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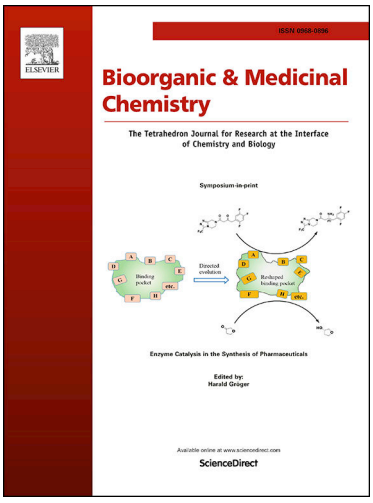

VEGFR-2 Inhibiting effect and Molecular Modeling of Newly Synthesized Coumarin Derivatives as Anti-breast Cancer Agents

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VEGFR-2 Inhibiting effect and Molecular Modeling of Newly Synthesized Coumarin Derivatives as Anti-breast Cancer Agents.

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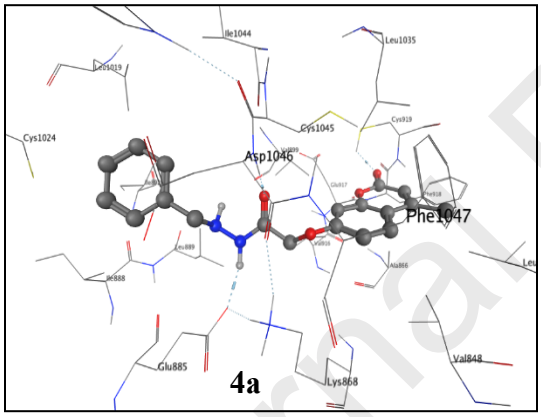
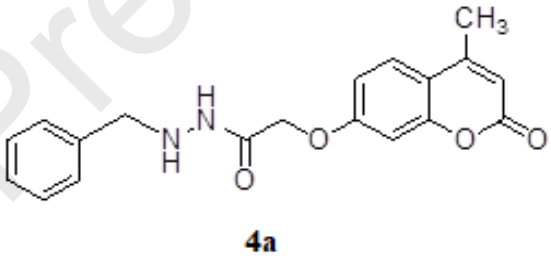
Graphical abstract





4a

Cytotoxic activity against MCF-7 of IC_{50} 1.24 µM

VEGFR-2 kinase inhibitory effect of IC_{50} 0.36 µM

Abstract

Twenty five newly synthesized coumarin scaffold based derivatives were assayed for their *in vitro* anticancer activity against MCF-7 breast and PC-3 prostate cancer cell lines and were further assessed for their *in vitro* VEGFR-2 kinase inhibitory activity. The *in vitro* cytotoxic studies revealed that most of the synthesized compounds possessed very promising cytotoxicity against MCF-7, particularly; compounds **4a** (IC_{50}=1.24 µM) and **3d** (IC_{50}=1.65 µM) exhibited exceptional activities superior to the positive control staurosporine (IC_{50}=8.81 µM). Similarly, the majority of the compounds exhibited higher antiproliferative activities compared to the reference standard with IC_{50} values ranging from 2.07 to 8.68 µM. The two cytotoxic derivatives

4a and **3d** were selected to evaluate their inhibitory potencies against VEGFR-2 kinase. Remarkably, compound **4a,** exhibited significant IC_{50} of 0.36 µM comparable to staurosporine (IC_{50}; 0.33 µM).Moreover, it was capable of inducing preG1 apoptosis, cell growth arrest at G2/M phase and activating caspase-9. On the other hand, insignificant cytotoxic activity was observed for all compounds towards PC-3 cell line. Molecular docking study was carried out for the most active anti-VEGFR-2 derivative **4a,** which demonstrated the ability of the tested compound to interact with the key amino acids in the target VEGFR-2 kinase binding site**.** Additionally, the ADME parameters and physicochemical properties of compound **4a** were examined *in silico*.

Key words: Coumarin; MCF-7; VEGFR-2 kinase; Molecular modeling.

1. Introduction

Despite the latest diagnostic and therapeutic advances, breast cancer remains one of the main causes of female death globally, and the second most common cancer in female population.[1] In recent years, the better understanding of breast cancer biology guided the evolution of drugs specifically aimed against tumorigenesis-associated molecular pathways.[2,3] Angiogenesis, the development of new blood vessels, is recognized as one of the malignancy hallmarks and breast vasculature has been reported to play a crucial role in controlling breast tumors.[4,5] Consequently, many anti-angiogenic drugs have been evaluated in breast cancer patients, including the oral vascular endothelial growth factor-receptor (VEGFR-2) tyrosine kinase inhibitor, sunitinib, which was approved by the Food and Drug Administration for treating different types of cancers.[6,7] Different classes of natural products reported to have significant effect in the treatment and prevention of breast cancer such as coumarins,[8] isoflavones,[9] and curcuminoids.[10] Coumarins are characterized by the presence of benzopyrone platform in their chemical structure and possess wide variety of biological properties.[11,12] The bio-pharmacological activities of coumarin scaffold are based mainly on the chemical structure and physicochemical properties of its heterocyclic ring, which permits easy binding to many target proteins. The 2H-chromen-2-one ring is aromatic, planar and lipophilic, and hence is capable of interacting with different biological counterparts. Furthermore, the lactone group of the coumarin grants the molecule the ability to make strong polar binding, like hydrogen bonds and to acylate protein targets.[13]

Recently, various studies reported the effect of coumarin derivatives in the treatment of breast cancer, particularly by targeting VEGFR-2 kinase.[14–16] The goal of this study is to design and develop novel derivatives of coumarin by incorporating specific functional groups of known VEGFR-2 inhibition.

2. Results and discussion

2.1. Chemistry

Provoked by the gained insight into the correlation between breast cancer and coumarin derivatives as VEGFR-2 inhibitors.[17–19] Literature survey revealed that certain types of functional groups exhibit anti-VEGFR-2 activity such as hydrazides,[20,21] Schiff's bases,[15] sulfonamides,[22] and amide derivatives.[15] Moreover, general structure-activity relationship (SAR) of coumarins divulged that alkylation at position 6 and ether linked substituent at position 7 augment the anticancer activity of the coumarin derivative, [23,24] as illustrated in **Figure 1**.

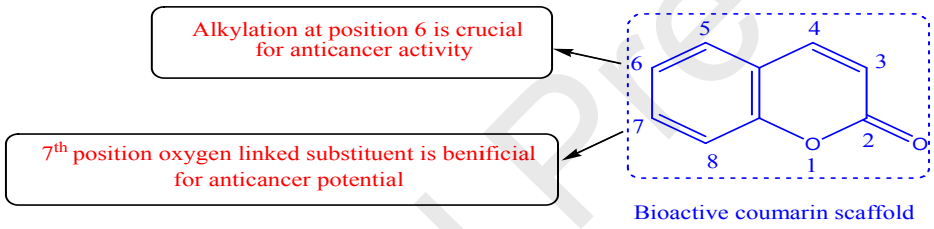
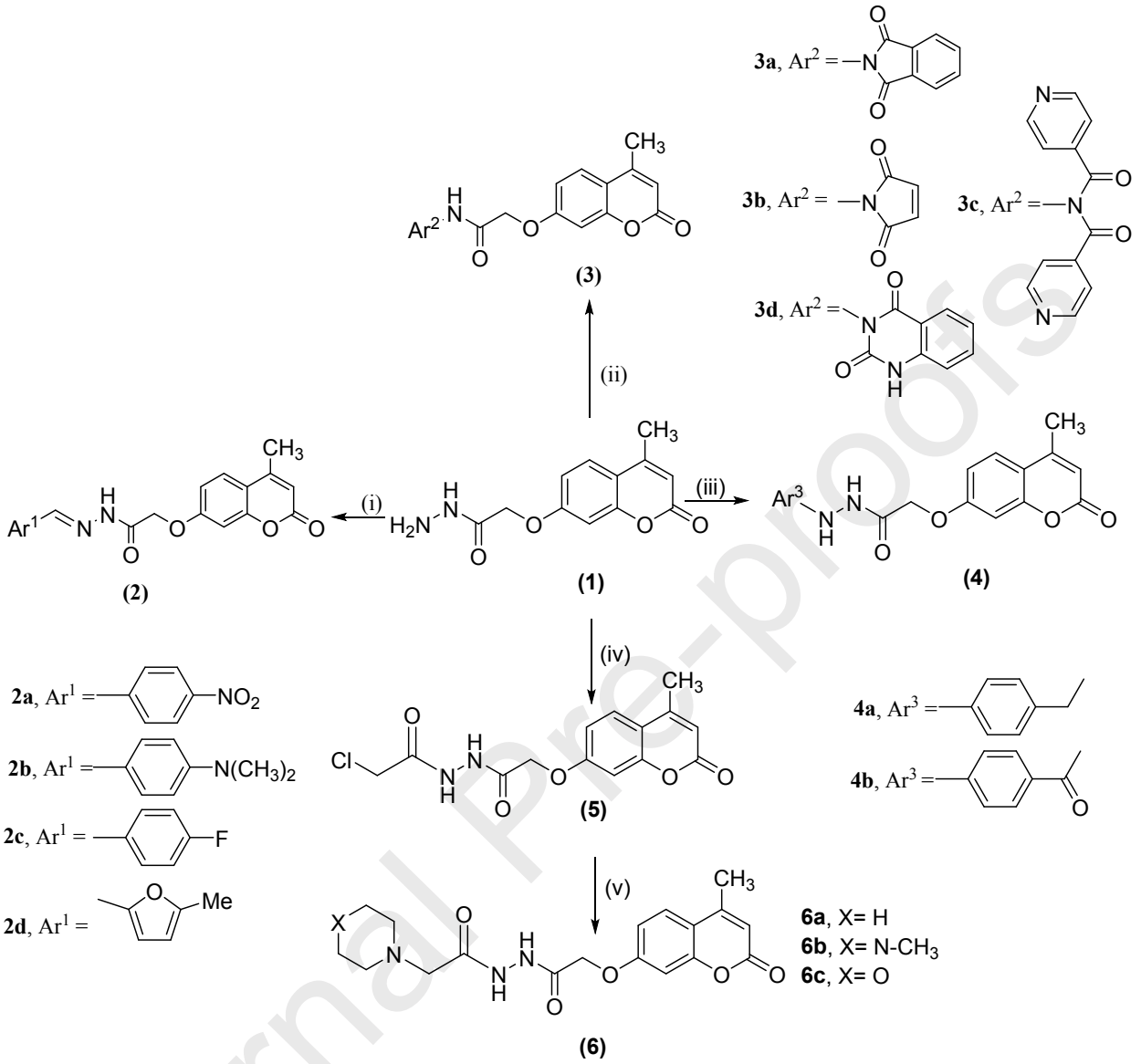


Figure 1. General structure-activity relationship of coumarins.

Considering this in mind, we designed novel coumarin derivatives bearing the aforementioned functional moieties at the positions indicated in the SAR studies.[23,24] Previously our research group disclosed the evaluation of coumarin-based hydrazides as VEGFR-2 inhibitors.[20,21] Expanding our knowledge in this area, we utilized the chemical reactivity of the coumarin-based hydrazides[25] toward diverse electrophiles to engender novel coumarin derivatives encompass imine, amide and sulfonamide moieties to be evaluated as VEGFR-2 inhibitors. The coumarin hydrazide derivative **1** was easily accessed from its ester derivative according to the reported literature procedure.[26] The newly prepared hydrazone derivatives **2a-d** were obtained in good yield through the reaction of the hydrazide **1** with 4-nitrobenzaldehyde, 4-dimethylaminobenzaldehyde, 4-fluorobenzaldehyde and 5- methyl furfural, respectively. The

structure assignment of **2b** was based on ^{1}H NMR data where the characteristic CH=N signal appeared at 8.1 ppm, singlet signal for six protons was observed at 2.97 ppm ($N(CH_3)_2$) and ^{13}C NMR showed a signal at 145 ppm. Reaction of the hydrazide derivative **1** with phthalic anhydride, maleic anhydride, isonicotinc anhydride and isatoic anhydride provided the corresponding N- substituted acetamide **3a-d** respectively as shown in **Scheme 1**.

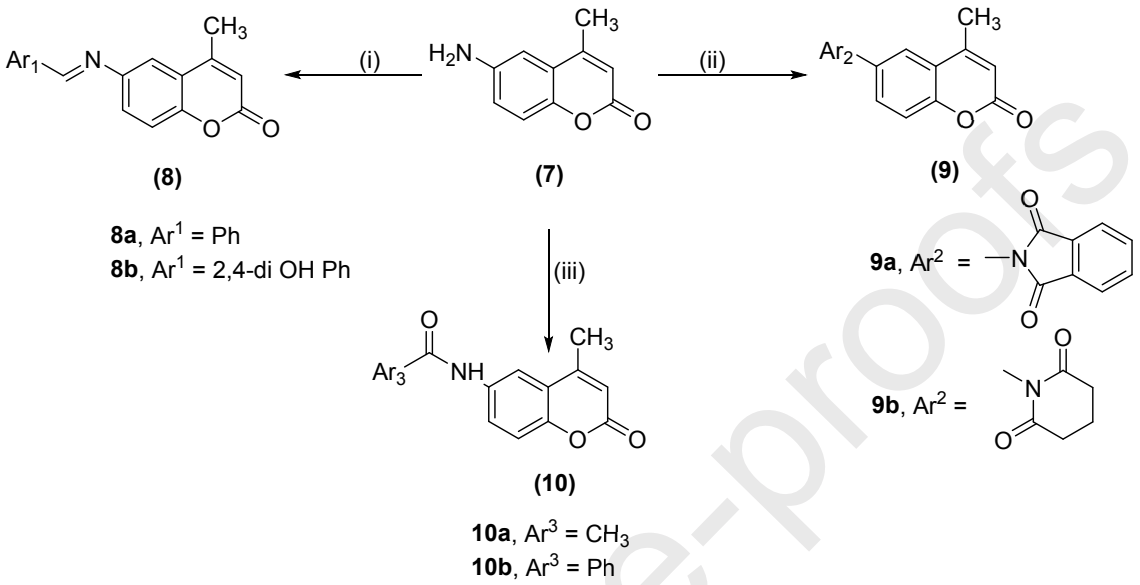
Nucleophilic attack on the benzyl chloride proceeded smoothly to give the benzyl derivative **4a**. The ^{1}H NMR spectra of **4a** showed a characteristic signal at 4.78 ppm as a singlet for the CH_2-N. Reaction of hydrazide **1** with benzoyl chloride and chloroacetylchloride afforded the corresponding hydrazides **4b** and **5** respectively. The newly formed amid bonds were detected in the ^{13}C NMR at 169.4 ppm for **4b** and 166.2 ppm for **5**. The chloroacetohydrazide **5** was allowed to react with different amines; piperidine, N-methyl piperazine and morphline to form the corresponding tertiary amine derivatives **6a-c** respectively, **Scheme 1**. The ^{1}H NMR spectral data for **6a** revealed multiplet signal at δ 2.18-3.01 suggesting the presence of 5 CH_2-piperidine, while in **6b** the appearance of a new singlet signal at 2.38 ppm indicates the presence of N-CH_3 in addition to the multiplet signal at 2.88-2.91 (10H, 4CH_2-piperazine, N-CH_2) and for the **6c** compound, a multiplet signal was detected at 2.48-3.66 ppm (10H, 4CH_2-morpholine, N-CH_2).



Scheme 1: Reagents and reaction conditions; i) appropriate ArCHO, methanol, reflux, 1-5 h. ii) appropriate acid anhydride, glacial AcOH, reflux 5-7 h iii) Acid chlorides, DMF, stirring, rt, 2 h iv) Chloroacetyl chloride, DMF, stirring, rt, 2 h. v) secondary amines, DMF, anhyd. K_2CO_3, stirring, rt, 1h.

Similarly, we envisioned new coumarin-based Schiff's bases and amid derivatives that could be prepared via treatment of 6-aminocoumarin derivative **7**[27] with different electrophiles. Imine derivatives **8a,b** were successfully obtained in moderate to good yield through the reaction of amine derivative **7** with benzaldehyde and/or 2, 4- dihydroxy benzaldehyde respectively. Also, the reaction of amine **7** with acid anhydrides; phthalic anhydride and maleic anhydride was

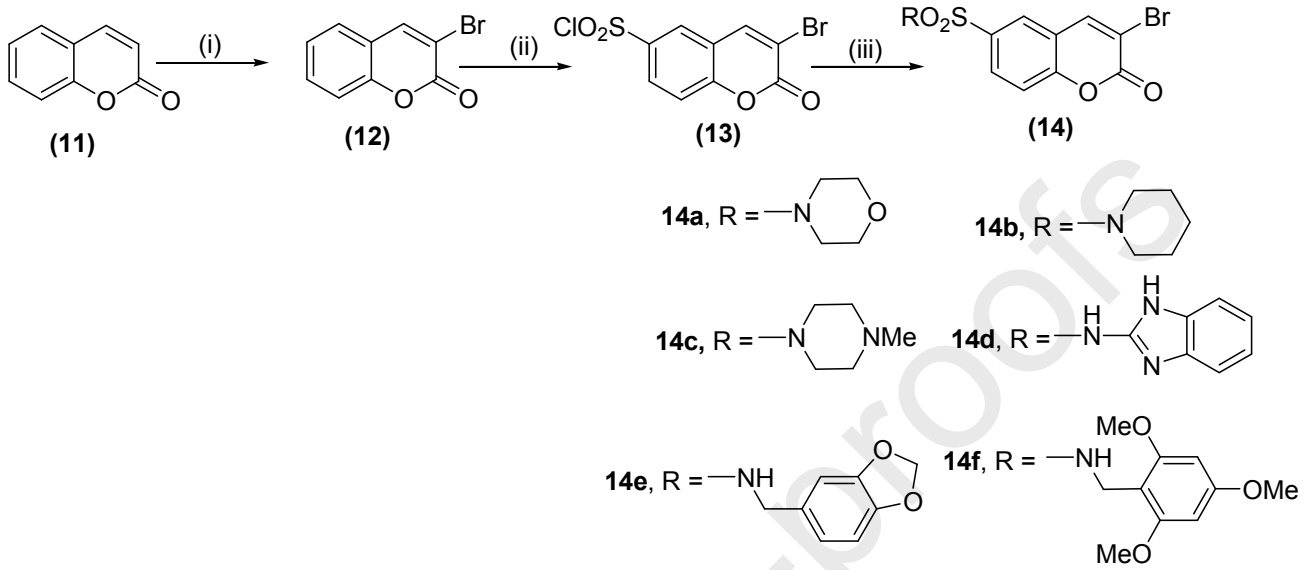
investigated as shown in **scheme 2** to produce **9a** and **9b**. The acetamide derivative **10a** was prepared according to Dawoud *et al*.,[27] by the reaction of amine **7** with acetyl chloride. In a similar fashion, the benzamide derivative **10b** was prepared in a decent yield.



Scheme 2 : Reagents and reaction conditions; i) appropriate ArCHO, glacial AcOH , reflux 7-9 h. ii) appropriate acid anhydride, glacial AcOH , reflux 5-7 h. iii) dry DMF, appropriate acid chloride, stirring 5-7 h.

To generate a series of sulfonamide-based coumarin derivatives, the commercially available coumarin **11** was transformed into 3-bromocoumarin **12**. The reported bromination of coumarin,[28] was modified by using acetic acid as a solvent to cleanly convert the coumarin into the brominated product which underwent elimination smoothly in presence of triethyl amine to give 3-bromocoumarin **12** in an excellent yield and single purification step. The chlorosulfonation of **12** was carried out according to the reported method,[29] to produce the known chlorosulfonyl derivative **13**. The possibility of synthesizing different sulfonamides was feasible by reacting the prepared chlorosulfonyl compound **13** with various amines; namely: morpholine, piperidine, N-methyl piperidine, 2-aminobenzoimidazole, piperonylamine and 2, 4, 6-trimethoxy benzyl amine, **Scheme 3**. The structure of the formed sulfonamides was confirmed using ^{1}H NMR data analysis particularly; in compound **14c** N-Me signal appeared at 2.21 ppm as a singlet while in **14e** two signals at 3.95 ppm as doublet for N-CH_2-Ar and singlet at 5.91 ppm

for $CH_2(O)_2$ were present. The presence of two singlet signals at 3.52 ppm (p-OMe) and the two (o-OMe) at 3.67 ppm confirmed the structure of compound **14f**.



Scheme 3: reagents and reaction conditions; i) bromine, AcOH, ii) chlorosulfonic acid, boiling; iii) Toluene, Et_3N, amine, stirring.

2.2. Biology

The new compounds were evaluated for their *in vitro* antitumor activities against MCF-7 and PC-3 cancer cell lines. The derivatives of the best cytotoxic activity against MCF-7 breast cancer cell line were subjected to VEGFR-2 kinase suppressing activity assay. Apoptosis, cell cycle arrest and detection of caspase-9 intensity against MCF-7 were performed for the most active compound as well.

2.2.1. *In vitro* cell proliferation assay:

We first investigated the growth inhibitory activities of compounds **2–14** against the two cancer cell lines, MCF-7.and PC-3 using MTT assay.[30,31] The two cell lines were selected since they express the target VEGFR-2 in this study.[15,32] The IC_{50} (μM) values of the cytotoxic compounds were calculated as the average of at least three independent experiments, **Table 1**.

The results revealed that most of the newly prepared derivatives showed higher cytotoxicity than the reference drug staurosporine (IC_{50}=8.81 μM) against MCF-7 breast cancer cell line with IC_{50} values ranging from 1.24-8.68 μM. In particular, the hydrazide **4a** (IC_{50}=1.24 μM) and the

acetamide **3d** (IC_{50}=1.65 μM) derivatives revealed the best cytotoxic activity against MCF-7 cell line, **Table 1**. However, the tested compounds showed insignificant cytotoxic activity against PC-3 cell line.

The anti-breast cancer activity of the synthesized compounds on MCF-7 cell line could be explained with regard to their structures. Starting with the hydrazone derivatives **2a-d** and the N-substituted acetamides **3a-d**, compounds **2d** and **3d** were the best in their series showing IC_{50} of 3.19 and 1.65 μM respectively, the rest of the compounds in the two series, except for compounds **2c** and **3a**, showed as well remarkable cytotoxicity ranging from 2.11 to 8.68 μM higher than the reference drug staurosporine (IC_{50}=8.81 μM). As for the hydrazide series and its amine derivatives **4-6**, compound **4a** (IC_{50}=1.24 μM) revealed exceptional cytotoxicity when compared to all the newly synthesized compounds. The aforementioned results, complies with the structure-activity relationship of coumarins that reported the importance of ether linked substitution, at the 7th position, for the anticancer activity.[23,24]

On the other hand, the acetamide derivatives **9a** and **10b** showed remarkable cytotoxicity (IC_{50}=4.17 and 4.60 μM) respectively and compounds **14b** and **14d-f** in the sulfonamide-based coumarin derivatives series showed significant cytotoxicity with IC_{50} ranging from 2.07 to 7.79 μM. As observed, most of the acetamides and sulfonamides showed remarkable cytotoxic activity conforming to the reported general SAR of coumarins which recognizes the significance of substitution at the 6th position for the anticancer activity.[23,24]

Overall, the results elects most of the newly synthesized compounds as promising candidates that could be utilized as lead compounds for optimizing new anticancer agents targeting breast cancer.

2.2.2. *In vitro* VEGFR-2 kinase activity assay

The two compounds **4a** and **3d** which showed the most cytotoxic activity on MCF-7 were evaluated for their effect on VEGFR-2 inhibition. The IC_{50} of the tested derivatives on VEGFR-2 as a marker for angiogensis is outlined in **Table 1**.

Compound **4a** (IC_{50}=0.36 μM) showed better anti-VEGFR-2 activity than compound **3d** (IC_{50}=0.66 μM), which is consistet with the cytotoxic results.

Table 1. The growth inhibitory activities (IC_{50}; μM) of the tested compounds, VEGFR-2 inhibition (μM) in MCF-7 breast cancer cell line.

Sample Code	cytotoxic activity IC_{50} (μM)	VEGFR-2 inhibition activity IC_{50} (μM)
2a	5.59±0.22	ND*
2b	5.54±0.27	ND
2c	9.78±0.38	ND
2d	3.19±0.14	ND
3a	14.68±0.57	ND
3b	2.11±0.07	ND
3c	8.68±0.34	ND
3d	1.65±0.07	0.66±0.019
4a	1.24±0.06	0.36±0.011
4b	7.89±0.33	ND
6b	6.68±0.26	ND
6c	37.33±1.6	ND
9a	4.17±0.19	ND
10b	4.60±0.18	ND
14a	13.20±0.55	ND
14b	2.07±0.11	ND
14c	31.38±1.31	ND
14d	3.65±0.17	ND
14e	7.79±0.36	ND
14f	5.76±0.23	ND
Staurosporine	8.81±0.39	0.33±0.011

ND*: Not Determined

2.2.3. Cellular mechanism of action

2.2.3.1. Cell apoptosis

Flow cytometry method was used to examine the effect of the most potent cytotoxic and anti-VEGFR-2 derivative, **4a**, on induction of apoptosis in MCF-7 cancer cells.[33] On subjecting the cells to derivative **4a**, there was an increase in the early and late cellular apoptosis, **Figure 2**.

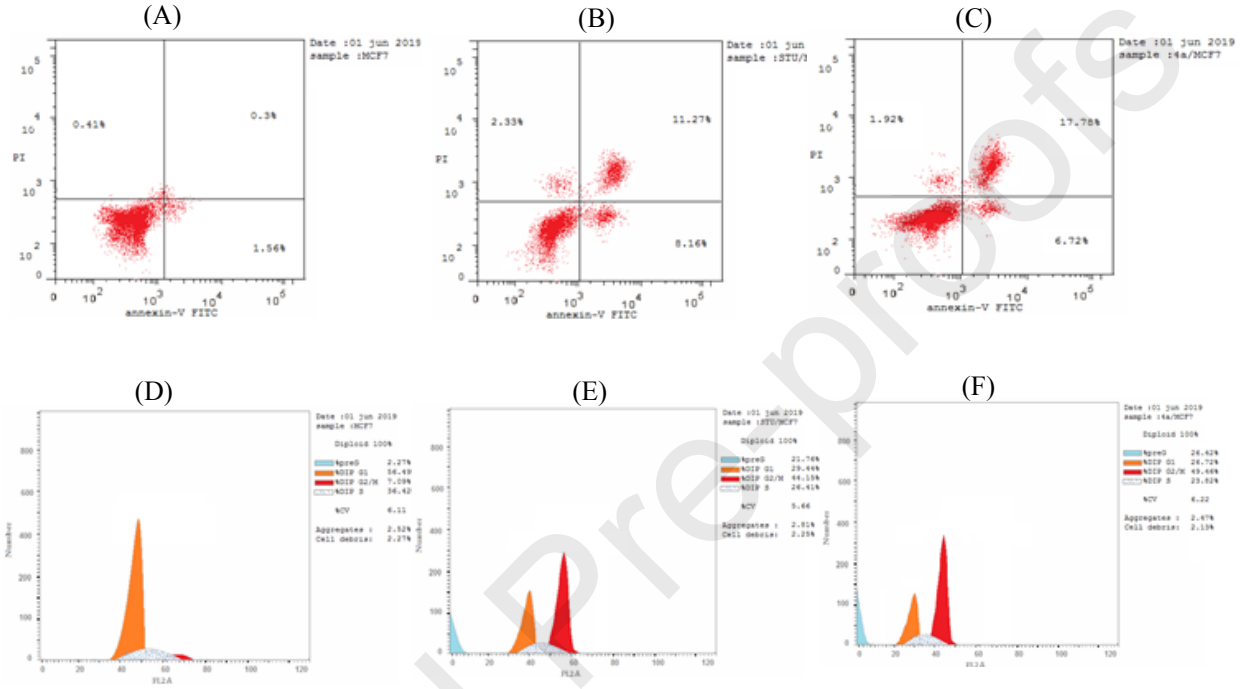


Figure 2. Cellular mechanism of action of compound 4a. (A, B, C) Induction of apoptosis by staurosporine and compound 4a. (D, E, F) Cell cycle analysis of MCF-7 after incubation with staurosporine and compound 4a for 24 h.

2.2.3.2. Cell cycle inhibition

To examine the effect of derivative **4a** on cell cycle, flow cytometry technique was utilized.[33] Accumulation of cells at G2/M phase was observed, as shown in **Figure 2**. The results showed that **4a** induced PreG1apoptosis and G2/M phase arrest preventing cells mitosis.

2.2.3.3. Upregulation of caspase-9

An increase in caspase-9 concentration, involved in apoptosis,[34] was observed on treating MCF-7 cells with **4a**, as shown in **Table 2**.

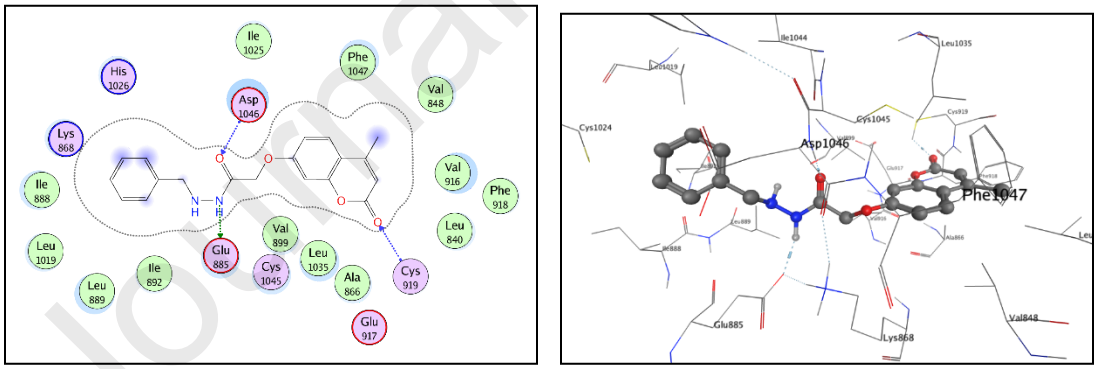
Table 2: Caspase-9 concentration in MCF-7 cells after treatment with compound **4a** in comparison with staurosporine.

Sample Code	Casp-9 (ng/ml)
4a	8.015
Staurosporine	8.41
Cont.MCF-7	1.223

2.3. Docking

Molecular modeling studies were carried out for the most active anti-VEGFR-2 compound to explore the significant interactions of the inhibitor and the amino acids residues in the VEGFR-2 kinase active site. The ability of compound **4a** to interact with the key amino acids in the binding site rationalizes its good activity as indicated by its docking score of −12.49 kcal/mol compared to that of sorafenib of −15.20 kcal/mol.

The tested compound interacts through hydrogen bonding with the key amino acids Asp1046 of the conserved DFG motif, the side chain carboxylate of Glu885 of the αC helix and Cys919 in the hinge region of the protein. In addition, through hydrophobic interaction with hydrophobic side chains lining the hydrophobic back pocket Ile888, Leu889, Ile892, Val898, Val899, Leu1019 and Ile1044 by its benzyl moiety, **Figure 3**.



(A) (B)

Figure 3. (A) 2D diagram, (B) 3D representation of compound **4a** in the VEGFR-2 binding site.

2.4. *In silico* physicochemical properties and pharmacokinetic parameters assessment

The pharmacokinetic parameters and the physicochemical properties of the active derivative were determined *in silico* to explore its drug like characteristics. Compound **4a** manifested the properties of a promising drug-like lead compound, **Table 3**. The compound showed zero violations of the Lipinski's rule of five, high GI absorption, 0.55 oral bioavailability score and zero PAINS alerts (pain-assay interference structural alerts) confirming its reliability for future optimization.

Table 3. Physicochemical and ADME characterization of compound **4a**

Compd	#Heavy atoms	#Rotatable bonds	#H-bond acceptors	#H-bond donors	MR	TPSA	WLOGP
4a	25	7	5	2	93.85	80.57	2.15
	SOL Class	GI absorption	BBB permeant	Lipinski #violations	Bioavailability Score	PAINS #alerts	Synthetic Accessibility
	Soluble	High	No	0	0.55	0	3.32

3. Conclusion

In this study, new coumarin derivatives incorporating different functional groups were synthesized and evaluated for their antiproliferative activity against MCF-7 breast cancer and PC-3 prostate cell lines. Compounds **2a, 2b, 2d, 3b-d, 4a, 4b, 6b, 9a, 10b, 14b, 14d-f** revealed remarkable cytotoxic activity on MCF-7 with IC_{50} values, ranging from 1.24 to 8.68 μM, higher than the reference standard staurosporine (IC_{50}=8.81 μM). Particularly, compounds **4a** (IC_{50}=1.24 μM) and **3d** (IC_{50}=1.65 μM) exhibited the best cytotoxic activities among the newly synthesized derivatives. Moreover, VEGFR-2 kinase assay was performed on compounds **3d** and **4a** and the results showed that compound **4a**, the most cytotoxic compound, had better anti-VEGFR-2 kinase activity than **3d** with IC_{50} value of 0.36 μM. Referring to both the cytotoxic results and VEGFR-2 kinase assay results, one may relate the cytotoxicity of compound **4a** to suppression of the target enzyme. The cellular mechanism of action studies on **4a**, revealed that it was able to induce preG1 apoptosis and cell growth arrest at G2/M phase and to activate caspase-9. However, none of the synthesized derivatives showed significant cytotoxic activity on PC-3 cell line. On the other hand, the ability of the most active anti-VEGFR-2 derivative **4a** to interact with the key amino acids in VEGFR-2 binding site was demonstrated by the performed

molecular docking study. Finally, compound **4a** was found to exhibit very good ADME properties and drug-like characteristics without violating Lipinski's rule of five.

4. Experimental

4.1. Chemistry

Melting points were determined on Electrothermal IA 9000 apparatus and were uncorrected. Elemental analyses were carried out at the Micro-analytical Laboratory, Central Services Laboratory, Faculty of Science, Cairo University, Egypt. ^{1}HNMR spectra were determined on Bruker 400 MHz at Faculty of Pharmacy, Cairo University, Egypt and ^{13}CNMR spectra were determined on Varian Mercury 100 MHz spectrometer at Faculty of Pharmacy, Cairo University, Cairo, Egypt, using tetramethylsilane (TMS) as the internal standard. The reactions were followed by TLC (silica gel, aluminum sheets 60 F254, Merck) using chloroform: methanol (9.5: 0.5 v/v) and toluene: ethyl acetate (8: 2 v/v) as eluents and sprayed with iodine-potassium iodide reagent. All the reactions were monitored by TLC and the purity of the newly synthesized compounds was assessed by TLC and elemental analysis.

4.1.1. General procedure for synthesis of (E)-2-(4-methyl-2-oxo-2H-chromen-7-yloxy)-N'-(substituted) acetohydrazide (2a-d)

A solution of aromatic and heterocyclic aldehydes namely, 4-nitrobenzaldehyde, 4-dimethylaminobenzaldehyde, 4-fluorobenzaldehyde and 5- methyl furfural, (0.01 mol) in methanol (5 mL) was added dropwise to a well-stirred solution of compound **1** (0.01 mol) in boiling methanol/ water (50.6 v/v). The reaction mixture was refluxed for 1-5 hours while stirring, then concentrated and cooled. The solid product was filtered, washed with dilute methanol and crystallized from ethanol.

4.1.1.1. (E)-2-(4-methyl-2-oxo-2H-chromen-7-yloxy)-N'-(4-nitrobenzylidene)acetohydrazide (2a)

Yield 70 %; m.p. 265-267 ºC. ^{1}H NMR (DMSO-d_6, δ, ppm): 1.82 (3H, s, CH_3), 4.52 (2H, s, OCH_2), 6.33 (1H, s, H-3 coumarin), 7.34-7.93 (7H, m, Ar-H), 8.1 (1H, s, CH=N), 10.54 (1H, s, NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, δ, ppm): 19.31, 61.44, 103.01, 110.43, 111.19.112.13, 124.24, 124.96, 125.11, 138.98, 143.71, 149.52, 151.27, 153.33, 160.88, 161.96, 171.25. Anal. calcd. For $C_{19}H_{15}N_3O_6$ (381.33): C, 59.84; H, 3.96; N, 11.02 Found: C, 59.81; H, 3.93; N, 11.05

4.1.1.2. (E)-2-(4-methyl-2-oxo-2H-chromen-7-yloxy)-N'-(4-(dimethylamino)benzylidene) acetohydrazide (2b)

Yield 70 %; m.p. 235-238 ºC; ^{1}H NMR (DMSO-d_6, δ, ppm): 2.40 (3H, s, CH_3), 2.97 (6H, s, $N(CH_3)_2$) , 4.76 (2H, s, OCH_2), 6.21 (1H, s, H-3 coumarin), 6.9-7.7 (7H, m, Ar-H), 8.1 (1H, s, CH=N), 11.38 (1H, s, NH, D_2O exchangeable). ^{13}C NMR (DMSO-d6, δ, ppm): 18.6, 41.69, 65.69, 102.14, 111.95, 113.76, 121.68, 126.84, 128.98, 145.31, 149.44, 151.89, 153.86, 155.03, 160.52, 161.90, 168.26. EIMS m/z (%):379 (M^+, 15), 190 (2), 162 (5), 146(21), 84 (61), 82(100), 77 (12), 57 (33). Anal. calcd. For $C_{21}H_{21}N_3O_4$ (379.40): C, 66.48; H, 5.58; N, 11.08 Found: C, 66.44; H, 5.55; N, 11.06.

4.1.1.3. (E)-N'-(4-fluorobenzylidene)-2-(4-methyl-2-oxo-2H-chromen-7-yloxy) acetohydrazide (2c)

Yield 75 %; m.p. 241-247 ºC; ^{1}H NMR (DMSO-d_6, δ, ppm): 1.97 (3H, s, CH_3), 4.34 (2H, s, OCH_2), 6.27 (1H, s, H-3 coumarin), 7.34-7.93 (7H, m, Ar-H), 8.32 (1H, s, CH=N), 9.96 (1H, s, NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, δ, ppm): 18.14, 68.43, 103.93, 111.78, 112.00, 112.96, 114.15, 125.83, 130.31, 131.18, 144.15, 152.76, 154.66, 159.88, 160.23, 165.27, 170.69. Anal. calcd. For $C_{19}H_{15}FN_2O_4$ (354.33): C, 64.40; H, 4.27; N, 7.9. Found: C, 64.37; H, 4.24; N, 7.88

4.1.1.4. (E)-2-(4-methyl-2-oxo-2H-chromen-7-yloxy)-N'-((5-methylfuran-2-yl) methylene) acetohydrazide (2d)

Yield 75 %; m.p. 268-274 ºC; ^{1}H NMR (DMSO-d6, δ, ppm): 1.87 (3H,s , CH3), 2.01 (3H, s, CH_3), 4.45 (2H, s, OCH_2), 6.38 (1H, s, H-3 coumarin), 7.34-7.93 (7H, m, Ar-H), 8.32 (1H, s, CH=N), 9.96 (1H, s, NH, D_2O exchangeable). ^{13}C NMR (DMSO-d6, δ, ppm): 14.34, 19.64, 69.19, 104.67, 105.78, 110.39, 111.92, 112.14, 125.18, 133.64, 146.73, 152.01, 152.98, 154.23, 155.99, 161.82, 162.86, 170.94. Anal. calcd. For $C_{18}H_{16}N_2O_5$ (340.33): C, 63.52; H, 4.74; N, 8.23. Found: C, 63.49; H, 4.71; N, 8.20.

4.1.2. General procedure for the synthesis of N-(substituted)-2-(4-methyl-2-oxo-2H-chromen-7-yloxy) acetamide (3a-d)

A mixture of compound **1** (0.01 mol) and the selected acid anhydride namely: phthalic anhydride, maleic anhydride, isonicotinc anhydride and isatoic anhydride (0.01 mol) in glacial acetic acid (50 mL) was heated under reflux for 5-7 hours. The obtained solid was filtered, washed with acetic acid and crystallized from glacial acetic acid.

4.1.2.1. N-(1,3-dioxoisoindolin-2-yl)-2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetamide (3a)

Yield 70 %; m.p. 208-210 ºC; ^{1}H NMR (DMSO-d_6, δ, ppm): 1.79 (3H, s, CH_3), 4.50 (2H, s, OCH_2), 6.33 (1H, s, H-3 coumarin), 7.01-7.83 (7H, m, Ar-H), 9.96 (1H, s, NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, δ, ppm): 18.94, 65.63, 103.05, 110.91, 111.82, 112.50, 122.37, 124.89, 131.78, 132.24, 151.27, 153.34, 161.80, 162.30, 164.73, 166.36. Anal. calcd. For $C_{20}H_{14}N_2O_6$ (378.33): C, 63.49; H, 3.73; N, 7.40 Found: C, 63.45; H, 3.70; N, 7.37

4.1.2.2. N-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-2-(4-methyl-2-oxo-2H-chromen-7-yloxy) acetamide (3b)

Yield 75 %; m.p. 167-170 ºC; ^{1}H NMR (DMSO-d_6, δ, ppm): 1.88 (3H, s, CH_3), 4.32 (2H, s, OCH_2), 6.25 (1H, s, H-3 coumarin), 7.23-7.99 (5H, m, Ar-H), 10.03 (1H, s, NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, δ, ppm): 20.14, 65.63, 104.63, 110.23, 111.42, 113.35, 124.58, 132.99, 152.57, 154.54, 160.36, 162.63, 164.12, 166.44. Anal. calcd. For $C_{16}H_{12}N_2O_6$ (328.27): C, 58.54; H, 3.68; N, 8.53. Found: C, 58.51; H, 3.65; N, 8.50.

4.1.2.3. N-isonicotinoyl-N'-(2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetyl) isonicotinohydrazide (3c)

Yield 75 %; m.p. 171-174 ºC; ^{1}H NMR (DMSO-d6, δ, ppm): 2.40 (3H, s, CH_3), 5.19 (2H, s, OCH_2), 6.2 (1H, s, H-3 coumarin), 7.10-8.17 (11H, m, Ar-H), 11.1 (1H, s, NH, D_2O exchangeable). ^{13}C NMR (DMSO-d6, δ, ppm): 18.61, 66.8, 102.22, 111.78, 113.15, 113.94, 120.86, 126.43, 134.72, 146.78, 153.21, 153.85, 155.06, 160.57, 161.04, 161.81. Anal. calcd. For $C_{24}H_{18}N_4O_6$ (458.42): C, 62.88; H, 3.96; N, 12.22. Found: C, 62.85; H, 3.93; N, 12.20.

4.1.2.4. N-(2,4-dioxo-1,2-dihydroquinazolin-3(4H)-yl)-2-(4-methyl-2-oxo-2H-chromen-7-yloxy) acetamide (3d)

Yield 70 %; m.p. 139-141 ºC; ^{1}H NMR (DMSO-d_6, δ, ppm): 1.88 (3H, s, CH_3), 4.74 (2H, s, OCH_2), 6.23 (1H, s, H-3 coumarin), 7.03-8.7 (7H, m, Ar-H), 9.8, 10.01 (2H, s, 2NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, δ, ppm): 19.72, 66.67, 101.67, 111.94, 113.02, 113.80, 114.13, 114.21, 121.78, 126.94, 126.99, 139.77, 139.99, 150.93, 160.53, 161.06, 161.10, 164.43, 164.51, 166.66. Anal. calcd. For $C_{20}H_{15}N_3O_6$ (393.34): C, 61.07; H, 3.84; N, 10.68 Found: C, C, 61.04; H, 3.80; N, 10.63.

4.1.3. General procedure for the synthesis of 2-(4-methyl-2-oxo-2H-chromen-7-yloxy)-N'-substituted hydrazide (4a,b and 5)

To a well stirred solution of acetohydrazide **1** (0.0024 mol) in dry dimethylformamide (3 mL), the appropriate acid chlorides namely: benzyl chloride, benzoyl chloride and chloroacetylchloride (0.0024 mol) were dropwisely added with continuous stirring on cold at 20 ºC for 2 hours. The solution was poured onto ice/water; the formed precipitate was collected, washed with water and crystallized from ethanol.

4.1.3.1. 2-(4-methyl-2-oxo-2H-chromen-7-yloxy)-N'-benzylacetohydrazide (4a),

Yield: 85%; mp: 211–213 ˚C. ^{1}H NMR (DMSO-d_6, δ, ppm): 1.95 (3H, s, CH_3), 4.78 (2H, s, CH_2-N), 5.12 (2H, s, OCH_2), 6.20 (1H, s, H-3 coumarin), 6.85-7.71 (8H, m, Ar-H), 10.28, 10.48 (2H, s, 2NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, δ, ppm): 18.58 (CH_3), 65.81, 66.68, 101.89, 111.64, 111.85, 112.67, 112.84, 113.71, 113.99, 126.81, 126.98, 152.14, 153.82, 154.99, 158.03, 160.57, 161.84 (C=O coumarin), 168.86 (C=O). EIMS m/z (%):338 (M^+, 0.14), 288 (23), 248 (2), 189(18), 148 (13), 113(100), 103 (35), 56 (19). Anal. calcd. for $C_{19}H_{18}N_2O_4$ (338.36): C, 67.44; H, 5.36; N, 8.28. Found: C, 67.51; H, 5.45; N, 8.29.

4.1.3.2. N'-(2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetyl)benzohydrazide (4b)

Yield (80 %); m.p. 94-96 ºC; ^{1}H NMR (DMSO-d_6, δ, ppm): 2.05 (3H, s, CH_3), 5.56 (2H, s, OCH_2), 6.10 (1H, s, H-3 coumarin), 6.59-8.00 (8H, m, Ar-H), 9.77, 10.14 (2H, s, 2NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, δ, ppm): 20.18 (CH_3), 69.14, 109.17, 112.22, 114.64, 116.11, 126.42, 126.99, 127.68, 135.40, 137.11, 155.14, 157.82, 161.17, 162.61 (C=O coumarin), 167.45 (C=O benzoyl), 169.32 (C=O). Anal. calcd. For $C_{19}H_{16}N_2O_5$ (352.34): C, 64.77; H, 4.58; N, 7.95, Found: C, 64.70; H, 4.48; N, 7.88.

4.1.3.3. N'-(2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetyl)-2-chloroacetohydrazide (5)

Yield (85 %); m.p. 233-235º C; ^{1}H NMR (DMSO-d_6, δ, ppm): 1.89 (3H, s, CH_3), 4.56 (2H, s, CH_2Cl), 4.91 (2H, s, OCH_2), 6.22 (1H, s, H-3 coumarin), 6.23 (1H, s, H-8 coumarin), 6.55 (1H, d, H-6 coumarin), 7.00 (1H, d, H-5 coumarin),10.77, 10.98 (2H, s, 2NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, δ, ppm): 19.42 (CH_3), 45.66, 68.19, 108.19, 111.13, 113.55, 115.92, 128.11, 151.33, 152.98, 161.17, 161.93 (C=O coumarin), 166.14, 166.87. Anal. calcd. For $C_{14}H_{13}ClN_2O_5$ (324.72): C, 51.78; H, 4.04; N, 8.63, Found: C, 51.71; H, 4.09; N, 8.59.

4.1.4. General procedure for the synthesis of N'-(2-(4-methyl-2-oxo-2H-chromen-7-yloxy) acetyl)-2-(substituted) acetohydrazide (6a-c)

To a well stirred solution of chloroacetohydrazide **5** (0.018 mol) in dry dimethylformamide (3 mL), different secondary amines namely: piperidine, N-methyl piperazine and morphline (0.002 mol) were added in the presence of (0.0022 mol) anhydrous potassium carbonate with continuous stirring on cold at 20 ºC for 1 hour. The solution was poured onto ice/water to give a precipitate which was collected, washed with water and crystallized from ethanol.

4.1.4.1. N'-(2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetyl)-2-(piperidin-1-yl)acetohydrazide (6a)

Yield (81%); m.p. 175-177º C; ^{1}H NMR (DMSO-d_6, δ, ppm): 2.03 (3H, s, CH_3), 2.18 - 3.01 (12H, m, 5CH_2-piperidine, N-CH_2), 4.80 (2H, s, OCH_2), 6.25 (1H, s, H-3 coumarin), 6.63 (1H, s, H-8 coumarin), 6.75 (1H, d, H-6 coumarin), 7.12 (1H, d, H-5 coumarin), 9.14,9.97 (2H, s, 2NH, D_2O exchangeable).^{13}C NMR(DMSO-d_6, δ, ppm): 19.60 (CH_3), 27.28, 28.00, 54.34, 60.10, 69.77, 109.63, 111.82, 112.65, 113.15, 128.46, 152.11, 153.28, 160.12, 160.98 (C=O pyrone), 165.66, 170.57. Anal. calcd. For $C_{19}H_{23}N_3O_5$ (373.4): C, 61.11; H, 6.21; N, 11.25, Found: C, 61.05; H, 6.24; N, 11.20.

4.1.4.2. N'-(2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetyl)-2-(4-methylpiperazin -1-yl) acetohydrazide (6b)

Yield: 80%; mp: 220–222 ˚C; ^{1}HNMR (DMSO-d_6, δ, ppm): 2.15 (3H, s, CH_3), 2.38 (3H, s, N-CH_3), 2.88 - 2.91 (10H, m, 4CH_2-piperazine, N-CH_2), 4.20 (2H, s, OCH_2), 6.15 (1H, s, H-3 coumarin), 6.72 (1H, s, H-8 coumarin), 6.85 (1H, d, H-6 coumarin), 7.62 (1H, d, H-5 coumarin), 8.55,8.95 (2H, s, 2NH, D_2O exchangeable).^{13}C NMR(DMSO-d_6, δ, ppm): 18.59 (CH_3), 46.19 (N-CH_3), 53.21, 53.42, 55.11, 68.90, 101.73, 110.91, 112.77, 113.06, 126.37, 154.02, 155.06, 160.80, 163.03, 166.18 (C=O pyrone), 169.48 (C=O). Anal. calcd. for $C_{19}H_{24}N_4O_5$ (388.42): C, 58.75; H, 6.23; N, 14.42. Found: C, 58.81; H, 6.32; N, 14.49.

4.1.4.3. N'-(2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetyl)-2-morpholinoacetohydrazide (6c)

Yield (82%); m.p. 163-165º C**;** ^{1}H NMR (DMSO-d_6, δ, ppm): 1.99 (3H, s, CH_3), 2.48-3.66 (10H, m, 4CH_2-morpholine, N-CH_2), 4.86 (2H, s, OCH_2), 6.11 (1H, s, H-3 coumarin), 6.54 (1H, s, H-8 coumarin), 6.85 (1H, d, H-6 coumarin), 7.23 (1H, d, H-5 coumarin), 10.01, 10.76 (2H, s, 2NH, D_2O exchangeable).^{13}C NMR(DMSO-d_6, δ, ppm): 20.63 (CH_3), 52.34, 59.22, 65,71, 66.97, 119.72, 111.93, 112.97, 113.89, 127.55, 151.14, 152.26, 161.01, 161.98 (C=O pyrone), 166.56,

171.36. Anal. calcd. For $C_{18}H_{21}N_3O_6$ (375.38): C, 57.59; H, 5.64; N, 11.19, Found: C, 57.53; H, 5.66; N, 11.22.

4.1.5. General procedure for the synthesis of (E)-6-(substituted benzylideneamino)-4-methyl-2H-chromen-2-one (8a,b)

To a well stirred solution of 6-amino coumarin **7** (0.001 mol) in chloroform, the appropriate aldehydes namely: benzaldehyde and 2, 4- dihydroxy benzaldehyde, were dropwisely added, then 2 mL glacial acetic acid was added. The reaction mixture was refluxed for 7- 9 hours. The solid obtained was crystallized from ethanol.

4.1.5.1. (E)-6-(benzylideneamino)-4-methyl-2H-chromen-2-one (8a)

Yield 67 %; m.p. 215-217 º C; ^{1}H NMR (DMSO-d_6, δ, ppm): 2.12 (3H, s, CH_3), 6.20 (1H, s, H-3 coumarin), 7.40-7.73 (8H, m, Ar-H), 8.14 (1H, s, HC=N). ^{13}C NMR (DMSO-d_6, δ, ppm): 18.40 (CH_3), 110.51, 116.82, 120.78, 121.34, 129.18, 130.44, 132.31, 135.42, 149.81, 152.01, 153.17, 159.79 (C=N), 161.18 (C=O). Anal. calcd. For $C_{17}H_{13}NO_2$ (263.29): C, 77.55; H, 4.98; N, 5.32, Found: C, 77.51; H, 4.93; N, 5.35.

4.1.5.2. (E)-6-(2,4-dihydroxybenzylideneamino)-4-methyl-2H-chromen-2-one (8b)

Yield 74 %; m.p. 234-236º C; ^{1}H NMR (DMSO-d_6, δ, ppm): 2.12 (3H, s, CH_3), 6.10 (1H, s, H-3 coumarin), 6.99-7.53 (6H, m, Ar-H), 8.87 (1H, s, HC=N), 9.72, 10.11 (2H, s, 2 OH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, δ, ppm): 19.11 (CH_3), 102.27, 107.76, 111.52, 112.11, 117.88, 120.12, 121.29, 132.89, 148.14, 151.32, 152.07, 161.06 (C=N), 161.86 (C=O), 163.24, 164.50. Anal. calcd. For $C_{17}H_{13}NO_4$ (295.28): C, 69.15; H, 4.44; N, 4.74, Found: C, 69.11; H, 4.38; N, 4.70.

4.1.6. General procedure for the synthesis (4-methyl-2-oxo-2H-chromen-6-yl) dione derivatives (9a, b)

A solution of 6-amino coumarin **7** (0.1 mol) and the appropriate acid anhydrides namely phthalic and glutaric anhydrides (0.1 mol) in glacial acetic acid (20 mL) were refluxed for 5-7 hours. The reaction mixture was concentrated, and then water was added. The precipitate was filtered, washed with water and crystallized from ethanol.

4.1.6.1. 2-(4-methyl-2-oxo-2H-chromen-6-yl)isoindoline-1, 3-dione (9a)

Yield 68 %; m.p. 233-235 º C; ^{1}H NMR (DMSO-d_6, δ, ppm): 1.91 (3H, s, CH_3), 6.31 (1H, s, H-3 coumarin), 7.20- 8.44 (7H, m, Ar-H). ^{13}C NMR (DMSO-d_6, δ, ppm): 17.44 (CH_3), 110.50,

114.41, 114.99, 121.32, 122.17, 125.74, 127.44, 130.22, 130.91, 148.82, 151.27, 160.08 (C=O), 168.47. Anal. calcd. For $C_{18}H_{11}NO_4$ (305.28): 70.82; H, 3.63; N, 4.59, Found: , 70.80; H, 3.65; N, 4.55.

4.1.6.1. 1-(4-methyl-2-oxo-2H-chromen-6-yl) piperidine-2, 6-dione (9b)

Yield 77 %; m.p. 240-142 º C; ^{1}H NMR (DMSO-d_6, δ, ppm): 1.99- 2.12 (6H, m, $3CH_2$ – piperidine dione), 2.31 (3H, s, CH_3), 6.12 (1H, s, H-3 coumarin), 7.15-7.90 (3H, m, Ar-H). ^{13}C NMR (DMSO-d_6, δ, ppm): 16.1, 18.94(CH_3), 30.19, 111.25, 115.64, 115.99, 120.32, 127.74, 131.09, 148.75, 152.19, 161.08 (C=O), 170.32. Anal. calcd. For $C_{15}H_{13}NO_4$ (271.26): C, 66.41; H, 4.83; N, 5.16, Found: C, 66.43; H, 4.80; N, 5.12.

4.1.7. General procedure for the synthesis of N-(4-methyl-2-oxo-2H-chromen-6-yl) amide derivatives (10a, b)

To a well stirred solution of 6-amino coumarin **7** (0.1 mol) in dry dimethylformamide (5 mL), the appropriate acid chloride namely: acetyl chloride and/or benzoyl chloride (0.1 mol) was dropwisely added with continuous stirring on cold for 5-7 hours. The solution was poured onto ice/water; the formed precipitate was collected, washed with water and crystallized from ethanol.

4.1.7.1. N-(4-methyl-2-oxo-2H-chromen-6-yl)benzamide (10b)

Yield 71 %; m.p. 250-252 º C; ^{1}H NMR (DMSO-d_6, δ, ppm): 2.32 (3H, s, CH_3), 6.13 (1H, s, H-3 coumarin), 7.47- 7.95 (8H, m, Ar-H), 8.83 (1H, s, NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, δ, ppm): 19.48 (CH_3), 111.37, 111.60, 113.16, 114.93, 129.01, 129.70, 131.23, 133.30, 154.82, 160.61, 163.08, 164.61, 165.38, 167.80. Anal. calcd. For $C_{17}H_{13}NO_3$ (279.29): C, 73.11; H, 4.69; N, 5.02, Found: C, 73.06; H, 4.62; N, 5.05.

4.1.8. Procedure for the synthesis of 3-bromocoumarin (12)

To a stirred solution of the known coumarin **11** (0.0342 mmol) in acetic acid (20 mL) at room temperature, a solution of bromine (0.0685 mmol) was added dropwisely over a time period of 1 hour. After the addition completed, the reaction was poured onto ice/water (300 mL) and neutralized with NaOH solution (10%). The formed precipitate was filtered and dissolved in DCM (150 mL) and was used in the next step without further purification. Triethyl amine was added dropwise to the ice cold solution of the dibromocoumarin in DCM. After 1 hour the reaction was quenched with HCl (10%, 100 mL), the bromocoumarin was extracted with DCM (20 mL, 3x), dried (anh, $CaCl_2$), concentracted in vacuo and the crude product was recrystallized

from ethanol (75%) to give 3-bromocoumarin **12** as colorless solid. (7.2 g, 94%); the spectral data are consistent with that reported in literature.[35]

4.1.9. General procedure for the synthesis of Coumarin-6-Sulfonamides (14a-f)

To a stirred solution of **13** (0.001 mol) in toluene (10 mL) at room temperature, one drop of triethyl amine was added followed by the addition of the selected amines, namely: morpholine, piperidine, N-methyl piperidine, 2-aminobenzoimidazole, piperonylamine and 2,4,6-trimethoxy benzyl amine (0.0011 mol). In a time period of 5 to 7 hrs, TLC showed the reaction is complete, the formed solid was filtered and washed with toluene.

4.1.9.1. 3-bromo-6-(morpholinosulfonyl)-2H-chromen-2-one (14a)

Yield 86 %; m.p. 183-185 º C; ^{1}H NMR (DMSO-d_6, δ, ppm): 2.76 (4 H, s, 2 N-CH_2 morpholine), 3. 7 (4 H, s, 2 O-CH_2 morpholine), 7.60 (1 H, d, J = 8.7 Hz, H-8 coumarin), 8.15 (1H, dd, J = 1.4, 8.7 Hz, H-7 coumarin), 8.23 (1 H, d, J= 1.4 Hz, H-5 coumarin), 8.32 (1 H, s, H-4 coumarin). ^{13}C NMR (DMSO-d_6, δ, ppm): 22.6, 32.5, 50.2, 56.6, 65.9, 120.9, 127.1, 127.2, 127.7, 128.25, 128.33, 128.5, 128.8, 138.8, 140.2, 146.7. Anal. calcd. For $C_{13}H_{12}BrNO_5S$ (374.21): C, 41.73; H, 3.23; N, 3.74, Found: C, 41.70; H, 3.17; N, 3.70

4.1.9.2. 3-bromo-6-(piperidin-1-ylsulfonyl)-2H-chromen-2-one (14b)

Yield 46 %; m.p. 221-224 ºC; ^{1}H NMR (DMSO-d_6, δ, ppm): 1.40- 1.60 (6 H, m, H-3, H-4 piperidine), 3.17 (4 H, m, H-2 piperidine), 7.33 (1 H, d, J = 8.7 Hz, H-8 coumarin), 8.25 (1H, dd, J = 1.4, 8.7 Hz, H-7 coumarin), 8.54 (1 H , d, J= 1.4 Hz, H-5 coumarin), 8.61(1 H , s, H-4 coumarin). ^{13}C NMR (DMSO-d_6, δ, ppm): 22.6, 32.5, 50.2, 56.6, 65.9, 120.9, 127.1, 127.2, 127.7, 128.25, 128.33, 128.5, 128.8, 138.8, 140.2, 146.7. Anal. calcd. For $C_{14}H_{14}BrNO_4S$ (374.21): C, 45.17; H, 3.79; N, 3.76, Found: C, 45.13; H, 3.71; N, 3.70.

4.1.9.3. 3-bromo-6-((4-methylpiperazin-1-yl)sulfonyl)-2H-chromen-2-one (14c)

Yield 45%; m.p. 191-192 º C; ^{1}H NMR (DMSO-d_6, δ, ppm): 2.21 (3 H, s, CH_3), 2.48 (4 H, s, H-3 piperazine), 2.97 (4 H, s, H-2 piperazine), 7.67 (1 H, d, J = 8.7 Hz, H-8 coumarin), 7.93 (1H, dd, J = 1.4, 8.7 Hz, H-7 coumarin), 8.19 (1 H , d, J= 1.4 Hz, H-5 coumarin), 8.76 (1 H , s, H-4 coumarin). ^{13}C NMR (DMSO-d_6, δ, ppm): 8.9 (CH_3), 45.8, 53.7, 113.2, 118.1, 120.4, 128.3, 131.1, 131.8, 144.7, 155.7, 156.4. Anal. calcd. For $C_{14}H_{15}BrN_2O_4S$ (387.25): C, 43.42; H, 3.90; N, 7.23, Found: C, 43.44; H, 3.91; N, 7.18.

4.1.9.4. N-(1H-benzo[d]imidazol-2-yl)-3-bromo-2-oxo-2H-chromene-6-sulfonamide (14d)

Yield 54% %; m.p. 273-275 º C; ^{1}H NMR (DMSO-d_6, δ, ppm): 7.10- 7.32 (4 H, m, CH-benzoimidazol), 7.70 (1 H, d, J = 8.7 Hz, H-8 coumarin), 8.12 (1H, dd, J = 1.4, 8.7 Hz, H-7 coumarin), 8.21 (1 H, d, J= 1.4 Hz, H-5 coumarin), 8.31(1 H, s, H-4 coumarin) , 8.55, 8.95 (2H, s, 2NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, δ, ppm): 111.6, 111.8, 116.4, 119.1, 123.5, 125.4, 130.1, 145.2, 145.7, 151.0, 153.1, 157.0. Anal. calcd. For $C_{16}H_{10}BrN_3O_4S$ (420.24): C, 45.73; H, 2.40; N, 10.00, Found: C, 45.70; H, 2.42; N, 10.05

4.1.9.5. N-(benzo[d][1,3]dioxol-5-ylmethyl)-3-bromo-2-oxo-2H-chromene-6-sulfonamide (14e)

Yield 60%; m.p. 173-176 º C; ^{1}H NMR (DMSO-d_6, δ, ppm): 3.95 (2 H , d, J= 6.1 Hz, CH_2NH), 5.91 (2 H, s, CH_2 dioxol), 6.65 (1 H , d, J = 7.9 Hz, H-6 benzodioxol), 6.71 (1 H, s, H-4 benzodioxol), 6.74 (1H, d, J = 7.9 Hz, H-7 benzodioxol), 7.57 (1 H ,d, J= 8.7 Hz, H-8 coumarin), 7.93 (1 H , dd, J= 1.4, 8.7 Hz, H-7 coumarin), 8.09 (1 H , d, J= 1.4 Hz, H-5 coumarin), 8.30 (1 H , t, J = 6.1 Hz, NH), 8.71 (1H , s, H-4 coumarin) ; ^{13}C NMR (DMSO-d_6, δ, ppm): 46.51, 101.34, 108.31, 108.62, 112.80, 117.86, 119.72, 121.62, 127.23, 130.15, 131.41, 137.82, 144.83, 146.72, 147.51, 154.99. Anal. calcd. For $C_{17}H_{12}BrNO_6S$ (438.25): C, 46.59; H, 2.76; N, 3.20, Found: C, 46.54; H, 2.72; N, 3.16

4.1.9.6. 3-bromo-2-oxo-N-(2, 4, 6-trimethoxybenzyl)-2H-chromene-6-sulfonamide (14f)

Yield 37%; m.p. 169-172 ºC; ^{1}H NMR (DMSO-d_6, δ, ppm): 3.52 (3 H , s, *p*-OCH3), 3.67 (6 H , s, 2 o-OCH_3), 4.01 (2 H , d, J = 6.0 Hz, CH_2N), 6.45 (2 H , s, H-3 phenyl), 7.53 (1H d, J = 8.7 Hz, H-8 coumarin), 7.91 (1 H , dd, J= 1.6, 8.7 Hz, H-7 coumarin), 8.10 (1 H , d, J= 1.6 Hz, H-5 coumarin), 8.32 (1 H , t, J= 6.0 Hz, NH, D_2O exchangeable), 8.70 (1H , s, H-4 coumarin) ; ^{13}C NMR (DMSO-d_6, δ, ppm): 47.01, 56.12, 60.23, 105.40, 112.74, 117.71, 119.58, 127.40, 130.29, 132.89, 136.71, 137.89, 144.80, 152.99, 154.87, 156.43. EIMS m/z (%): 485/483 (M^{+1}, 15), 360 (86), 329 (66), 302(93), 288 (25), 224(18), 195 (93), 116(39), 88(100), 61 (65). Anal. calcd. For $C_{19}H_{18}BrNO_7S$ (484.32): C, 47.12; H, 3.75; N, 2.89, Found: C, C, 47.09; H, 3.71; N, 2.83.

4.2. Biology

4.2.1. *In vitro* cytotoxicity assay on MCF-7

Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan.[30,31]

The cells used in cytotoxicity assay were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Cells suspended in the medium (2 x 10^4 cells/mL) were plated in 96-well culture plates and incubated at 37 °C in a 5% CO_2 incubator. After 12 h, the test sample (2 µL) was added to the cells (2 x 10^4) in 96-well plates and cultured at 37 °C for 3 days. The cultured cells were mixed with 20 µL of MTT solution and incubated for 4 h at 37 °C. The supernatant was carefully removed from each well and 100 µL of DMSO were added to each well to dissolve the formazan crystals which were formed by the cellular reduction of MTT. After mixing with a mechanical plate mixer, the absorbance of each well was measured by a microplate reader using a test wavelength of 570 nm.

4.2.2. *In vitro* VEGFR-2 kinase activity assay by ELISA

ELISA (Enzyme Linked Immunosorbent Assay) kit according to manufacturer's instructions (Cell Signaling Technology, Inc., USA) was used. The microtiter plate provided in this kit had been pre-coated with horseradish peroxidase (HRP) labeled secondary antibody (anti-rabbit IgG) specific to VEGFR-2. Samples were added to the appropriate microtiter plate wells. Tetramethyl benzidine (TMB) substrate was added and incubated at room temperature and the stop solution was then added. The color change was measured colorimetrically at a wavelength of 450 nm using a microtiter plate reader. The concentration of test compounds causing 50% inhibition (IC_{50}) was calculated from the concentration inhibition response curve and compared to the reference standard staurosporine.[36]

4.2.3. Cell cycle analysis and apoptosis assay

Cell cycle analysis and apoptosis detection were carried out by flow cytometry as reported.[33] MCF-7 cells were seeded at 8x10^4 and incubated overnight at 37 °C and supplied with 5% CO_2. After 48 h of treatment, cell pellets were collected and centrifuged at 300 *g* for 5 min. For cell cycle analysis, cell pellets were fixed in 70% ethanol on ice for 15 min. The collected pellets were incubated with propidium iodide (PI) staining solution (50 µg/mL PI, 0.1 mg/mL RNaseA and 0.05% Triton X-100) at room temperature for 1 h. Apoptosis detection was performed by FITC Annexin-V/PI kit (Becton Dickenson, Franklin Lakes, NJ, USA) following the manufacture's protocol. The samples were analyzed by fluorescence-activated cell sorting

(FACS) with a Gallios flow cytometer (Beckman Coulter, Brea, CA, USA) within 1 h from the staining. Data were analyzed using Kaluza v1.2 (Beckman Coulter).

4.2.4. Caspase-9 enzyme assay

The activity of caspase-9 was measured using Human CASP9 (Caspase- 9) ELISA Kit (*Catalog# MBS2505226, Invitrogen Corporation, USA)* according to the manufacturer instructions.

4.3. Docking

All the molecular modeling studies were carried out using Molecular Operating Environment (MOE, 10.2008) software. All minimizations were performed with MOE until an RMSD gradient of 0.1 kcal·mol^{-1}Å^{-1} with MMFF94x force field and the partial charges were automatically calculated. The X-ray crystallographic structure of vascular endothelial growth factor receptor 2 (VEGFR-2) co-crystallized with the ureide derivative sorafenib [IC_{50} = 90nM] (PDB ID: 4ASD) was downloaded from the protein data bank.[37] Water molecules and ligands that are not involved in binding were removed. Then, the protein was prepared for docking study using *Protonate 3D* protocol in MOE with default options. The co-crystalized ligand was used to define the binding site for docking. Triangle Matcher placement method and London dG scoring function were used for docking. Docking setup was first validated by re-docking of the co-crystallized ligand (sorafenib) in the vicinity of the binding site of the enzyme with energy score (S) = −15.20 kcal/mol and RMSD of 0.467 Å and by the ability of the docking pose to reproduce all the key interactions accomplished by the co-crystallized ligand with the hot spots in the active site (Glu885, Cys919 and Asp1046). The validated setup was then used in predicting the ligand-receptor interactions at the binding site for the compound of interest.

4.4. *In silico* physicochemical properties and pharmacokinetic parameters assessment

The drug-likeness and ADME properties of the most active compound in this study, were tested *in silico* with the online Swiss ADME web tool.[38] Swiss ADME employs a set of robust models for prediction of physicochemical properties, pharmacokinetics, drug-likeness and medicinal chemistry friendliness.

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Authors declared that there are no actual or potential conflicts of interest, and have approved the article.

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Highlights

- Twenty five new coumarin derivatives featuring a variety of bioactive functional groups were synthesized.
- Fifteen of the newly synthesized derivatives exhibited exceptional *in vitro* cytotoxicity against MCF-7.
- The hydrazide coumarin derivative **4a**, as the most cytotoxic compound, showed significant anti-VEGFR-2 activity, induced apoptosis and activated caspase-9.
- Molecular modeling of **4a**, showed the ability of the derivative to interact with the key amino acids in VEGFR-2 binding site.
- Compound **4a** exhibited very good ADME properties and drug-like characteristics without violating Lipinski's rule of five.

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