doses) or in combination with cisplatin on A549 cells were investigated by lactate dehydrogenase (LDH) release, oxidant/antioxidant status, DNA fragmentation, cell cycle arrest, apoptotic gene expressions, and antiangiogenic analysis.

Experimental

Chemistry

Compounds **1a-f** and **2** were synthesized according to a previously reported study [9]. Coumarin-triazole derivatives were synthesized from the reaction of compounds **1a-e** with compound **2**

under reflux in benzene (Supporting Information File 1). Spectral investigations of synthesized compounds were in accordance with the proposed structures of target molecules (Figure 1). All the chemicals were supplied from Merck, and Alfa Aesar. The melting points were determined on capillary tubes on the Stuart SMP30 melting point apparatus and uncorrected. ^{1}H and ^{13}C NMR spectra (400 and 100 MHz, respectively) were obtained using a Varian-Mercury. The mass spectra were recorded on Agilent 1260 Infinity series Accurate-Mass Time-of-Flight (TOF) LC/MS spectrometer (Supporting Information File 2).

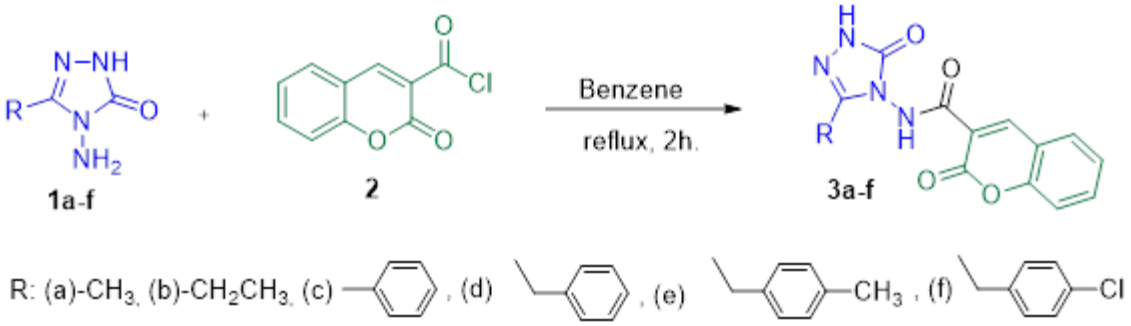

Figure 1. Synthetic pathways for the synthesis of triazole-coumarin hybrids

Cell viability

Cell viability was determined on the human lung cancer (A549) (ATCC CCL-185), breast cancer cell line (MCF-7) (ATCC-HTB-22), cervix cancer (HeLa) (ATCC CCL-2), and human embryonic kidney cells 293 (HEK293) (ATCC-CRL-1573) cell lines and were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The cells maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/L streptomycin (Biochrom AG, Berlin, Germany) at 37 °C in a humidified atmosphere of 5 % CO_2.

Cytotoxic effect was determined by MTT (3-(4, 5- dimethyl-2-thiazolyl) -2, 5-diphenyl-2H-tetrazolium bromide)) cell viability assay) assay. Cells were seeded at an initial concentration of $1x10^5$ cells/mL in 96-well microplates for 48 h. Cells were treated with different concentrations

(0.5, 5, 50 mg/L) of the compounds (3a-f). After the incubation period, the formed formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and the optical density (OD) of compounds was measured at 570 nm using a spectrophotometer (BMG Labtech, Ortenberg, Germany). Cytotoxicity was expressed as an increase of the mean percentage of cytotoxicity relative to the unexposed control ± standard deviation (SD). Control values were set at 0% cytotoxicity. IC50 was calculated by fitting the data to a sigmoidal curve and using a four parameters logistic model and presented as an average of three independent measurements. The IC50 values were reported at a 95% confidence interval and calculation was performed using GraphPad Prism software (San Diego, USA). The values of the blank wells were subtracted from each well of treated and control cells and inhibition of growth 50% was calculated in comparison with untreated controls. Cisplatin (10 µmol/L) was used as a positive control. Each sample was tested in triplicate.

Lactate dehydrogenase (LDH) assay

LDH leakage assay was determined using the LDH Assay Kit (Cat no. ab102526, Abcam, Cambridge, UK) on the culture medium of a new set of A549 cells exposed to the cisplatin and calculated IC50 values of the compounds (3c-f) and a combination of these compounds with cisplatin for 48 h. 100 µL of culture medium was transferred to a new 96 well plate. 100 µL of LDH reaction solution to each well was added and absorbance was measured at 490 nm using an ELISA plate reader (BMG Labtech, Ortenberg, Germany) after 30 min.

Total antioxidant capacity (TAC) and total oxidative stress (TOS) activities

TAC and TOS levels were measured in cellular media using a commercial kit (Rel Assay Diagnostics®, Gaziantep, Turkey) according to the manufacturer's instructions. A549 cells for these experiments were treated with the cisplatin and calculated IC50 values of the compounds

(3c-f) and a combination of compounds with cisplatin and incubated at 37 °C in a humidified 5 % CO_2 for 2 h.

In TAC assay, potential antioxidants in culture medium cause a reduction of ABTS (2,2′ azino-bis 3-ethyl benzothiazoline-6-sulfuric acid) radical. Briefly, 500 µL of the Reagent 1 solution in the kit content was added to the quartz cuvette containing 30 µL of plasma sample, and the initial absorbance was measured at 660 nm after 30 s. Then 75 µL of Reagent 2 solution was added to the same cuvette and the absorbance was measured at 660 nm after 5 min incubation. The assay was calibrated with Trolox and the results were expressed in terms of mMTrolox equivalent per liter (mmoLTroloxEquiv/L).

TOS assay was based on the conversion of ferrous ion–chelator complex to ferric ion via oxidants present in the culture medium. To determine the TOS level, 500 µL of Reagent 1 was mixed with 75 µL of each plasma sample and the absorbance of each sample was measured at 530 nm after 30 s. Then, 15 µL of Reagent 2 was added to the mixture and the absorbance was again read at 530 nm.

Measurement of DNA fragmentation

DNA fragmentation was determined by Cell Death Detection kit (Sigma Aldrich, UK) according to the manufacturer's instructions. Briefly, the cells (at 1×10^5 cells/well) were seeded in a 96 well plate, and then the A549 cells were exposed to cisplatin and calculated IC50 values of the compounds (3c-f) and a combination of compounds with cisplatin. 20 mL of the cytoplasmic fractions were transferred into a streptavidin-coated 96-well with anti- DNA antibodies and incubated for 2 h at room temperature. After the washing period, 2,2-azino-di-(3-ethylbenzthiazoline sulphonate) diammonium salt was added and absorbances were measured at 405 nm using an ELISA reader (BMG Labtech, Ortenberg, Germany).

Cell cycle analysis

A549 cells were seeded in 6 well plates at 1×10^4 cells/ml for 48 h and treated cisplatin and calculated IC50 values of the compounds (3c-f) and a combination of compounds with cisplatin. The cell cycle phase was realized using a Muse™ Cell Cycle Assay Kit (Merck Millipore, Germany) according to the manufacturer's instructions. Briefly, cells were trypsinized with PBS and fixed by 70 % cold-ethanol. Muse cell cycle reagent was added to the obtained cell pellet and incubated for 30 min. The G0/G1, S, and G2/M percentage of cells was calculated by the Muse cell cycle analyzer (Merck Millipore, Germany).

cDNA synthesize and Quantitative Real-Time PCR analysis

The effects of compounds 3c-f on the expressions of Bax, Bıd, Bcl-2, caspase-3, caspase-8, caspase-9, FAS, P53, DFFA, NFkB1, and VEGF genes were determined by RT-qPCR analysis. Briefly, A549 cells treated with IC50 value of compounds 3c-f, cisplatin and their combinations for 48 h were harvested and total RNA was isolated using the TriPure isolation reagent (Roche, Basel, Switzerland, Cat. no. 11 667 157 001). The quality of the isolated RNA was controlled by NanoDrop (NanoDrop ND-2000c, Thermo Scientific, Waltham, MA, USA). First-strand cDNA was synthesized from total RNA with the Transcriptor First Strand cDNA Synthesis kit (Roche, Cat. no. 04 379 012 001). Real-time polymerase chain reaction (RT-PCR) analysis was conducted on the LightCycler v.1.5 instruments (Roche Applied Science) and performed with SYBR Green PCR Master Mix (Qiagen). The real-time PCR mixture contained 5 µl SYBR Green PCR Master Mix, 0.5 µl cDNA, and 0.3 µM primer pairs in a total volume of 10 µl. Cycling conditions for the PCR reaction were as follows: initially 10 min at 95 °C, followed by 40 cycles of cyclic denaturation at 95°C for 15 s, annealing at 59°C for 1 min, and extension 13 s at 72 °C. The beta-actin was used as an endogenous control. Relative ratios were calculated by normalizing gene expression levels of each sample and the experiment was performed with three duplicates. Results were calculated by using the Ct method ($2^{-\Delta\Delta Ct}$ method)[10].

186 *Antiangiogenic activity*

187 The anti-angiogenic potential of compounds 3c-f, cisplatin, and their combinations were
188 determined by a chorioallantoic membrane model on fertilized hen eggs with a slight change of
189 the procedure of [11]. Fertile Leghorn chicken eggs weighing 50-60 g purchased from
190 commercial sources (Giresun, Turkey). Fertilized hens' eggs were placed into an incubator with a
191 conveyor rotation system at 37 ± 1 °C and 80 ± 2% humidity for 7 days. On day 7, the eggs were
192 opened on the snub side sucked off through a hole in the pointed side and then around the piece
193 of shell (3-4 cm diameter) was removed carefully with forceps. Then, the inner membrane was
194 carefully removed with forceps, without injury to the blood vessel. After that, the eggs were
195 divided into six groups as follows: Group I.; DMSO as a negative control, Group II.; suramin (10
196 mM) as a positive control, Group III-VI.; treatment with IC50value of compounds 3c-f.; Group
197 VII.; Cisplatin.; Group VII-XI.; a combination of compounds 3c-f with cisplatin. The samples
198 were loaded onto a Whatman filter paper and were applied to CAM and were incubated. At the
199 end of the 24 h incubation period, antiangiogenic effects were assayed following the scoring table
200 under the stereomicroscope. The scoring as followed: 0 score.; there was no effect, 0.5 score.; a
201 weak effect (reduced capillary area), 1 score (small and capillary-free).; a moderate effect, 2
202 score.; a strong effect (a capillary-free area). The average score was calculated as follows:
203 Average score = (number of eggs [score 2] × 2 + number of eggs [score 1]) × 1/(total number of
204 eggs [score 0, 1, 2]). According to the results of this formula, a score <0.5 indicated that there
205 was no antiangiogenic effect, 0.5-1 showed a moderate antiangiogenic effect, and >1 showed a
206 strong antiangiogenic effect.

207 *Statistical analysis*

208 Statistical analysis was performed using SPSS 20.0 (SPSS, Chicago, IL, USA). The experimental
209 data were analyzed by one-way analysis of variance (ANOVA) and Duncan's test was performed

to examine whether there were any differences between the application and control groups. Pearson's r coefficient was used to determine correlations between data. The results are presented as means ± SD of at least three independent experiments and $p < 0.05$ was accepted as significant. All assays were run in triplicate.

Results

Cell viability

To assess the possible cytotoxic effects of novel synthesized coumarin compounds, we first evaluated the cytotoxicity of these molecules on different human cancer and normal cell lines by using an MTT assay. The IC50 (mg/L) values of tested compounds (**3a-f**) and cisplatin which was used as a positive control, were shown in Table 1. Synthesized compounds **3a** and **3b** exhibited high cytotoxic effect ($p < 0.05$) with 8.21 ± 0.71 and 28.3 ± 1.11 mg/L values of IC50 against HeLa and MCF-7 cancer cells, respectively. Compound **3c** significantly inhibited ($p < 0.05$) the viability of lung cancer (IC50 = 14.6 ± 0.78). Compound **3d** caused a significant decrease ($p < 0.05$) with 38.2 ± 1.02 and 20.2 ± 0.98 mg/L values of IC50 in the viability of lung and cervix cancers, respectively. Compounds **3e** and **3f** were most effective compounds with 3.2 ± 0.78 and 2.5 ± 0.61 values of IC50 on lung cancer compared to the cisplatin, respectively while decreasing viability on breast cancer cells with 35.1 ± 1.25 and 26.5 ± 1.17 values of IC50, respectively.

Table 1. Calculated IC50 values on different cancer and normal cells after treatmentwith synthesized coumarin derivatives (3a-f) (mg/L).

Compound	Lung cancer (A 549)	Breast cancer (MCF 7)	Cervix cancer (HeLa)	Human embriyonic kidney (HEK 293)
3a	58.2 ± 1.31	8.21 ± 0.71	28.3 ± 1.11	17.6 ± 0.81
3b	63.8 ± 0.81	34.3 ± 1.32	12 ± 1.29	19.3 ± 0.73
3c	14.6 ± 0.78	51.3 ± 0.95	66.8 ± 1.21	>100
3d	38.2 ± 1.02	>100	20.2 ± 0.98	68.2 ± 1.04

3e	3.2 ± 0.78	35.1 ± 1.25	56.7 ± 0.92	58.1 ± 1.23
3f	2.5 ± 0.61	26.5 ± 1.17	58.3 ± 0.87	67.5 ± 1.25
Cisplatin	4.6 ± 0.93	9.6 ± 0.88	2.12 ± 0.75	3.4 ± 0.87

To reveal a selective effect against cancer cells, all compounds were also tested for in vitro cytotoxicity on human normal HEK293 cells. Results revealed that IC50 values of compounds **3c-f** were>50 mg/L against HEK293 cells. However, compounds **3a** and **3b** caused cytotoxicity ($p < 0.05$) on HEK-293 cells with the IC50 < 50 mg/L. This remarkable selective cytotoxic effect of compounds **3c-f**, having a phenyl group at the positions 5 of the triazole nucleus, on A 549 lung cancer cells prompted us to investigate their effects at molecular levels for whether lung cells could overcome their acquired resistance to cisplatin.

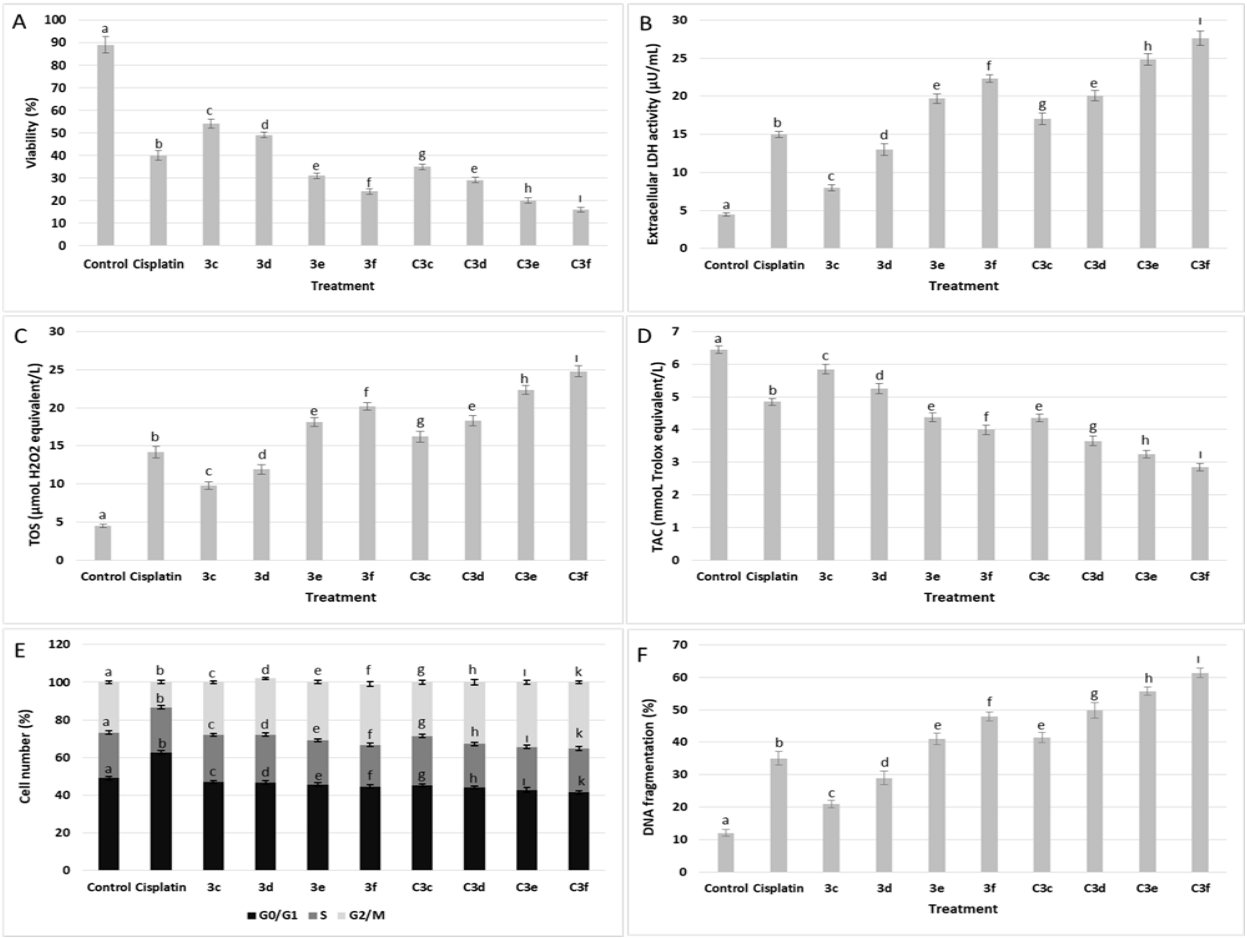

Figure 2. Effects of coumarin compounds (treated with IC50 values) alone or in the combination with cisplatin (10 μmol/L) on (A) Cell viability.; (B) LDH levels.; (C) TAC levels.; (D) TOS levels.; (E) Cell cycle arrest.; and (F) DNA fragmentation on human lung cancer cells. Values represent means ± SD of at least three experiments. Bars indicated by the different letters (a, b, c, d, e, f, g, h, ı, k) show significant differences from each other at the $p<0.05$ level. Data values obtained from analysis in triplicate

*Compounds **3c-f** decrease cell proliferation and increase LDH activity*

To evaluate the cytotoxicity of coumarin compounds against acquired resistance to cisplatin on A549 cells, the second set of A549 cells were treated with calculated IC50 values(14.6 ± 0.78, 38.2 ± 1.02, 3.2 ± 0.78, and 2.5 ± 0.61, respectively) of compounds **3c-f**. Cell viability results showed that compounds **3c-f** significantly inhibited ($p < 0.05$) with a fold change of 1.64, 1.81, 2.87, and 3.70 the viability on A549 cells, respectively. Treatments combined of compounds **3c-f** with cisplatin caused the more cytotoxic effect on cell viability with a fold change of 2.54, 3.06, 4.45, and 5.56 compared to the cisplatin (2.23-fold change), respectively (Figure 2A).

LDH results revealed that the compounds **3c-f** significantly increased ($p < 0.05$) with a fold change of 1.64, 1.81, 2.87, and 3.70 the LDH level on A549 cells, respectively (Figure 2B). Positive control cisplatin caused a significant increase ($p < 0.05$) in LDH release with a 3.3-fold change. Compounds **3c-f** medium supplemented with cisplatin exhibited higher LDH levels than with a fold change of 3.7, 4.4, 5.5, and 6.2 cisplatin alone (3.3-fold change). There was a significant linear correlation between cell viability and LDH release results (R^2= -0.99, $p < 0.05$).

Compounds 3c-f increase oxidative stress and decrease antioxidant activity

Oxidative effects and antioxidant status of compounds synthesized bearing coumarin were determined by TOS and TAC assay, respectively. Oxidant status results of A549 exposed to compounds showed that TOS levels significantly increased ($p < 0.05$) after treatments of

compounds **3c-f** with a fold-increase of 2.1, 2.64, 4.02, and 4.48 compared to untreated controls, respectively (Figure 2C). Combination treatments of compounds **3c-f** with cisplatin increased ($p < 0.05$) more TOS levels than with values of 3.6, 4.06, 4.95, and a 5.5-fold increase compared to the cisplatin alone (3-fold increase), respectively.

As seen Figure 2D, compounds **3c-f** significantly decreased ($p < 0.05$) the antioxidant levels with 1.1, 1.23, 1.47, and 1.61-fold change increase, respectively. Compounds **3c-f** after the addition of cisplatin caused more decrease ($p < 0.05$) in TAC levels than the cisplatin alone (1.32-fold change) with 1.48, 1.76, 1.98, and 2.26-fold changes, respectively.

Compounds 3c-f increase DNA fragmentation and G2/M cell arrest

After treatments of compounds **3c-f** cells in the G2/M population significantly increased ($p < 0.05$) with a value of 1.01, 1.12, 1.16, and 1.20-fold changes compared to the control following by a decrease of the cell population in the G0/G1 phase (Figure 2E). Cisplatin alone significantly decreased ($p < 0.05$) with a 2-fold change in the cell population of the G2/M cycle. Combination treatments of compounds **3c-f** with cisplatin significantly increased ($p < 0.05$) the cell population in the G2/M cycle with a value of 2.14, 2.45, 2.58, and 2.63- fold changes compared to the cisplatin alone.

DNA fragmentation results showed that compounds **3c-f** significantly increased ($p < 0.05$) DNA fragmentation with a value of 1.75, 2.41, 3.4, and 4 compare to the untreated control (Figure 2F). Simultaneously treatments of **3c-f** with cisplatin caused more increase ($p < 0.05$) with a value of 3.45, 4.15, 4.65, and 5.2 compared to the cisplatin alone (2.91-fold change).

*Compounds **3c-f** regulate apoptotic gene expression*

Compounds **3c-f** significantly upregulated ($p < 0.05$) pro-apoptotic Bax, Bıd, caspase-3, caspase-8, caspase-9, FAS, and P53 gene expressions (Figure 3). However, simultaneously treatments of **3c-f** with cisplatin caused more upregulation ($p < 0.05$) in the expression of these pro-apoptotic

genes compared to the cisplatin alone. Antiapoptotic DFFA, NFkB1, and Bcl-2 gene significantly downregulated ($p < 0.05$) after treatments of compounds **3c-f** alone. Furthermore, the combination of **3c-f** with cisplatin caused more downregulation ($p < 0.05$) in the expression of these anti-apoptotic genes compared to the cisplatin alone treatment.

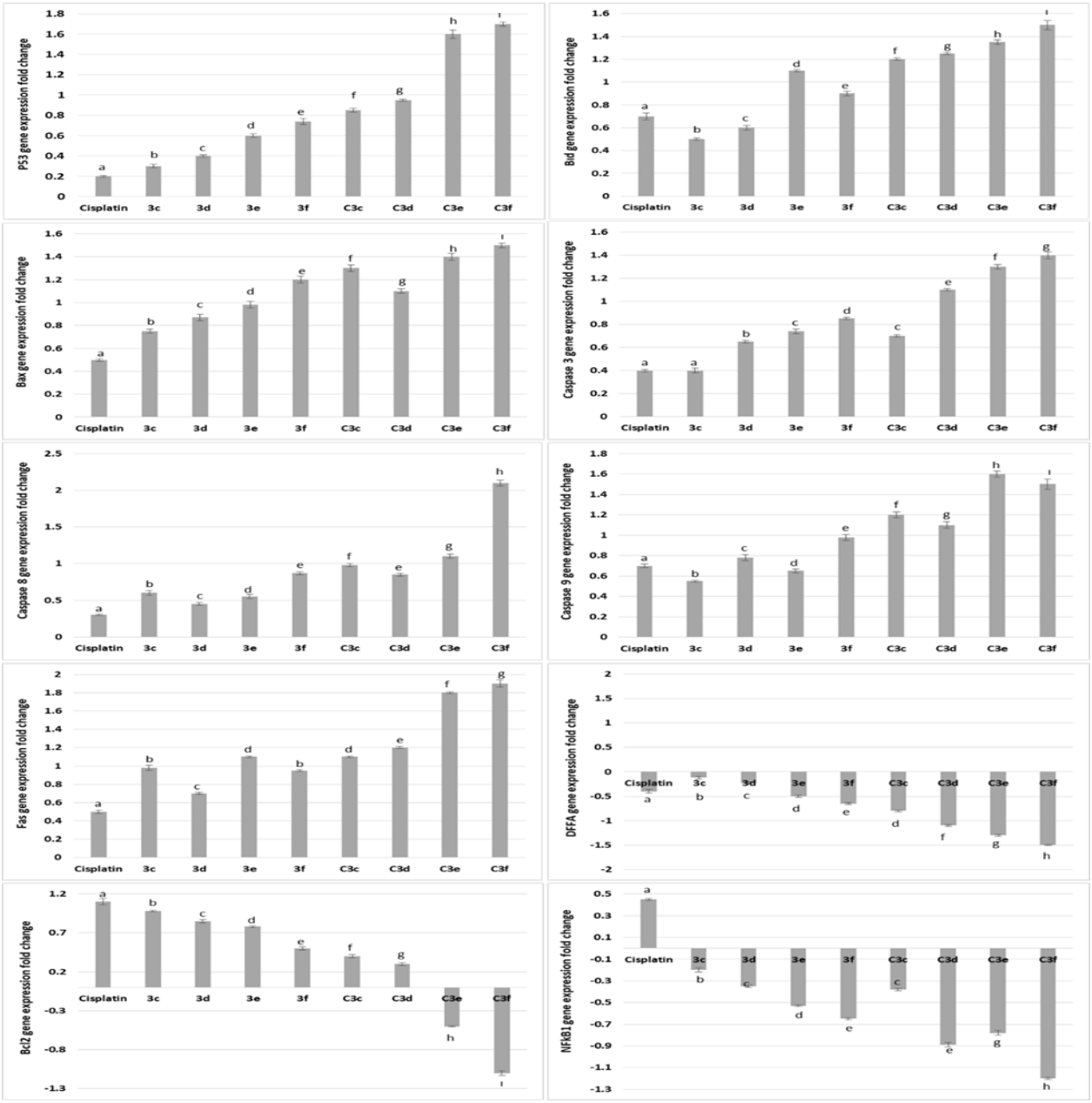

Figure 3. Quantifying changes in gene expression after treatments of coumarin compounds (treated with IC50 values) alone or in the combination with cisplatin (10 μmol/L) on human lung

cancer cells by Real-time PCR analysis. Values represent means ± SD of at least three experiments. Bars indicated by the different letters (a, b, c, d, e, f, g, h, ı) show significant differences from each other at the $p<0.05$ level. Data values obtained from analysis in triplicate

*Compounds **3c-f** inhibit VEGF gene expression and CAM surface vessel formation*

As seen in Figure 4A, VEGF expression on A549 cells treated with compounds **3c-f** significantly downregulated with a value of 1.4, 1.3, 1.1, and 1.02-fold changes. Treatments combined of **3c-f** with cisplatin caused a significant decrease in gene expressions with a value of 0.9, 0.78, 0.67, and 0.55-fold changes as compared to cisplatin alone (1.7-fold change). Besides, antiangiogenic effects and vascular damage on CAM surface after treatments of compounds **3c-f** were shown in Figure 4B and -C. Suramin had a strong antiangiogenic effect ($p < 0.05$) with a value of 1.8 ± 0.06 while DMSO had no antiangiogenic effect. Compounds **3c-e**, cisplatin, and combination of **3c** with cisplatin caused a moderate antiangiogenic effect with a value of 0.65 ± 0.02, 0.75± 0.03, 0.95± 0.02, 0.85± 0.03, and 0.9 ± 0.02, respectively. Compound **3f** and combination of compounds **3d**, **3e**, and **3f** with cisplatin showed a strong antiangiogenic activity with a value of 1.1± 0.06, 1.02± 0.06, 1.15± 0.06, and 1.3± 0.06, respectively.

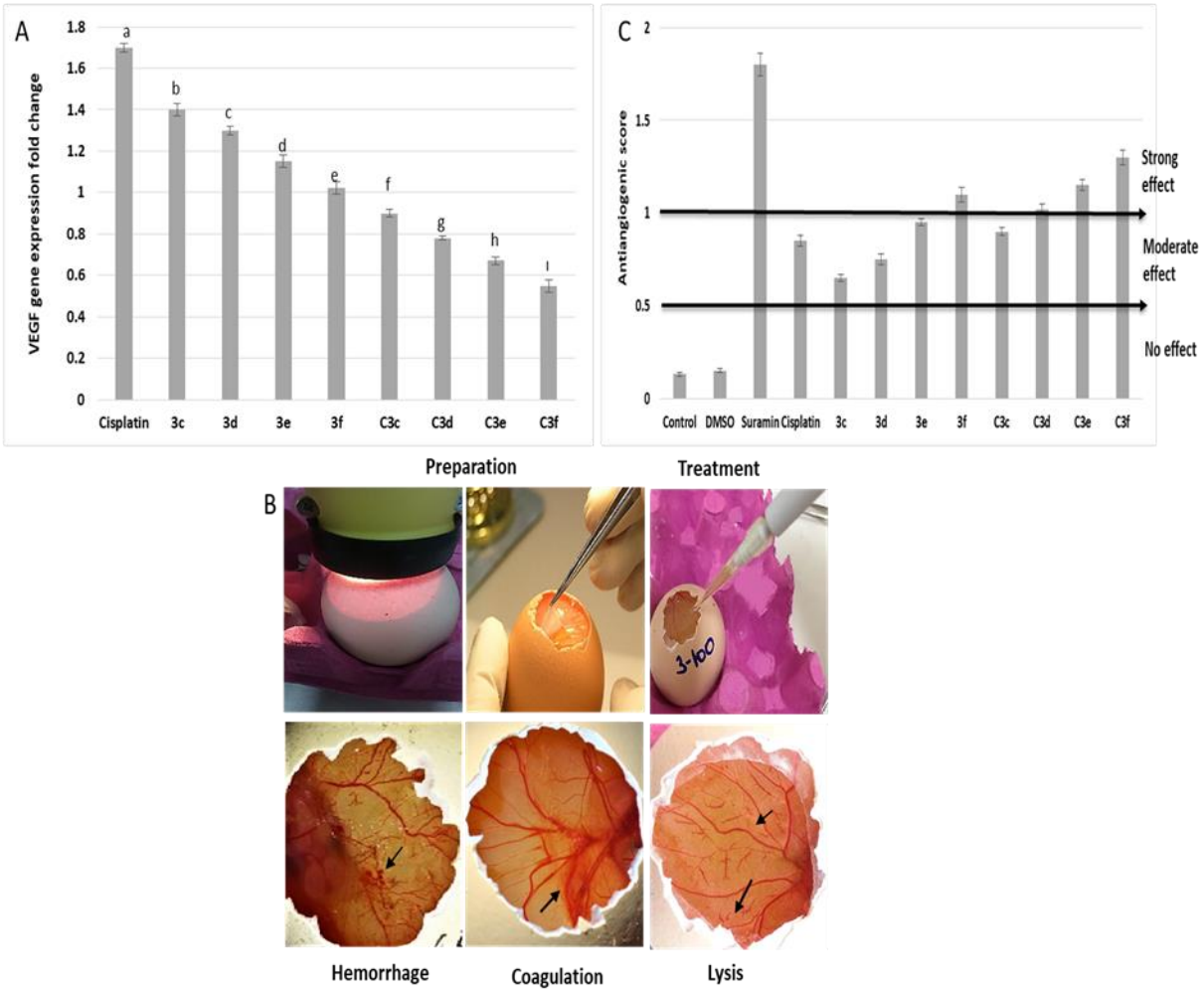

Figure 4. Antiangiogenic assay. (A) Effects of coumarin compounds.; cisplatin and their combinations on VEGF gene expression in A549 cells, (B) Pictures indicating different vascular damages following coumarin, cisplatin and their combinationtreatments, (C) Anti-angiogenic scores after treatments of coumarin compounds, cisplatin.; and their combinations on CAM membrane surface. Values represent means ± SD of at least three experiments. DMSO and suramin were used as a negative and positive control, respectively

Discussion

Novel chemotherapeutic strategies aim to increase the effectiveness of existing methodologies and to eliminate their toxic effects on normal cells. Coumarin and its derivates attached to

different heterocyclic moieties can simply react with biomacromolecules and able to regulate their activity [12]. This present study revealed the anticancer effectiveness of novel synthesized coumarin compounds alone and in combination with cisplatin on human lung cancer cells. Our results showed that coumarin-triazole compounds 3c, d, e, and f significantly inhibited the proliferation of A549cells. Previous studies showed that coumarin-derivated compounds could cause cytotoxicity on various human cancer cell lines [4,13]. Yu et al., [14] showed that coumarin compounds bearing triazole moiety exhibited an important cytotoxic activity against human breast (MDA-MB-231), colorectal (HT-29 and HCT-116), and lung A549 cells. However, compounds 3e and –f were great potency against A549 cells, which were comparable with cisplatin. Sinha et al., [15] have been shown that compound **5d** which was a hybrid form of coumarin and triazole exhibits a more potent effect than cisplatin with 17.5 ± 1.22 and 9.83 ± 0.69 values of IC50 against cervix and breast cancer cells, respectively. Combination treatments with coumarin derivates to increase the effectiveness cytotoxic of cisplatin showed more successful results compared to the cisplatin alone on cancer cells [16]. Supporting these results, combination treatments of **3c-f** compounds bearing coumarin and triazole with cisplatin significantly inhibited the viability of A549 cells as compared to standard drug cisplatin alone treatment. Furthermore, these compounds showed low cytotoxic effects (IC50>50) against HEK293 cells. The structure-activity relationship showed significant differences in activity depending on the substituent in position 5 of the triazole moiety. Compounds **3a** and **3b** contain an aliphatic group in position 5 of the triazole ring when compared with compounds **3c-f** having phenyl group at the 5th position of the triazole ring showed potent anti-cancer activity. These results suggest that the combinational regimen of **3c-f** compounds with cisplatin selectively increase the inhibition of cell proliferation as a result of synergistic efficacy on A549 cells [17]

and these ring structure of coumarin is a crucial strategy to overcome acquired cisplatin resistance [18].

LDH is a cytoplasmic enzyme and its increased levels in the cellular environment are an important sign of cytotoxicity following the loss of membrane integrity [19]. Our results confirmed the cytotoxic effect on lung cancer cells. The compounds induced LDH leakage, and among the compounds examined, compounds **3e** and **3f** were found to be more potent in inducing LDH leakage into the culture. Kalaiarasia et al., [20] synthesized a novel series of coumarin for anticancer activity on MCF-7 and A549 and showed that complexes caused more induce cytotoxicity and LDH leakage as compared to cisplatin. Combination treatments with cisplatin of compounds **3c-f** induced more LDH release than cisplatin. Previously studies showed that combination treatment of cisplatin enhanced cytotoxic effects of cisplatin on cancer cells by regulating drug and protein transporters through increased LDH [21].

Moderate levels of reactive oxygen species (ROS) are essential for the maintenance of cell proliferation and differentiation. The balance between production and scavenging of ROS is an important marker to the complex link between cancer and ROS levels. Cancer initiation and progression are needed slight increases in ROS levels. At high levels of ROS, cancer cells more sensitive to external stimuli that promote the production of ROS, and thus, cells tend to be damaged and die [22,23]. Cisplatin, which is used in anticancer treatments, mainly aims to induce oxidative stress in cancer cells [24]. Our result showed that cisplatin caused an increase in TOS level on A549 cells. Increased ROS levels are also thought to affect drug resistance against cancer cells [25] and cancer cells balance their increased ROS levels through enhanced antioxidant defense mechanisms [26]. Recent studies have attempted to induce cancer cell death by disrupting the antioxidant response in cancer cells [27]. Combination treatment of cisplatin with an antioxidant inhibitor can be considered as a strategic move to overcome cisplatin

resistance in cancer cells. Sivalingam et al., [21] showed that neferine, is an alkaloid derivate, could be enhanced the effectiveness of cisplatin through the increase in ROS levels and inhibition of cellular antioxidant enzymes in lung cancer cells. Our coumarin compounds could potentiate for the first time the efficacy of cisplatin by inhibiting antioxidant levels in lung cancer cells.

p53 gene is an important transcription factor regulating the expression of genes responsible for antioxidant and oxidative mechanisms. Indeed, slightly increased ROS level downregulates p53 gene expression, while higher ROS levels induce upregulation of p53 expression [22]. Combination treatments of **3c-f** coumarin compounds with cisplatin significantly upregulated P53 gene expression between 4.2 to 8.5 -fold increase as compared to cisplatin alone and lung cancer cells draw to apoptosis. ROS may impact the expressions of different signaling pathways involved in apoptosis and cell proliferation by interacting with cellular proteins [28]. Combination treatments of coumarin compounds with cisplatin significantly upregulated the pro-apoptotic gene expressions in extrinsic (Fas, Caspase 8 and Bıd genes) and intrinsic (Bax and caspase-9) pathways. Upregulation in caspase 9 expression and downregulation in DFFA expression confirmed that lung cancer cells undergo apoptosis. Downregulations in Bcl-2, DFFA, and NFkB1 gene expressions suggested that our treatments promoted apoptosis via inhibition of anti-apoptotic pathways. Over-expression of Fas genes reverses cisplatin resistance through enhanced cell sensitivity to apoptosis in human lung cancer [29] and resistance to cisplatin in cancer cells can be overcome by upregulating the TRAIL receptor [30]. Zhu et al., [31] showed that coumarin complex could induction apoptosis through the upregulation of p53 and Bax and downregulation of the Bcl-2 gene in lung adenocarcinoma cells. Briefly, our results have shown that coumarin compounds sensitize lung cancer cells to cisplatin through both caspase-dependent pathways involving extrinsic and intrinsic/mitochondrial and caspase-independent pathways.

Anticancer efficacy of cisplatin is that it causes cytotoxicity, mainly via the formation of DNA intrastrand adducts and interstrand cross-links in cancer cells. Inducing DNA damages induce cell cycle arrest in the G2/M checkpoint [32]. But, excessive increases in DNA damage are tolerated by the DNA repair mechanism of cancer cells and this process causes acquired resistance to cisplatin of cancer cells involving reduced G2/M cell cycle arrest and apoptotic responses [33]. Previous studies showed that acquired resistance of lung cancer cells against cisplatin could be attributed to decreased G2/M cell cycle arrest [34] and DNA damages [35] similar to our results. Combination treatments of cisplatin with coumarin compounds may enhance the sensitivity of lung cancer cells through stimulation of DNA damage and regulation of cell cycle arrest[18,21]. In a study on the anticancer effects of coumarin conjugates bearing triazole ring, compounds were shown to induce apoptosis through a decrease in cell population in the G1 phase and an increase in the cell population of the G2 phase in A549 cells [12]. Supporting these results, A549 cells treated by combination treatments of cisplatin with compounds **3c-f** cells remarkably accumulated (between 2.1 to 2.6-fold increase) in the G2/M phase of the cell cycle and, DNA fragmentation levels was significantly increased (between 3.4 to 5.2 fold increase).

Angiogenesis is a process that describes the formation of new blood vessels from pre-existing vessels in the development of normal physiological processes such as embryonic development, wound healing, and inflammation. Angiogenesis has also a critical role in the invasive growth and metastasis of cancer cells [36]. Many studies have shown that the intensity of angiogenesis is increased in a variety of human tumors and therefore inhibition of angiogenesis or its signal pathways is one of the most important strategies in antitumor treatments. Platinum-based chemotherapeutics can target block the tumor vascularization that carries nutrients and oxygen to the tumor as well as cytotoxic activity [37]. Cisplatin has been

shown to inhibit tumor growth in various cell carcinomas by inducing antiangiogenic factors or by decreasing vascular density [38, 39]. But, tumor cells develop resistance to antiangiogenic therapies thanks to their increased metastatic abilities, desire for revascularization, and compact vascularization structures [40]. VEGF is an effective inducer in tumor angiogenesis and its expression is upregulated in tumors. Inhibition of VEGF signaling is a strategic step to overcome resistance to antiangiogenic treatments [41]. Combination treatments of **3c-f** compounds of coumarin with cisplatin significantly downregulated the VEGF gene expressions compared to the cisplatin alone. Similar results were demonstrated with CAM findings. Cisplatin alone treatment showed a moderate antiangiogenic effect with a value of 0.85± 0.03 while antiangiogenic score after combination treatments with **3c-f** compounds of its indicated a strong effect (between 1.1 to 1.3). The antiangiogenic effects of the various compounds derived from coumarin have been demonstrated by many studies showing both a marked reduction in the number of blood vessels in the CAM model and the inhibition of human umbilical vein endothelial cells (HUVECs) induced by VEGF [42-44].

The present study provides molecular evidence that synthesized **3c-f** coumarin-triazole hybrid compounds sensitize lung cancer cells to cisplatin. A combination of cisplatin with these compounds showed anticancer effects more efficiently than cisplatin alone. The proliferation of A549 cells treated by combination treatments was significantly inhibited via an increase in LDH release, ROS, G2 cell cycle arrest, and DNA fragmentation levels as well as a decrease in antioxidant levels. Combination treatments induce apoptosis through upregulations in the expression of pro-apoptotic genes in the extrinsic and mitochondrial pathways and down regulations in antiapoptotic gene expressions. Furthermore, these treatments enhanced the antiangiogenic effect of cisplatin following vascular damages in the CAM model and reduction in VGEF expression. Altogether our results suggest that using designed compounds with a cisplatin

combined regimen improves the efficacy of cisplatin on lung cancer cells and, considering low cytotoxic effects of coumarin compounds on normal cells, reduces the dose-associated adverse effects of cisplatin in chemotherapy.

Supporting Information

Supporting Information File 1; Synthesize of compounds 3a-f

Supporting Information File 2; NMR data for compounds 3a-f

Declaration of competing interest

The authors declare that they have no disclosed any financial or personal relationships that could have appeared to influence the work reported in this manuscript.

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