Polycyclic Aromatic Compounds

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/gpol20

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To cite this article: Serdar Burmaoglu, Arzu Gobek, Derya Aktas Anil, Mehmet Abdullah Alagoz, Adem Guner, Cem Güler, Ceylan Hepokur, N. Ulku Karabay Yavasoglu & Oztekin Algul (2024) Assessing the Antiangiogenic Effects of Chalcones and Their Derivatives, Polycyclic Aromatic Compounds, 44:1, 51-66, DOI: 10.1080/10406638.2023.2167216

To link to this article: https://doi.org/10.1080/10406638.2023.2167216

Published online: 19 Jan 2023.

POLYCYCLIC AROMATIC COMPOUNDS
2024, VOL. 44, NO. 1, 51–66
https://doi.org/10.1080/10406638.2023.2167216

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Assessing the Antiangiogenic Effects of Chalcones and Their Derivatives

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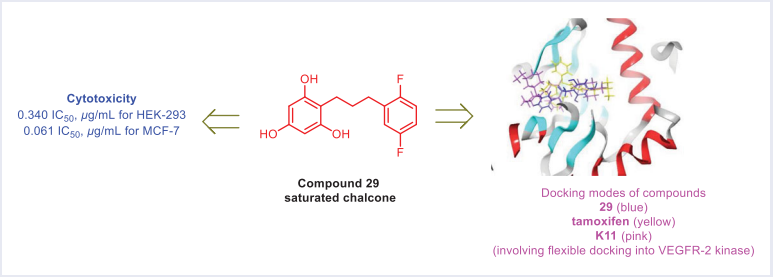
ABSTRACT

Pathological angiogenesis plays a critical role in tumorigenesis and tumor progression, and anti-angiogenesis therapies have evinced promising antitumor effects in solid tumors. Chalcone skeleton has been regarded as a potential antitumor agent that also targets angiogenesis. In this study, we designed twenty-one non-fluoro-substituted chalcones (13–18, 24–27) and saturated chalcone derivatives (19–23, 28–33) as anti-angiogenic compounds. During the initial stage, these compounds were assessed for their anti-cancer activities against MCF-7 cancer cell lines according to the MTT assay. The compounds revealed satisfactory anti-proliferative capability. An *ex vivo* fertilized hens' egg-chorioallantoic membrane (HET-CAM) angiogenic study was conducted for the compounds to gauge their mortality and toxicity, which, in turn, revealed a potent anti-angiogenic effect. Eight compounds (**16**, **17**, **21**, **24**, **26**, **27**, **29**, and **31**) significantly reduced densities of capillaries on CAM, whereas compounds **27** and **29** were the most effective anti-angiogenic agents, when compared with Suramin. Moreover, RT-qPCR analysis demonstrated that the anti-angiogenic activity was associated with the fold changes of VEGFR2. Molecular docking studies were conducted for compounds to investigate their mode of interaction within the binding site of VEGFR-2 kinases. This work provided a basis for further design, structural modification, and development of chalcone derivatives as new anti-angiogenic agents.



ARTICLE HISTORY

Received 16 June 2022
Accepted 5 January 2023

KEYWORDS

Chalcone; anti-angiogenic activity; anti-proliferative activity; RT-qPCR; molecular docking

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Introduction

As cancer chemotherapy has not reached the desired level, continual intensive studies are being conducted to develop more potent and less toxic novel anticancer drugs. In anticancer drug development studies, the effect of novel compounds on antiangiogenic activity is of paramount importance. Antiangiogenic therapy has been extensively accepted as a feasible and efficacious approach for anticancer drug development recently.[1]

Angiogenesis, a physiological process through which new capillary blood vessels are formed, plays a critical role in various other physiological processes, such as embryonic development, wound healing, and the reproductive cycle. It is a highly regulated phenomenon under different physiological conditions. However, pathological angiogenesis is an abnormal and unregulated process, which is considered a key step in tumor growth and progression.[2] Thus, suppression of pathological angiogenesis may provide therapeutic strategies for cancer treatment. Various pro-angiogenic factors, including vascular endothelial growth factor (VEGF), exert their action through endothelial receptor tyrosine kinases (RTKs). Although antiangiogenic therapy has made considerable progress in protracting the survival of patients with cancer, only a few satisfactory results have been reported due to certain limitations, including insignificant clinical effects, concerns about safety, tumor recurrence, and drug resistance.[3,4] Such factors have necessitated the development of ingenious, safer, and multi-targeted inhibitors. In particular, the use of small-molecule angiogenesis inhibitors proves to be an efficacious approach for cancer treatment and plausible prevention. Extensive efforts have been directed toward antiangiogenic therapies that combat cancer by preventing it from accessing the blood supply that is critical for tumor growth and survival.[5–7] The antiangiogenic inhibitors approved by the FDA can be classified into three groups, as depicted in Figure 1.[8] Chalcones, a group of plant-derived compounds, have been regarded as potential antitumor agents that mainly target angiogenesis. Antiangiogenic properties of naturally occurring and synthetic chalcones continue to amass considerable interest.[1,9–12] Moreover, several rigorous studies with diversified cellular and animal models have suggested that certain chalcones can inhibit tumor initiation as well as tumor progression.[12–14]

Based on previous pharmacological investigation of chalcones, it was found that there were certain reports on the antiangiogenic activities of substituted chalcones. These studies have provided us with an impetus to explore its chemical constituents and bioactivities further. The rational design of our fluoro-substituted chalcone derivatives primarily depends on the aforementioned significant inhibitory activities of chalcones and flavonoids against VEGFR-2 and the process of angiogenesis, as shown in Figure 2.[15,16]

In a previously conducted study, we reported a series of fluoro-substituted chalcone derivatives with potent cytotoxic activities in various tumor cell lines; some of them exhibited modest selectivity toward cancer cell lines.[17,18] Those compounds with selective cytotoxicity may have the potential to be selective angiogenesis inhibitors.

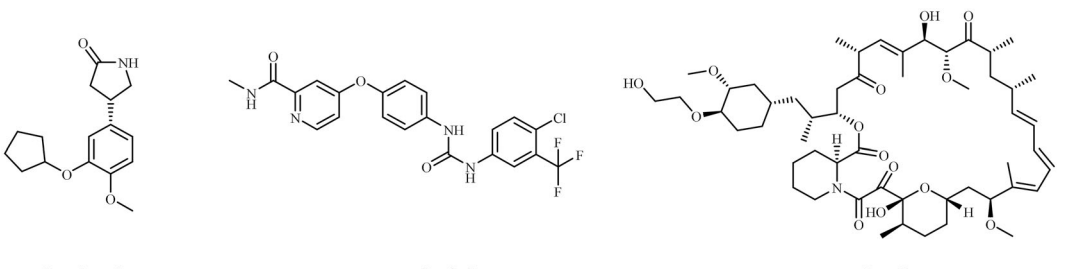


Figure 1. The antiangiogenic inhibitors approved by FDA.

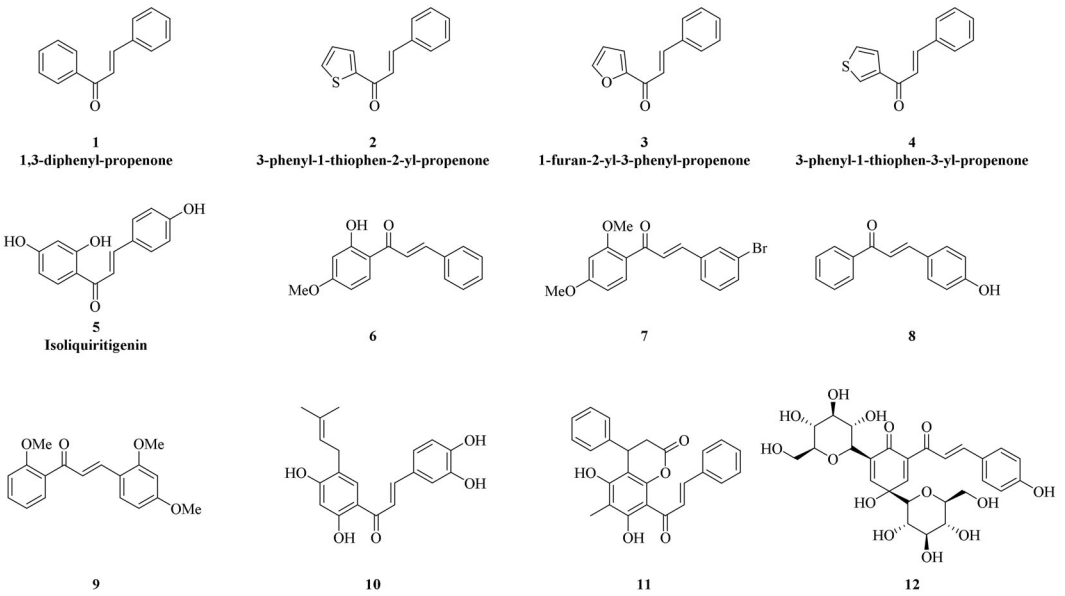


Figure 2. List of some chalcone derivatives as VEGF inhibitors.

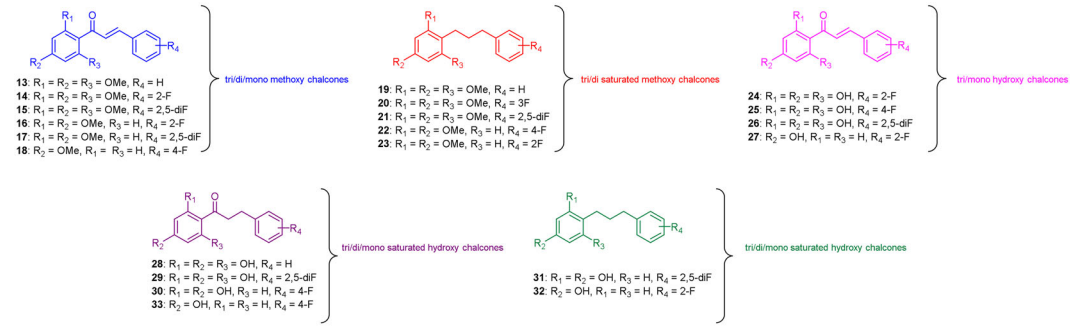


Figure 3. Structures of synthesized chalcone and saturated chalcone derivatives.

In this study, we conducted a bioactivity-guided investigation of 21 fluoro-substituted chalcone derivatives and saturated chalcone derivatives, as shown in Figure 3, to evaluate their *ex vivo* anti-angiogenic activities using hen's egg-chorioallantoic membrane (HET-CAM) method. The principal objective of the study was to assess the role of these compounds in antiangiogenic activities, as the linkers responsible for molecule flexibility markedly affect both the selectivity and potency positively or negatively. It is worth noting that the prediction of the interaction of VEGF receptor activities of all the compounds and molecular docking studies were also performed. In addition, the anti-angiogenic activity of active molecules was shown to be correlated with VEGFR2 by RT-qPCR analysis.

Material and methods

Chemistry

The synthesis of the chalcone/saturated chalcone derivatives was performed according to the procedure described in our previous studies.[17–21]

Pharmacological/biological assays

Cell culture

HEK-293 cell line was selected to determine the cytotoxicity of compounds. The anticancer drug studies have been carried out on the concentrations that were determined as non-cytotoxic. MCF-7 cell lines (human breast adenocarcinoma cells) were obtained from the Department of Virology, Faculty of Veterinary, Ankara (Turkey). The cell cultures were maintained in RPMI-1640 media supplemented with 10% fetal calf serum, 100 µg/mL penicillin, and 100 µg/mL streptomycin at 37 °C in an atmosphere of 5% CO_2.

Experimental design

The control cells (HEK-293) and cancer cells (MCF-7) were exposed to various concentrations of compounds for a period of 96 h. At the end of the incubation period, cell morphology, as well as cytotoxic responses of the cells, were assessed using an MTT assay.

Compounds and culturing

The compounds were not completely soluble in maintaining media; therefore, stock solutions of all compounds were dissolved in the nontoxic dimethyl sulfoxide (DMSO) concentration. DMSO concentration of 1% was detected as non-cytotoxic. The final concentration of chemicals reached at 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.078 µg/mL in micro plaques. In order to evaluate the effects of the compounds, the cells of cancer lines were inoculated into well flat bottom plates at a density of 1 × 105 cells per plate. The cells were then incubated for 8 h in the attachment plates. Finally, chemicals were inoculated at different concentrations into each well to grow for additional 96 h. All experiments were performed thrice.

Cytotoxicity assay

Cell viability was determined by quantitative colorimetric MTT assay. The assay is based on the principle of the cleavage of the MTT tetrazolium ring in active mitochondria.[22] 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, first described by Mosmann, is a practical method commonly used to determine cell viability. MTT is a substance that actively absorbs into cells and is reduced to a colored insoluble formazan product due to activity in mitochondria. The color intensity of the Forazman dye is correlated to the number of viable cells. In the study, effects on cell proliferation were assessed by MTT cell proliferation method in cell cultures treated with chemical synthesis at different concentrations.

In MTT method, after adding chemicals to the cell cultures, the cells were incubated at 37 °C in an atmosphere of 5% carbon dioxide for 96 h. MTT solution of 10 mL was added to each well, and plates were incubated under the same condition for 4 h. Absorbance measurements were made at 570 nm. Proliferation rate was expressed as the ratio of cells in the wells treated with chemicals in proportion to the control group. IC_{50} values were calculated using Statistical Package for the Social Science (SPSS. Inc, Chicago).

Ex vivo anti-angiogenesis assay

A chick chorioallantoic membrane (HET-CAM) assay was conducted to evaluate the anti-angiogenic activities of synthesized compounds. Antiangiogenic effects of samples were carried out on fertile Leghorn chicken eggs weighing 50–60 g obtained from commercial sources (Izmir Veterinary Control Institute, Izmir, TURKEY) by using the HET-CAM method modified by Güner et al.[23] Fertilized hens' eggs were placed in an incubator with a conveyor rotation system at 37 ± 1 °C and 80 ± 2% humidity for six days. On day 6, the eggs were opened on the snub side,

Table 1. Semi-quantitative score system of anti-angiogenic effect on CAM after treatment.

Scores	Anti-angiogenic effect	Effects observed on CAM after treatment
0	Inactive	No change (normal embryo growth)
0.50	Weak	No capillary free area. Area with reduced density of capillaries around the pellet not larger than its own area
1	Strong	Small capillary free area or area with significantly reduced density of capillaries. Effects not larger than double the size of the pellet
2	Very strong	Capillary free area around the pellet at least doubles the size of the pellet

and before that, 10 mL of albumin was sucked off through a hole on the pointed side, and then a round piece of shell (3–4 cm diameter) was removed carefully with forceps. The opened Cavity was sealed with laboratory film and incubated in same condition. When reaches 2 cm the diameter of CAM (approximately 72 h), the freshly prepared sample that dissolved in DMSO (0.05%) was placed onto it as 1,25 mg/100 µL (1 pellet/egg). The eggs were incubated for one additional day, and scores were calculated using a formula for scoring, as shown in Table 1. According to the results of the formula, a score of < 0.5 indicated that there was no antiangiogenic effect, 0.5–1 showed a low antiangiogenic effect, and > 1 showed a strong antiangiogenic effect. Scores were evaluated as the average of 3 eggs. All samples were tested in triplicate at different intervals. Also, 0.9% NaCl as negative control, and 0.1 N NaOH and Suramin (50 µg/pellet) as an antiangiogenic agent approved by the Federal Drug Administration (FDA) as a positive controls were tested.

Isolation of RNA and cDNA synthesis

The total RNA was extracted from MCF-7 cell lines collected by using the RNA isolation kit (GeneAll, Hybrid-R, Cat 305–101) according to the manufacturer' recommendations (1 mL RiboEx was added on 1×10^7 culture cells and mixed by pipetting). It was incubated 5 min at room temperature. The mixture was centrifuged at 11.000 rpm, 10 min, 4 °C and the supernatant was transferred to another tube. 200 µL of chloroform was added to the mixture and mixed, 2 min. incubated. It was centrifuged at + 4 °C at 12,000×*g* for 15 min. The supernatant was transferred to a clean tube. RB1 Buffer was added as much as the volume of the supernatant. It was mixed by pipetting. 700 µL of the mixture was transferred to the column. 30 s at 10,000×*g* centrifuge was done. Collection tube was replaced with a new one. Processes 7 and 8 were repeated in case of remaining amount from the mixture. 500 µL of SW1 Buffer was added on the column. 30 s at 10.000×*g* centrifuge was done. Collection tube was replaced with a new one. 500 µL of RNW Buffer was added onto the column. 30 s at 10,000×*g* centrifuge was done. Collection tube was replaced with a new one. 1 min at 10,000×*g* to remove wash solutions remaining in the column and centrifuge was done. The column was then moved into a clean tube. 50 µL of RNase-free water was added onto the column and 1 min. kept at room temperature. Then at 10,000×*g* for 1 min. mRNA samples obtained by centrifugation were frozen at −80 °C]. The obtained RNA samples were frozen at −80 °C until the cDNA synthesis step. cDNA synthesis using a hyperscript first strand synthesis kit (Wizbio; WizScript™ cDNA Synthesis Kit (High Capacity); W2211), according to manufacturer's protocols.

RT-qPCR

After the cDNA was obtained, the Real-Time qPCR stage was started. The brand, name and catalog number of the kit used for Real-Time qPCR are indicated (Wizbio; WizPure™ qPCR Master (SYBR); W1711). The Real-Time qPCR reaction was performed on the Applied Biosystems™ 7500 Fast Real-Time PCR instrument. The quantitation of RNA expressions was normalized to the control group using the ACTB transcript as a reference. The following primers were used:

VEGFR2 forward primer 5′-GTGACCAACATGGAGTCGTG −3′, VEGFR2 revers primer 5′-CC AGAGATTCCATGCCACTT-3′, ACTB-F forward primer 5′-GGCTGTATTCCCCTCCATCG-3′, ACTB-F revers primer 5′-CCAGTTGGTAACAATGCCATGT-3′. All genes expression levels were normalized to the amount of ACTB in the same sample. The PCR reaction mixtures were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. Ct (threshold cycle) values were determined by the $2^{-\Delta\Delta Cp}$ Ct method.[24]

Statistical analysis

Kolmogorov–Smirnov test was used to test compatibility with normal distribution. All statistical analysis was performed by SPSS 22.0, and significant difference was set at $p < 0.05$.

Molecular docking

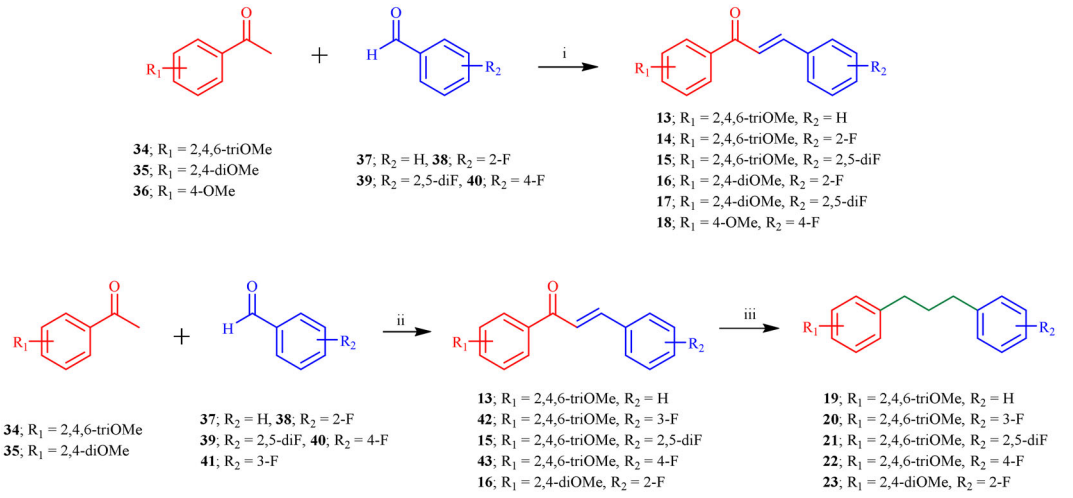
3D models of the compounds were designed in 2 D Sketcher of Maestro 11.8 (Schrödinger, LLC, NY) software and MacroModel (Schrödinger, LLC, NY) software using OPLS-2005 force field parameters and optimized by conjugate gradient method. Later, the enantiomers of the ligand models with appropriate tautomer and ionization conditions (if any) were determined using LigPrep (Schrödinger, LLC, NY) software.[25,26] Crystal structures of the 3EWH PBD encoded protein (Crystal structure of the VEGFR-2 kinase domain in complex with a pyridyl-pyrimidine benzimidazole inhibitor) were downloaded from the RCSB Protein Data Bank (www.rcsb.org).[27] Maestro version of protein preparation wizard was used for docking studies. In this context, unwanted solvent molecules in crystal structures, ligands, and segments were deleted, missing amino acid atoms and hydrogens (if any) were added, charges assigned, orientations of polar hydrogens, and waters were adjusted. Grid maps of proteins were created using Maestro's receptor grid generation module. Ligands were docked on these maps 100 times in extra precision mode using Glide (Schrödinger, LLC, NY) software. Ligand protein interactions were obtained using Schrodinger's XP visualizer.[25,26,28]

Results and discussion

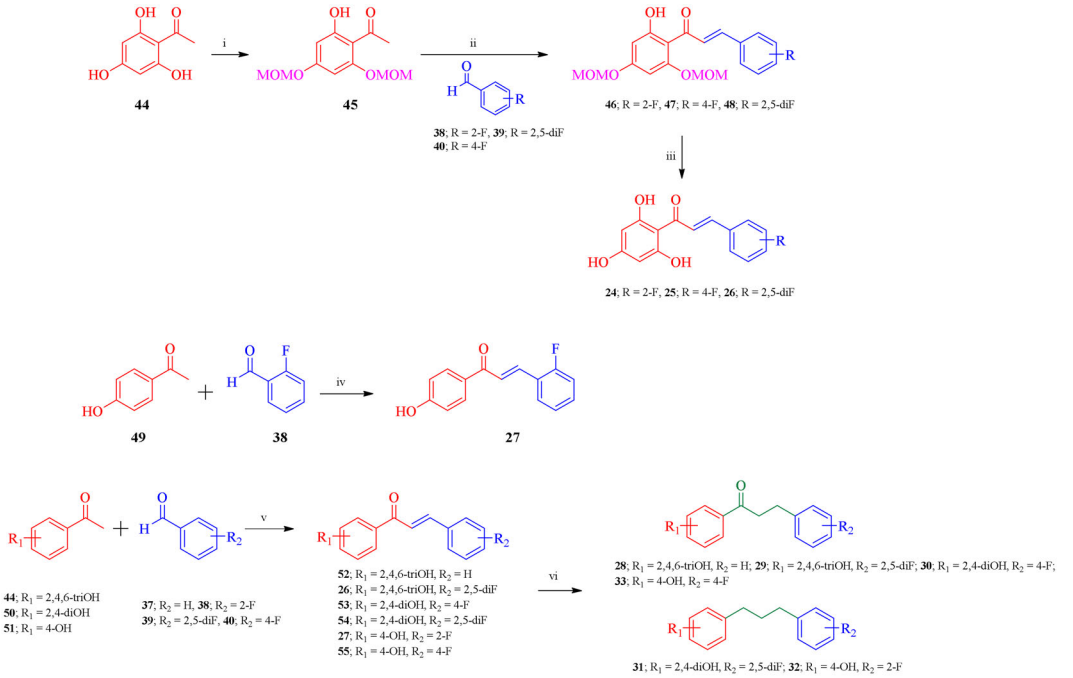
Chemistry

We previously reported the synthesis of nonsubstituted and fluoro-substituted/saturated chalcone derivatives.[17–21] The condensation reaction of appropriate acetophenones (34–36) with benzaldehydes (37–40) in an aqueous solution of KOH resulted in related chalcones (13–18) in 80–98% yields by adopting a general synthesis protocol. Hydrogenation of trimethoxychalcones **13**, **15**, **16**, **42**, and **43** over Pd/C gave related fully saturated chalcones (19–23) in 74%–87% yields, as described in Scheme 1[17,18].

For the synthesis of trihydroxy chalcone derivatives (24–26), compound **44** was first treated with chloromethyl methyl ether (MOMCl) to obtain compound **45**. Then, MOM-protected chalcone derivatives (46–48) were obtained by condensation of benzaldehydes (38–40) with compound **45**. Finally, by removing the protecting group in an acidic medium, trihydroxy chalcone derivatives (24–26) were obtained in 40%-70% yields. Preparation of fluoro-substituted monohydroxy chalcone (27) was summarized in Scheme 2. For this, we did not need any protecting group to protect the hydroxyl group. Condensation of **49** with benzaldehydes **38** gave related chalcone **27** in 40% yield. Hydrogenation of trihydroxy chalcones **26**, **27**, **52**, **53**, **54**, and **55** on Pd/C afforded related fully saturated chalcone derivatives **28**–**33** in 74%–87% yields, as summarized in Scheme 2[17,18].



Scheme 1. Preparation of tri/di/mono methoxy chalcones and tri/di saturated methoxy chalcones. Reagents and conditions: (i) 50% KOH aqueous solution, MeOH, room temperature, 15 h (ii) 50% KOH aqueous solution, MeOH, room temperature, 15 h, (iii) H_2, Pd-C, EtOH, room temperature, 16 h.



Scheme 2. Preparation of tri/mono hydroxy chalcones and tri/di/mono saturated hydroxy chalcones. Reagents and conditions: (i) MOMCl, DIPEA, DCM, 0 °C, 3 h; (ii) KOH, MeOH, room temperature, 15 h; (iii) HCl, room temperature, 12 h; (iv) KOH, MeOH, room temperature, 15 h; (v) KOH, MeOH, room temperature, 15 h; (vi) H_2, Pd-C, EtOH, room temperature, 16 h.

Biology

A plethora of studies targeting VEGFR-2 and angiogenesis have garnered immense interest, which prompted us to synthesize a series of fluoro-substituted chalcones, as shown in Schemes 1 and 2. The synthesized compounds include two groups of chalcone derivatives with the mono/di/tri OMe ring A or non/F ring B attached to the chalcone structures.

Table 2. Cytotoxicity of synthesized chalcones in MCF-7 tumor cell line.

	Cytotoxicity (IC_{50}, μM)	
Compound no	HEK-293	MCF-7
13	1.12 ± 0.02	0.45 ± 0.01
14	1.99 ± 0.04	1.67 ± 0.03
15	1.79 ± 0.06	1.57 ± 0.04
16	2.99 ± 0.04	0.87 ± 0.04
17	13.51 ± 0.15	3.27 ± 0.02
18	3.99 ± 0.07	17.09 ± 0.17
19	5.81 ± 0.17	34.08 ± 0.74
20	4.99 ± 0.16	0.86 ± 0.04
21	4.81 ± 0.18	1.19 ± 0.02
22	5.99 ± 0.16	24.15 ± 0.16
23	4.01 ± 0.19	13.80 ± 0.15
24	6.99 ± 0.13	0.35 ± 0.01
25	8.69 ± 0.04	15.80 ± 0.42
26	7.99 ± 0.03	1.47 ± 0.16
27	10.65 ± 0.17	2.13 ± 0.03
28	8.99 ± 0.18	47.22 ± 1.89
29	1.16 ± 0.04	0.21 ± 0.04
30	9.99 ± 0.13	2.48 ± 0.07
31	4.33 ± 0.11	1.07 ± 0.02
32	10.99 ± 0.21	44.56 ± 1.54
33	10.73 ± 0.27	19.51 ± 1.23
Tamoxifen	11.99 ± 0.14	1.47 ± 0.04

The experiments were run with six ($n = 6$) repetitions and standard deviations were calculated and given as ±.

Table 3. Anti-angiogenic effects of compounds on HET-CAM. Scores are presented mean ± SEM.

Compound no	Scores	Anti-angiogenic effect	Compound no	Scores	Anti-angiogenic effect
13	0.87 ± 0.01	Mild	**25**	0.10 ± 0.01	No effect
14	0.30 ± 0.01	No effect	**26**	1.67 ± 0.11	Very Strong
15	0.65 ± 0.04	Mild	**27**	2.01 ± 0.16	Very Strong
16	1.84 ± 0.06	Very Strong	**28**	0.44 ± 0.01	No effect
17	1.95 ± 0.11	Very Strong	**29**	2.14 ± 0.02	Very Strong
18	0.30 ± 0.01	No effect	**30**	0.86 ± 0.08	Mild
19	0.30 ± 0.01	No effect	**31**	1.80 ± 0.11	Very Strong
20	0.96 ± 0.12	Mild	**32**	0.20 ± 0.01	No effect
21	1.58 ± 0.10	Very Strong	**33**	0.62 ± 0.02	Mild
22	0.60 ± 0.02	Mild	**NaCl**[a]	0.10 ± 0.01	No effect
23	0.20 ± 0.01	No effect	**NaOH**[b]	2.21 ± 0.15	Very Strong
24	1.46 ± 0.18	Very Strong	**Suramin**[c]	1.32 ± 0.05	Very Strong

[a]NaCl (0.9%): negative control; [b]0.1 N NaOH and [c]Suramin (50 μg/pellet): positive controls.

A HET-CAM assay was conducted to evaluate the antiangiogenic activities of the synthesized compounds. First, these compounds were investigated for their cytotoxic activity by the MTT assay in a two-cell panel, as shown in Table 2. After that, the compounds were screened by the HET-CAM assay to check their antiangiogenic effects Table 3, Figure 4). Then, VEGFR2 inhibitory activities of the most active molecules (**27** and **29**) were investigated by RT-qPCR analysis (Figure 5).

Cytotoxicity assays

The cytotoxicity activities of 21 chalcone derivatives (**13**–**18**, **24**–**27** and **19**–**23**, **28**–**33**) were evaluated. The primary objective of the synthesis of the chalcone derivatives was to determine their potency against MCF-7 cancer cells compared with tamoxifen.

To appraise the specificity of the compounds, their toxicity was tested against normal cells using human embryonic kidney cells (HEK-293). The nontoxic concentrations of all the compounds were determined on the HEK-293 cell line; after that, the cytotoxicity activity studies

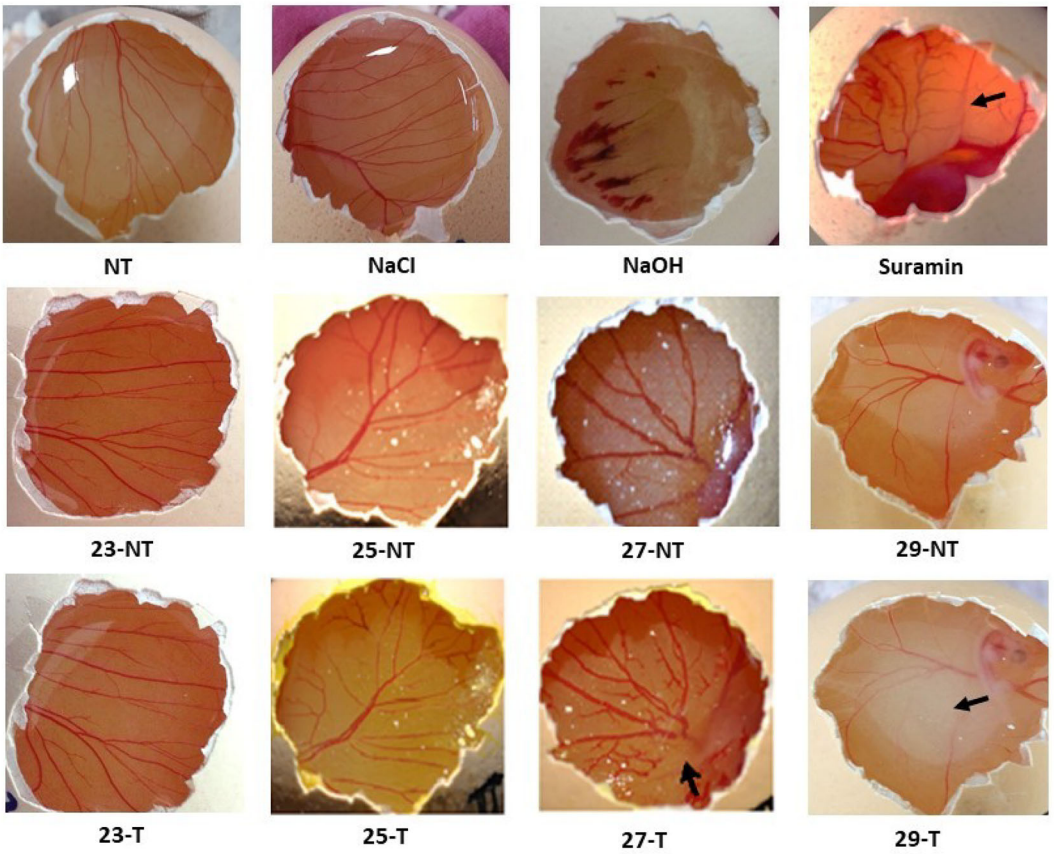


Figure 4. Anti-angiogenic effects of compounds on HET-CAM. NT: Non-treated, T: treated. NaCl (0.9%): negative control; NaOH (0.1 N) and Suramin (50 μg/pellet) positive controls.

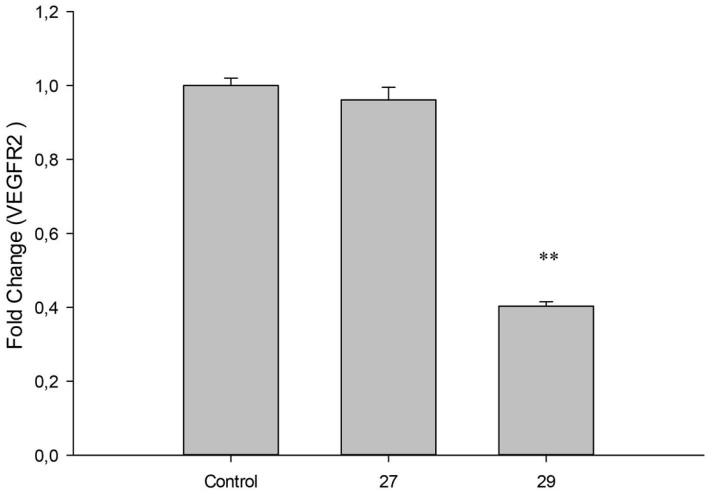


Figure 5. Kolmogorov-Smirnov gene expression analysis of VEGFR2 by RT-qPCR. The compound **29** down-regulated VEGFR2 ($^{**}p < 0.01$).

were conducted at nontoxic concentrations on the MCF-7 cell line. The compounds and tamoxifen were exposed for a period of 0–96 h for each cancer cell line. At the end of the incubation, all cells were evaluated and subjected to the assessment of their cytotoxic responses using an MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.[29]

The synthesized compounds were methoxy- or hydroxyl-substituted analogs of chalcone with varying components: fluorine atoms, ketone groups, or alkyl chains. Accordingly, a structure-activity relationship (SAR) was established by comparing the cytotoxic activities of the different chalcone groups. Among these compounds, **13–16, 20, 21, 24, 26, 27, 29,** and **31** exhibited the most potent activity. In contrast, compounds **17–19, 22, 23, 25, 28, 30,32** and **33** with saturated or non- or mono-substituted compounds displayed the weakest activity against all the tested cancer cells. The resultant data are presented in Table 2, which demonstrates that all the active compounds had significant activity against the cancer cell line with 0.21–2.13 µg/mL. The most active agent against MCF-7 cells was **29** with an IC_{50} value of 0.21 µg/mL. Almost all the tri-fluoro-substituted compounds (**13–15, 20, 21, 25, 26** and **29**), except compound **19, 22, 25** and **28** exhibited improved activity against the cancer cell lines compared with mono- or non-fluoro compounds (**17, 18, 23, 30, 32** and **33**). Also, the saturated chalcone derivatives (**19–23**, and **28–33**) did not considerably improve the activities against the cancer cell line except the di and tri methoxy or hydroxyl-substituted compounds (**20, 21, 29**, and **31**).

Effect of compounds on chick embryonic angiogenesis

The 21 chalcone analogs were examined for antiangiogenic and vascular disrupting properties using the HET-CAM assay.[23] In this test, the vascular system of a fertilized chick embryo is used as a model. Figure 4 demonstrates the effects of compounds **23**, **25**, **27**, and **29** on the development and densities of embryonic blood vessels compared to negative and positive controls.

All the compounds showed promising scores in the HET-CAM assay, as shown in Table 3. The results suggest that some compounds (**16**, **17**, **21**, **24**, **26**, **27**, **29**, and **31**) are potent inhibitors of angiogenesis, with compound **29** being more effective than others and antiangiogenic agent Suramin in suppressing new vessel growth, but it could reflect potential in transport or other pharmacokinetic differences of the analogs. The respective impacts of the inactive, strong, and very strong antiangiogenic compounds in the study are illustrated in Figure 4.

Isolation of RNA, cDNA synthesis and quantitative RT-PCR (RT-qPCR) studies

The quantitative Real-time Polymerase Chain Reaction (RT-qPCR) is the most common technique used to compare the expression levels of genes under different experimental conditions. In this study, the total RNA (GeneAll, Hybrid-R, Cat 305–101) was extracted from MCF-7 cell lines and were frozen at −80 °C until the cDNA synthesis step (Wizbio; WizScript™ cDNA Synthesis Kit (High Capacity); W2211). After the cDNA was obtained, the Real-Time qPCR stage was started.

The two most active molecules (**27** and **29**) were selected for RT-qPCR analysis. The results suggest that the compound **29** was observed to be very effective in VEGFR2 on (MCF-7) breast cancer cells, by down- regulating it by half compared to the control group. There is a statistically significant difference between control and compound **29** ($^{**}p < 0.01$) (Figure 5). Otherwise, the effect of the compound **27** was not determined much in VEGFR2 according to control on (MCF-7) breast cancer cells ($p > 0.05$).

Molecular docking

Angiogenesis plays an effective role in the metastasis and growth of tumors. The process is regulated by various growth factors.[30–32] Among these factors, the most effective signal protein in

stimulating angiogenesis is vascular endothelial growth factor (VEGF).[33] The binding of VEGF to its own receptor family (VEGFR) may stimulate proliferation, survival, and progression of cancer cells. VEGFR-2, a part of the VEGF receptor family, is considered the most important receptor that mediates the cellular responses to VEGF. Hence, antiangiogenic activities can be promoted by means of VEGFR-2 inhibition.[34,35]

In molecular docking studies, the protein encoded 3EWH PDB (Crystal structure of the VEGFR-2 kinase domain in complex with a pyridyl-pyrimidine benzimidazole inhibitor) was used as a target protein.[36] The interactions of the synthesized compounds with the target protein were compared with 4-hydroxytamoxifen, the active metabolite of tamoxifen, (98% inhibition) a potent angiogenesis inhibitor and sorafenib, validated VEGFR-2 inhibitor.[36,37] Maestro 11.8 was used in all molecular modeling studies. In docking studies, native protein tyrosine kinase (PTK) inhibitor (K11), a part of the crystal structure of the 3EWH encoded protein, was extracted, randomized, and docked into the active site of the protein for validation, as shown in Figure 6.

The RMSD value of the K11 molecule was calculated as 1.37 Å. A significant correlation was found between the scoring system of antiangiogenic effects and docking scores with a correlation coefficient of 0.8517, as shown in Figure 7.

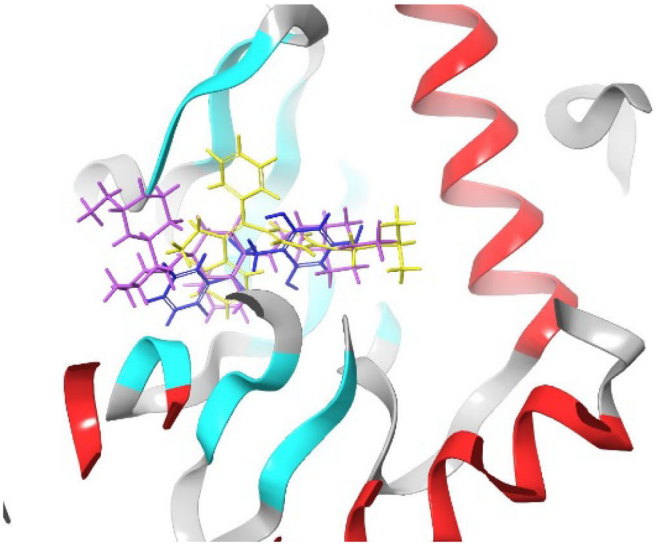


Figure 6. Comparative docking modes of compounds **29** (blue), 4-hydroxytamoxifen (yellow) and K11 (pink) involving flexible docking into VEGFR-2 kinase (3EWH).

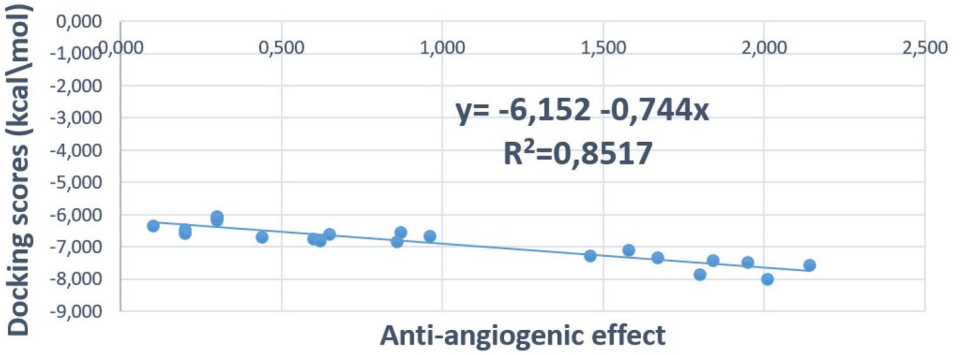


Figure 7. The correlation between anti-angiogenic effect docking scores for compounds.

Although the docking score of 4-hydroxytamoxifen and sorafenib were −7.395 kcal/mol and −7.139 respectively, the docking scores of the synthesized compounds ranged between −6.061 and −7.994. The docking scores of the compounds **29**, **31**, **27**, **16** were higher than the docking score of both 4-hydroxytamoxifen and sorafenib. As shown in Table 4, with the docking score of −7.994 kcal/mol, compound **29** presented a high potential binding affinity to the binding site of the 3 D VEGFR-2 kinase.

In addition, the interactions of compounds and tamoxifen with residues in the active region of the protein were investigated. As shown in Figure 8, 4-hydroxytamoxifen had cation-pi interaction with LYS868; pi-pi stacking with PHE1047; had hydrogen bonding with CYS919; charged (negative) interaction with ASP1046, GLU885, charged (positive) interaction with ARG842; hydrophobic interaction with PHE845, VAL848, CYS1045, LEU889, VAL899, LEU1035, PHE918, CYS919, LEU840. Sorafenib had cation-pi interaction with LYS868; pi-pi stacking with PHE1047; charged (negative) interaction with ASP1046, GLU885, GLU917; charged (positive) interaction with LYS868; hydrophobic interaction with ILE888, ILE892, PHE918, VAL848, CYS1024, LEU1044, LEU1019, LEU1035, CYS919, ALA866, LEU840.

Compound **29** had hydrogen bond with GLU885, LYS868, THR916; charged (negative) interaction with ASP1046, GLU885; hydrophobic interaction with VAL848, CYS1045, LEU889, VAL899, LEU1035, PHE918, CYS919, ALA866, LEU840, PHE1047. Compound **31** had hydrogen

Table 4. Docking scores of compounds (kcal/mol) against 3EWH PDB encoded protein.

Compounds	Docking scores (kcal/mol)	Compounds	Docking scores (kcal/mol)
13	−6.557	**25**	−6.354
14	−6.175	**26**	−7.345
15	−6.606	**27**	−7.584
16	−7.420	**28**	−6.690
17	−7.376	**29**	−7.994
18	−6.089	**30**	−6.849
19	−6.061	**31**	−7.863
20	−6.661	**32**	−6.571
21	−7.104	**33**	−6.826
22	−6.744	**4-Hydroxytamoxifen**	−7.395
23	−6.457	**K11**	−7.988
24	−7.282	**Sorafenib**	−7.139

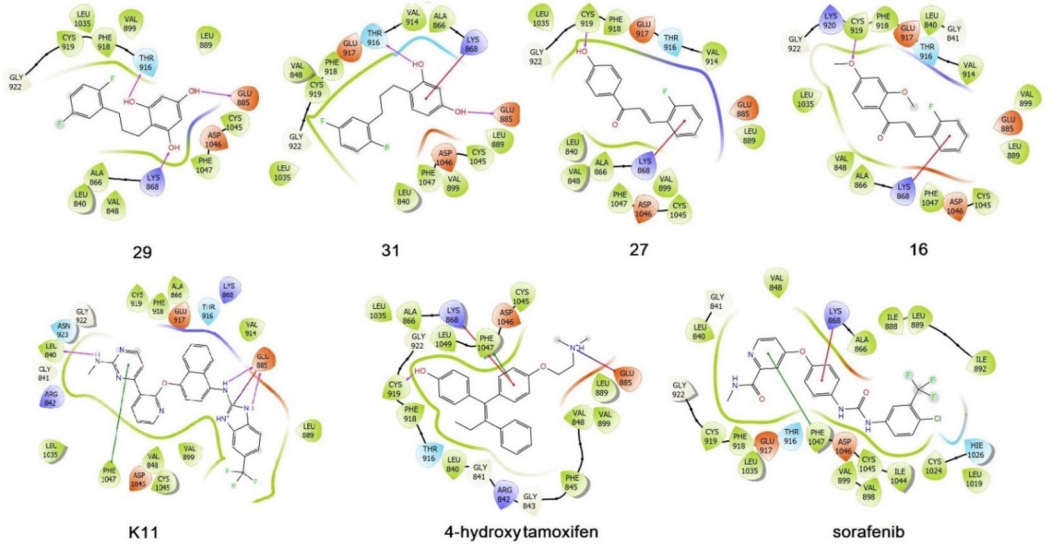


Figure 8. 2 D interaction compounds **29**, **31**, **27**, **16**, K11, 4-hydroxytamoxifen, and sorafenib with active site of protein (3EWH).

bond with GLU885, THR916; had cation-pi interaction with LYS868, charged (negative) interaction with ASP1046, GLU885, GLU917; hydrophobic interaction with VAL848, CYS1045, LEU889, VAL899, LEU1035, PHE918, CYS919, ALA866, LEU840, PHE1047, VAL914. Compound **27** had hydrogen bond with THR919; cation-pi interaction with LYS868; charged (negative) interaction with ASP1046, GLU885, GLU917; hydrophobic interaction with VAL848, CYS1045, LEU889, VAL899, LEU1035, PHE918, CYS919, ALA866, LEU840, PHE1047, VAL914. Compound **16** had hydrogen bond with THR919; had cation-pi interaction with LYS868, charged (negative) interaction with ASP1046, GLU885, GLU917; charged (negative) interaction with LYS920; hydrophobic interaction with VAL848, CYS1045, LEU889, VAL899, LEU1035, PHE918, CYS919, ALA866, LEU840, PHE1047, VAL914; polar interaction with THR916. It was determined that compounds with the highest docking score 4-hydroxytamoxifen, and sorafenib have similar interactions with residues in the active site of the protein. Interaction with residues of LYS868, GLU885, THR916, ASP1046, PHE1047, and CYS919 was found to be very important in VEGFR-2 kinase inhibition. Compounds **29**, **31**, **27**, and **16** are thought to be candidate anticancer compounds because of their high antiangiogenic effects and high VEGFR-2 kinase inhibition in *in silico* studies.

Conclusion

The current study aimed to synthesize chalcone derivatives due to their useful biological properties and lack of toxicity at reasonable plasma concentrations, have been used in the treatment of various diseases.[38] In addition, the antiangiogenic activities of synthetic chalcone derivatives have also been reported.[39] However, to the best of our knowledge, this is the first report regarding the synthesis of fluoro- substituted chalcones using a simple and effective method, demonstrating robust antiangiogenic activity mediated by RTK inhibition.

This study compared the antiangiogenic activities of fluoro-substituted chalcone derivatives. The results revealed that fluoro-substituted chalcones are more potent antiangiogenic compounds among the tested non-substituted chalcone derivatives. Furthermore, differences in antiangiogenic activities of the compounds indicate the importance of the hydroxyl substitution at different positions on the phenyl ring in chalcone moiety. The analogs' activities with 3-atom linker varied widely depending on the phenyl ring substitutions, but the most potent compounds are exclusive with the hydroxy-containing phenyl ring A and saturated linker configuration and two fluoro substitutions in phenyl ring B. The progressive improvement of activity from **28**, **24**, and **26** to **29** demonstrates, at least qualitatively, that the 3-atom linker containing saturation and di-fluoro atoms represent the more favorable linker structure.

RT-qPCR study shows that the compound **29** was observed to be very effective in VEGFR2 on (MCF-7) breast cancer cells, by down-regulating it by half compared to the control group. Molecular modeling analysis indicates similar results that all three analogs bind to the VEGFR-2 active site with **16**, **27**, **29**, and **31** showing greater binding affinity. Competitive binding assay confirmed that the mono- or di-hydroxyl group on ring B and two OMe or tri OH group on ring A analogs bind to the VEGFR-2 site with affinities similar to 4-hydroxytamoxifen.

The design and development of ingenious antitumor compounds with different mechanisms of action are domains of persistent and exacting research for drug developers around the globe. The use of small-molecule angiogenesis inhibitors promises to be an effective method for cancer treatment and possible prevention. Many antiangiogenic compounds are currently at various stages of laboratory evaluations and clinical trials.

The findings of the study suggest that the fluoro-substituted chalcones are worthwhile as antiangiogenic and anticancer agents. These research findings are anticipated to guide the search for new chalcone derivatives with anti-proliferative and antiangiogenic attributes.

Disclosure statement

The authors declared that they have no conflict of interest in the present study.

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References

1. J. Chen, C. F. Liu, and G. W. Rao, "Progress in the Synthesis, Angiogenesis Activity and Mechanism of Chalcone Derivatives," *Mini-Reviews in Organic Chemistry* 17 (2020): 814–27. doi:10.2174/1570193X17666191223161941
2. D. Hanahan, and J. Folkman, "Patterns and Emerging Mechanisms of the Angiogenic Switch during Tumorigenesis," *Cell* 86, no. 3 (1996): 353–64. doi:10.1016/S0092-8674(00)80108-7
3. F. Fan, A. Schimming, D. Jaeger, and K. Podar, "Targeting the Tumor Microenvironment: focus on Angiogenesis," *Journal of Oncology* 2012 (2012): 281261. doi:10.1155/2012/281261
4. Sofie Frandsen, Sascha Kopp, Markus Wehland, Jessica Pietsch, Manfred Infanger, and Daniela Grimm, "Latest Results for Anti-Angiogenic Drugs in Cancer Treatment," *Current Pharmaceutical Design* 22, no. 39 (2016): 5927–42. doi:10.2174/1381612822666160715130419
5. G. G. Yue, B. C. Chan, H. F. Kwok, Y. L. Wong, H. W. Leung, C. J. Ji, K. P. Fung, P. C. Leung, N. H. Tan, and C. B. S. Lau, "Anti-Angiogenesis and Immunomodulatory Activities of an Anti-Tumor Sesquiterpene Bigelovin Isolated from Inula Helianthus-Aquatica," *European Journal of Medicinal Chemistry* 59 (2013): 243–52. doi:10.1016/j.ejmech.2012.11.029
6. W. Chen, G. Zhang, L. Guo, W. Fan, Q. Ma, X. Zhang, R. Du, and R. Cao, "Synthesis and Biological Evaluation of Novel Alkyl Diamine Linked Bivalent β-Carbolines as Angiogenesis Inhibitors," *European Journal of Medicinal Chemistry* 124 (2016): 249–61. doi:10.1016/j.ejmech.2016.08.050
7. A. Markowska, S. Sajdak, J. Markowska, and A. Huczyński, "Angiogenesis and Cancer Stem Cells: New Perspectives on Therapy of Ovarian Cancer," *European Journal of Medicinal Chemistry* 142 (2017): 87–94. doi:10.1016/j.ejmech.2017.06.030
8. C. Wang, L. Li, D. Fu, T. Qin, Y. Ran, F. Xu, X. Du, H. Gao, S. Sun, T. Yang, et al, "Discovery of Chalcone-Modified Estradiol Analogs as Antitumour Agents That Inhibit Tumour Angiogenesis and Epithelial to Mesenchymal Transition," *European Journal of Medicinal Chemistry* 176 (2019): 135–48. doi:10.1016/j.ejmech.2019.04.071
9. L. Mirossay, L. Varinská, and J. Mojžiš, "Antiangiogenic Effect of Flavonoids and Chalcones: An Update," *International Journal of Molecular Sciences* 19 (2018): 27. doi:10.3390/ijms19010027
10. E. Nuti, B. Bassani, C. Camodeca, L. Rosalia, A. Cantelmo, C. Gallo, D. Baci, A. Bruno, E. Orlandini, S. Nencetti, et al, "Synthesis and Antiangiogenic Activity Study of New Hop Chalcone Xanthohumol Analogues," *European Journal of Medicinal Chemistry* 138 (2017): 890–9. doi:10.1016/j.ejmech.2017.07.024
11. S. R. Lemes, L. A. Júnior, D. da Silva Manoel, M. A. M. de Sousa, R. D. Fonseca, R. S. Lima, C. Noda-Perez, P. R. de Melo Reis, C. G. Cardoso, E. de Paula Silveira-Lacerda, et al, "Optical Properties and Antiangiogenic Activity of a Chalcone Derivate, Spectrochim," *Spectrochimica Acta. Part A, Molecular and Biomolecular Spectroscopy* 204 (2018): 685–95. doi:10.1016/j.saa.2018.06.099
12. V. Kumar, S. Kumar, M. Hassan, H. Wu, R. K. Thimmulappa, A. Kumar, S. K. Sharma, V. S. Parmar, S. Biswal, and S. V. Malhotra, "Novel Chalcone Derivatives as Potent Nrf2 Activators in Mice and Human Lung Epithelial Cells," *Journal of Medicinal Chemistry* 54, no. 12 (2011): 4147–59. doi:10.1021/jm2002348
13. Z. Rozmer, T. Berki, and P. Perjési, "Different Effects of Two Cyclic Chalcone Analogues on Cell Cycle of Jurkat T Cells," *Toxicology In Vitro* 20, no. 8 (2006): 1354–62. doi:10.1016/j.tiv.2006.05.006
14. T. Akihisa, H. Tokuda, D. Hasegawa, M. Ukiya, Y. Kimura, F. Enjo, T. Suzuki, and H. Nishino, "Chalcones and Other Compounds from the Exudates of Angelica k Eiskei and Their Cancer Chemopreventive Effects," *Journal of Natural Products* 69, no. 1 (2006): 38–42. doi:10.1021/np058080d
15. J. S. Lee, Y. Kang, J. T. Kim, D. Thapa, E. S. Lee, and J. A. Kim, "The Anti-Angiogenic and Anti-Tumor Activity of Synthetic Phenylpropenone Derivatives is Mediated through the Inhibition of Receptor Tyrosine Kinases," *European Journal of Pharmacology* 677, no. 1-3 (2012): 22–30. doi:10.1016/j.ejphar.2011.12.012
16. D. K. Mahapatra, S. K. Bharti, and V. Asati, "Anti-Cancer Chalcones: Structural and Molecular Target Perspectives," *European Journal of Medicinal Chemistry* 98 (2015): 69–114. doi:10.1016/j.ejmech.2015.05.004

17. S. Burmaoglu, O. Algul, D. A. Anıl, A. Gobek, G. G. Duran, R. H. Ersan, and N. Duran, "Synthesis and Anti-Proliferative Activity of Fluoro-Substituted Chalcones," *Bioorganic & Medicinal Chemistry Letters* 26, no. 13 (2016): 3172–6. doi:10.1016/j.bmcl.2016.04.096
18. S. Burmaoglu, D. Aktas Anil, A. Gobek, D. Kilic, D. Yetkin, N. Duran, and O. Algul, "Design, Synthesis and Antiproliferative Activity Evaluation of Fluorine-Containing Chalcone Derivatives," *Journal of Biomolecular Structure & Dynamics* 40, no. 8 (2022): 3525–50. doi:10.1080/07391102.2020.1848627
19. S. Burmaoglu, A. O. Yilmaz, M. F. Polat, R. Kaya, İ. Gulcin, and O. Algul, "Synthesis of Novel Tris-Chalcones and Determination of Their Inhibition Profiles against Some Metabolic Enzymes," *Archives of Physiology and Biochemistry* 127, no. 2 (2021): 153–61. doi:10.1080/13813455.2019.1623265
20. S. Burmaoglu, S. Ozcan, S. Balcioglu, M. Gencel, S. A. A. Noma, S. Essiz, B. Ates, and O. Algul, "Synthesis, Biological Evaluation and Molecular Docking Studies of Bis-Chalcone Derivatives as Xanthine Oxidase Inhibitors and Anticancer Agents," *Bioorganic Chemistry* 91 (2019): 103149. doi:10.1016/j.bioorg.2019.103149
21. S. Burmaoglu, E. A. Kazancioglu, R. Kaya, M. Kazancioglu, M. Karaman, O. Algul, and I. Gulcin, "Synthesis of Novel Organohalogen Chalcone Derivatives and Screening of Their Molecular Docking Study and Some Enzymes Inhibition Effects," *Journal of Molecular Structure.* 1208 (2020): 127868. doi:10.1016/j.molstruc.2020.127868
22. A. J. McGahon, S. J. Martin, R. P. Bissonnette, A. Mahboubi, Y. Shi, R. J. Mogil, W. K. Nishioka, and D. R. Green, "The End of the (Cell) Line: Methods for the Study of Apoptosis In Vitro," *Methods Cell Biol* 46 (1995): 153–85. doi:10.1016/S0091-679X(08)61929-9
23. A. Güner, A. Nalbantsoy, A. Sukatar, and N. Ü. K. Yavaşoğlu, "Apoptosis-Inducing Activities of *Halopteris scoparia* L. Sauvageau (Brown Algae) on Cancer Cells and Its Biosafety and Antioxidant Properties," *Cytotechnology* 71, no. 3 (2019): 687–704. doi:10.1007/s10616-019-00314-5
24. K. J. Livak, and T. D. Schmittgen, "Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T)) Method," *Methods (San Diego, Calif.)* 25, no. 4 (2001): 402–8. doi:10.1006/meth.2001.1262
25. O. Algul, R. H. Ersan, M. A. Alagoz, N. Duran, and S. Burmaoglu, "An Efficient Synthesis of Novel di-Heterocyclic Benzazole Derivatives and Evaluation of Their Antiproliferative Activities," *Journal of Biomolecular Structure & Dynamics* 39, no. 18 (2021): 6926–38. doi:10.1080/07391102.2020.1803966
26. M. A. Alagoz, "New Molecule Design with In-Silico Methods for Covid-19 Treatment," *Bioorganic and Medicinal Chemistry Reports* 3 (2020): 32–40.
27. A. Temirak, Y. M. Shaker, F. A. Ragab, M. M. Ali, S. M. Soliman, J. Mortier, G. Wolber, H. I. Ali, and H. I. El Diwani, "Synthesis, Biological Evaluation, and Docking Studies of New 2-Furylbenzimidazoles as anti-Angiogenic Agents: Part II," *Archiv Der Pharmazie* 347, no. 4 (2014): 291–304. doi:10.1002/ardp.201300356
28. R. H. Ersan, M. A. Alagoz, A. Dogen, N. Duran, S. Burmaoglu, and O. Algul, "Bisbenzoxazole Derivatives: Design, Synthesis, In Vitro Antimicrobial, Antiproliferative Activity, and Molecular Docking Studies," *Polycyclic Aromatic Compounds* 42, no. 6 (2020): 3103-3123. doi:10.1080/10406638.2020.1852589
29. T. Mosmann, "Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays," *Journal of Immunological Methods* 65, no. 1–2 (1983): 55–63. doi:10.1016/0022-1759(83)90303-4
30. X. Jiang, J. Wang, X. Deng, F. Xiong, S. Zhang, Z. Gong, X. Li, K. Cao, H. Deng, Y. He, et al, "The Role of Microenvironment in Tumor Angiogenesis," *Journal of Experimental & Clinical Cancer Research* 39, no. 1 (2020): 204. doi:10.1186/s13046-020-01709-5
31. R. Lugano, M. Ramachandran, and A. Dimberg, "Tumor Angiogenesis: Causes, Consequences, Challenges and Opportunities, Cell," *Cellular and Molecular Life Sciences : CMLS* 77, no. 9 (2020): 1745–70. doi:10.1007/s00018-019-03351-7
32. C. Aslan, S. Maralbashi, F. Salari, H. Kahroba, F. Sigaroodi, T. Kazemi, and P. Kharaziha, "Tumor-Derived Exosomes: Implication in Angiogenesis and Antiangiogenesis Cancer Therapy," *Journal of Cellular Physiology* 234, no. 10 (2019): 16885–903. doi:10.1002/jcp.28374
33. A. Uccelli, T. Wolff, P. Valente, N. Di Maggio, M. Pellegrino, L. Gürke, B. Andrea, and G. B. Roberto, "Vascular Endothelial Growth Factor Biology for Regenerative Angiogenesis, Swiss," *Swiss Medical Weekly* 149 (2019): w20011. doi:10.4414/smw.2019.20011
34. Velma Ganga Reddy, T. Srinivasa Reddy, Chetna Jadala, M. Soumya Reddy, Faria Sultana, Ravikumar Akunuri, Suresh K. Bhargava, Donald Wlodkowic, P. Srihari, and Ahmed Kamal, "Pyrazolo-Benzothiazole Hybrids: Synthesis, Anticancer Properties and Evaluation of Antiangiogenic Activity Using In Vitro VEGFR-2 Kinase and In Vivo Transgenic Zebrafish Model," *European Journal of Medicinal Chemistry* 182 (2019): 111609. doi:10.1016/j.ejmech.2019.111609
35. N. M. Elsayed, R. A. Serya, M. F. Tolba, M. Ahmed, K. Barakat, D. A. Abou El Ella, and K. A. Abouzid, "Design, Synthesis, Biological Evaluation and Dynamics Simulation of Indazole Derivatives with Antiangiogenic and Antiproliferative Anticancer Activity, Bioorg," *Bioorganic Chemistry* 82 (2019): 340–59. doi:10.1016/j.bioorg.2018.10.071

36. S. Salerno, E. Barresi, A. N. García-Argáez, S. Taliani, F. Simorini, G. Amendola, S. Tomassi, S. Cosconati, E. Novellino, F. Da Settimo, et al, "Discovery of Pyrido[3,2:5,6]Thiopyrano[4,3-d]pyrimidine-Based Antiproliferative Multikinase Inhibitors," *ACS Medicinal Chemistry Letters* 10, no. 4 (2019): 457–62. doi:10.1021/acsmedchemlett.8b00499
37. K. L. Blackwell, Z. A. Haroon, S. Shan, W. Saito, G. Broadwater, C. S. Greenberg, and M. W. Dewhirst, "Tamoxifen Inhibits Angiogenesis in Estrogen Receptor-Negative Animal Models," *Clinical Cancer Research* 6 (2000): 4359–64.
38. D. I. Batovska, and I. T. Todorova, "Trends in Utilization of the Pharmacological Potential of Chalcones," *Current Clinical Pharmacology* 5, no. 1 (2010): 1–29. doi:10.2174/157488410790410579
39. M. Pilatova, L. Varinska, P. Perjesi, M. Sarissky, L. Mirossay, P. Solar, A. Ostro, and J. Mojzis, "In Vitro Antiproliferative and Antiangiogenic Effects of Synthetic Chalcone Analogues," *Toxicology in Vitro : An International Journal Published in Association with BIBRA* 24, no. 5 (2010): 1347–55. doi:10.1016/j.tiv.2010.04.013