**MATERIALS AND METHOD**

**Chemistry**

All reactions were carried out in aerobic conditions at room temperature. Acetonitrile was distilled and kept under an inert atmosphere. All glass were oven-dried at 120 °C for at least 24 h prior to use. The starting materials **3a-f** have been prepared as described in the literature. All melting points are uncorrected and measured using Electro‐Thermal IA 9100 apparatus (Shimadzu, Japan). The Infrared spectra were recorded as potassium bromide pellets on a JASCO spectrophotometer between 4000 cm-1 and 400 cm-1. 1H NMR spectra were recorded in deuterated dimethylsulfoxide (DMSO-d6) on a Brucker spectrometer (400 MHz) at 25 °C. The chemical shifts were expressed as parts per million (δ values, ppm) using the solvent (DMSO = 2.51, water signal at 3.34) as reference.

**General procedure for the synthesis of *bis*-chalcone derivatives 5-10.**

A mixture of 1,3-diphenyl-1*H*-pyrazole-4-carbaldehyde, **4** (2 mmol) and *bis*-(acetyl) derivatives (**3a-f**) (1 mmol) was dissolved in ethanol (20 mL). To this mixture, potassium hydroxide (20%, 5 ml) was added at 0-5 oC. The reaction mixture was stirred at room temperature for 5 h, then poured over ice containing HCl. The obtained yellow solid was then filtered, washed with water, and dried. The crude product was crystallized from the proper solvent to afford *bis*-chalcone as yellow crystals of **5-10**.

***2.2.1. 1,1'-((Ethane-1,2-diylbis(oxy))bis(4,1-phenylene))bis(3-(1,3-diphenyl-1H-pyrazol-4-yl)prop-2-en-1-one) (5)***

m.p = 200-202 °C (EtOH-Dioxane). Yield 77 %. IR (KBr, cm-1): 1668 (C=O). 1H NMR (400 MHz, DMSO-*d*6): δ, ppm: 4.05 (s, 4H, CH2), 710-8.13 (m, 32H, Ar-H + vinyl-H), 9.43 (s, 2H, pyrazole-H5). 13C NMR (100 MHz, DMSO-*d*6): δ, ppm: 67.1, 115.1, 118.4, 119.1, 119.5, 121.9, 127.6, 128.9, 129.2, 129.4, 130.2, 131.1, 131.2, 139.5, 153.3, 162.7, 187.4. Anal. Calcd. for C50H38N4O4 (758.88): C, 79.14; H, 5.05; N, 7.38; Found: C, 79.22; H, 5.19; N, 7.37.

***2.2.2. 1,1'-((Ethane-1,2-diylbis(oxy))bis(2,1-phenylene))bis(3-(1,3-diphenyl-1H-pyrazol-4-yl)prop-2-en-1-one) (6)***

m.p = 178-181 °C (EtOH-Dioxane). Yield 82 %. IR (KBr, cm-1): 1670 (C=O). 1H NMR (400 MHz, DMSO-*d*6): δ, ppm: 4.51 (s, 4H, CH2), 6.85-7.76 (m, 32H, Ar-H + vinyl-H), 8.82 (s, 2H, pyrazole-H5). 13C NMR (100 MHz, DMSO-*d*6): δ, ppm: 68.1, 113.9, 117.9, 119.1, 121.3, 126.9, 127.4, 128.2, 128.7, 128.8, 129.0, 129.1, 129.9, 130.1, 132.4, 133.3, 139.4, 153.1, 157.4, 191.6. Anal. Calcd. for C50H38N4O4 (758.88): C, 79.14; H, 5.05; N, 7.38; Found: C, 79.20; H, 5.02; N, 7.47.

***2.2.3. 1,1'-((Propane-1,3-diylbis(oxy))bis(4,1-phenylene))bis(3-(1,3-diphenyl-1H-pyrazol-4-yl)prop-2-en-1-one) (7)***

m.p = 98-100 °C (EtOH). Yield 80 %. IR (KBr, cm-1): 1682 (C=O). 1H NMR (400 MHz, DMSO-*d*6): δ, ppm: 2.26 (m, 2H, CH2), 4.29 (m, 4H, CH2), 7.13-8.10 (m, 32H, Ar-H + vinyl-H), 9.41 (s, 2H, pyrazole-H5). 13C NMR (100 MHz, DMSO-*d*6): δ, ppm: 28.9, 65.1, 114.9, 116.9, 118.4, 119.1, 119.5, 121.8, 127.6, 128.9, 129.2, 129.3, 130.2, 131.0, 131.1, 132.5, 133.7, 139.5, 153.3, 162.9, 187.4. Anal. Calcd.for C51H40N4O4 (772.91): C, 79.25; H, 5.22; N, 7.25; Found: C, 79.30; H, 5.20; N, 7.33.

***2.2.4. 1,1'-((Propane-1,3-diylbis(oxy))bis(2,1-phenylene))bis(3-(1,3-diphenyl-1H-pyrazol-4-yl)prop-2-en-1-one) (8)***

m.p = 152-154 °C (EtOH). Yield 83 %. IR (KBr, cm-1): 1672 (C=O). 1H NMR (400 MHz, DMSO-*d*6): δ, ppm: 2.12 (m, 2H, CH2), 4.15 (m, 4H, CH2), 6.89-7.89 (m, 32H, Ar-H + vinyl-H), 9.19 (s, 2H, pyrazole-H5). 13C NMR (100 MHz, DMSO-*d*6): δ, ppm: 29.0, 65.4, 112.9, 117.9, 119.1, 119.4, 121.0, 127.0, 127.6, 128.8, 129.1, 129.2, 129.6, 129.7, 130.1, 132.3, 132.8, 133.7, 139.4, 153.2, 156.8, 192.8. Anal. Calcd. for C51H40N4O4 (772.91): C, 79.25; H, 5.22; N, 7.25; Found: C,79.32; H, 5.38; N, 7.31.

***2.2.5. 1,1'-((Butane-1,4-diylbis(oxy))bis(4,1-phenylene))bis(3-(1,3-diphenyl-1H-pyrazol-4-yl)prop-2-en-1-one)*** *(****9****)*

m.p = 115-117 °C (EtOH). Yield 70 %. IR (KBr, cm-1): 1668 (C=O). 1H NMR (400 MHz, DMSO-*d*6): δ, ppm: 1.91 (m, 4H, CH2), 4.17 (m, 4H, CH2), 7.03-8.1 (m, 32H, Ar-H + vinyl-H), 9.41 (s, 2H, pyrazole-H5). 13C NMR (100 MHz, DMSO-*d*6): δ, ppm: 25.7, 68.0, 114.7, 114.9, 118.4, 119.1, 119.5, 121.9, 128.9, 129.2, 129.3, 130.2, 130.9, 131.1, 132.5, 133.7, 139.5, 153.3, 163.1, 172.5, 187.4. Anal. Anal. Calcd.for C52H42N4O4 (786.93): C, 79.37; H, 5.38; N, 7.12; Found: C, 79.41; H, 5.55; N, 7.19.

***2.2.6. 1,1'-((Butane-1,4-diylbis(oxy))bis(2,1-phenylene))bis(3-(1,3-diphenyl-1H-pyrazol-4-yl)prop-2-en-1-one) (10)***

m.p = 114-115 °C (EtOH). Yield 84 %. IR (KBr, cm-1): 1673 (C=O). 1H NMR (400 MHz, DMSO-*d*6): δ, ppm: 1.81 (m, 4H, CH2), 3.95 (m, 4H, CH2), 6.92-7.90 (m, 32H, Ar-H + vinyl-H), 9.17 (s, 2H, pyrazole-H5). 13C NMR (100 MHz, DMSO-*d*6): δ, ppm: 25.8, 68.0, 113.0, 117.9, 119.1, 119.4, 120.9, 127.3, 127.5, 128.8, 129.1, 129.2, 129.4, 129.8, 130.1, 132.4, 133.1, 133.5, 139.3, 153.1, 157.1, 172.5, 192.6. Anal. Calcd. for C52H42N4O4 (786.93): C, 79.37; H, 5.38; N, 7.12; Found: C, 79.45; H, 5.47; N, 7.17.

**Anticancer evaluation**

**Cell line culture**

In our study, MTT protocol was utilized to assay cell viability after treatment with the tested compounds. The following cell lines (PC3, A431, A549, and BJ1) were cultured and grown in Roswell Park Memorial Institute (RPMI‐1640) media. The BJ1 cells were reserved in DMEM‐F12 media, and the two media were supplemented with 10% FBS), 1% antibiotic–antimycotic mixture, and 1% L‐glutamine. Cancer cell lines were kept at 37 °C and 5% CO2. Counted cancer cells (10 × 103) of A431, A549, and PC3 and the same count of normal skin cells (BJ1) were cultured in a 96‐well plate. The cultured cells were incubated at 37 °C and 5% CO2 overnight. After 24h, the media was discarded, then 100 µL of different concentrations of the tested compounds dissolved in fresh medium without serum, with final concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 mg/Ml. The treated cells were then incubated for 48 hours. 20 µL of MTT was applied to the residual living cells in each well for 4 hours for the formation of formazan crystals. 200 µl of 10% (SDS) was added into each well and the plates were incubated overnight at room temperature. A microplate reader was used to read the optical density at 575 nm, and the growth inhibition value (%) was calculated according to our previously published paper. The dose-response curve was drawn to calculate the IC50 values for each compound. To examine the possibility of our new structures, doxorubicin, a commercial classical anticancer drug, was used as a reference or standard drug (positive control). Negative cell culture control was involved in this assay.

**Gene expression analysis**

**Quantitative Real Time PCR method**

#### *RNA isolation and Reverse Transcription (RT) Reaction*

Total RNA from skin and lung cancer cell lines was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) supplemented with DNaseI (Qiagen) digestion step according to the manufacturer’s protocol. Isolated total RNA was treated with one unit of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, resuspended in DEPC-treated water, and quantified photospectrometrically at 260 nm. The purity of total RNA was assessed at 260/280 nm ratio which was between 1.8 and 2.1. Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis.

Total Poly(A)+ RNA isolated from skin and lung cancer cell lines was reverse transcribed into cDNA in a total volume of 20 µl using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, Germany). An amount of total RNA (5 µg) was used with a master mix consisting of 50 mM MgCl2,10x RT buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3), 10 mM of each dNTP, 50 µM oligo-dT primer, 20 IU ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 IU MuLV reverse transcriptase. The mixture of each sample was centrifuged for 30 sec at 1000 xg and transferred to the thermocycler. The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C, and finished with a denaturation step at 99°C for 5 min. Afterwards, the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for gene expression quantification using quantitative real time- polymerase chain reaction (qRT-PCR).

#### *Quantitative real time- PCR (qPCR)*

StepOne™ Real-Time PCR System from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA USA) was used to measre the Skin and lung cancer cell line cDNA copy number. qPCR Reactions were set up in 25 μL reaction mixtures containing 12.5 μL 1× SYBR**®** Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 μL 0.2 μM sense primer, 0.5 μL 0.2 μM antisense primer, 6.5 μL distilled water, and 5 μL of cDNA template. The reaction program was allocated to 3 steps. The first step was at 95 °C for 3 min. The second step consisted of 40 cycles in which each cycle divided to 3 steps: (a) at 95°C for 15 sec; (b) at 55°C for 30 sec; and (c) at 72°C for 30 sec. The third step consisted of 71 cycles which started at 60°C and then increased about 0.5°C every 10 sec up to 95°C. At the end of each qRT-PCR a melting curve analysis was performed at 95°C to check the quality of the used primers. Each experiment included a distilled water control. According to Edqvist *et al*. [[61](#_ENREF_61)] and Kim *et al*. [[62](#_ENREF_62)], the sequences of specific primers of the skin and lung cancer cell line related genes used are listed in Table 1. The relative quantification of the target to the reference was determined by using the 2*−*ΔΔCT method.

**Table 1.** Primer sequences used for *qRT-PCR* of skin cancer cell lines.

|  |  |  |
| --- | --- | --- |
| Gene | Forward | Reverse |
| *DSP* | 5'-gatgtactattctcggcgcg-3' | 5'- caattcaggctgcacgatga-3' |
| *SFN* | 5'-gggagaaggtggagactgag-3' | 5'- gctgagtcaatgatgcgctt-3' |
| *DDIT3* | *5'- CCA AAA TCA GAG CTG GAA CC-3'* | *5'- CCA TCT CTG CAG TTG GAT CA -3* |
| *MAFF* | *5'- AGA CGC GCG TGT GTG A -3'* | *5'- CTG GAT AGG GGA TCC ACA GA-3'* |
| *GAPDH* | 5'-CCTCAACTACATGGTTTACATGTTCC-3' | 5'-ATG GGA TTT CCA TTG ATG ACA AG-3' |

DSP: desmoplakinhuman. SFN: Stratifin. DDIT3: DNA damage-inducible transcript 3. MAFF: MAF, bZIP transcription factor F. GAPDH: Glyseraldehyde-3-phosphate dehydrogenase.

***DNA damage using the comet assay***

The DNA damage using comet assay was determined using skin and lung cancer cell lines as stated by Olive *et al*.[[63](#_ENREF_63)] After the trypsin treatment to produce a single cell suspension, approximately 1.5×104 cells were embedded in 0.75% low-gelling-temperature agarose and rapidly pipetted onto a pre-coated microscope slide. Samples were lysed for 4 h at 50 °C in 0.5% SDS, 30 mM EDTA, pH 8.0. After rinsing overnight at room temperature in Tris/borate/EDTA buffer, pH 8.0, the samples were electrophoresed for 25 min at 0.6 V/cm, and then stained with propidium iodide. Slides were viewed using a fluorescence microscope with a CCD camera, and 150 individual comet images were analyzed from each sample for tail moment, DNA content, and percentage DNA in tail. For each sample, about 100 cells were examined to define the percentage of cells with DNA damage that appear like comets. The nonoverlapping cells were randomly selected and were visually assigned a score on an arbitrary scale of 0–3 (i.e., class 0 = no detectable DNA damage and no tail; class 1 = tail with a length less than the diameter of the nucleus; class 2 = tail with a length between 1× and 2× the nuclear diameter; and class 3 = tail longer than 2× the diameter of the nucleus) based on perceived comet tail length migration and relative proportion of DNA in the nucleus (Collins *et al*. [[64](#_ENREF_64)]).

***DNA fragmentation assay***

The fragmentation rates of the DNA were performed according to the premises established by Yawata [[65](#_ENREF_65)] with some modifications. Briefly, after 24 h of exposure of skin and lung cancer cell lines to the tested substances (D3) in different Petri-dishes (60 × 15 mm, Greiner), the cells were trypsinized, suspended, homogenized in 1 ml of medium, and centrifuged (10 min at 800 rpm).Low-molecular-weight genomic DNA was extracted as described in Yawata.[[65](#_ENREF_65)] Approximately, 1 × 106 cells were plated and treated with the tested substances in various treatments (1575/ 100, 1653/ 100, and 1832/ 100). All cells (including floating cells) were harvested by trypsinization and washed with Dulbecco`s Phosphate Buffered Saline. Cells were lysed with the lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 10000 xg for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol: chloroform: isoamyl alcohol mixture (25: 24:1) and examined electrophoretically on 2% agarose gels containing 0.1 μg/ml ethidium bromide.

***Statistical analysis***

All tests were done via the statistical package for the social science program (SPSS version 20.0). The data of this study was shown as mean ± standard error (SE) by using one-way ANOVA. Percentage difference, indicating the percentage of variation regarding the corresponding control group, was also calculated by the following formula: % change = treated - control value/control value × 100. The relative gene expression of target genes (DSP, SFN, DDIT3, MAFF and GAPDH) as compared to the reference gene was performed with the 2*−*ΔΔCT method as follows:

ΔCT(test)*=* CT(target, test)*−* CT(reference, test) ,

ΔCT (calibrator) *=*CT(target, calibrator)*−* CT(reference, calibrator) ,

ΔΔCT *=*ΔCT(Test)*−* ΔCT(calibrator) .

**Molecular docking study**

In an attempt to theoretically predict the mechanistic pathways of our novel prepared compound against different proteins, a molecular docking study was used. Herein, we used two docking programs, MOE and BIOVIA Discovery Studio programs. The role of the MOE program is to illustrate the binding affinity of target novel compounds to the active sites of selected proteins (DHFR, cdk2, Bcl2-xl, cIAP1-BIR3, and MDM2). In which co-crystallized standard ligands complexed with these proteins were replaced by our target compound after calculating and comparing the binding energy of it with that of our target. BIOVIA Discovery Studio program was used at the end to visualize all binding modes of interaction in 2D and 3D dimensions between our novel and the active sites of the selected protein set. All measurements were carried out in details according to our previous literature.[[45](#_ENREF_45), [66](#_ENREF_66)] Where the selected protein codes (1dls, 2c6o, 2w3t, 4kmn, and 4wt2,) respectively were downloaded from protein database (www.pdb.org) in complex with standard co-crystallized ligands (**methotrexate,** triazolopyrimidine, Phenyl Tetrahydroisoquinoline Amide Complex, (**(2*S*)-*N*-{(2*R*)-1-[(2*R*,4*S*)-2-{[6,6'-difluoro-3'-({(2*R*,4*S*)-4-hydroxy-1-[(2*S*)-2-{[(2*S*)-2-(methylamino)propanoyl]amino}butanoyl]pyrrolidin-2-yl}methyl)-1*H*,1'*H*-2,2'-biindol-3-yl]methyl}-4-hydroxypyrrolidin-1-yl]-1-oxobutan-2-yl}-2 (methylamino)propanamide)**, and AM-7209) respectively.