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Design, synthesis and biological evaluation of novel 3-substituted 4-anilino-coumarin derivatives as antitumor agents

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Abstract

Various 3-substituted 4-anilino-coumarin derivatives have been designed, synthesized and their anti-proliferative properties have been studied. The *in vitro* cytotoxicity screening was performed against MCF-7, HepG2, HCT116 and Panc-1 cancer cell lines by MTT assay. Most of the synthesized compounds exhibited comparable anti-proliferative activity to the positive control 5-Fluorouracil against these four tested cancer cell lines. Among the different substituents at C-3 position of coumarin scaffold, 3-trifluoroacetyl group showed the most promising results. Especially, compounds **33d** (IC_{50}=16.57, 5.45, 4.42 and 5.16 μM) and **33e** (IC_{50}=20.14, 6.71, 4.62 and 5.62 μM) showed excellent anti-proliferative activities on MCF-7, HepG2, HCT116 and Panc-1 cell lines respectively. In addition, cell cycle analysis and apoptosis activation revealed that **33d** induced G2/M phase arrest and apoptosis in MCF-7 cells in a dose-dependent manner. Low toxicity of compounds **33d** and **33e** was observed against human umbilical vein endothelial cells (HUVECs), suggesting their acceptable safety profiles in normal cells. Furthermore, the results of *in silico* ADME studies indicated that both **33d** and **33e** exhibited good pharmacokinetic properties.

Keywords: coumarin derivatives; synthesis; antitumor; apoptosis; selectivity index.

Cancer is a worldwide disease that affects around fourteen million people every year. The American Cancer Society estimated that 1,685,210 new cancer cases and 595,690 cancer deaths will occur in the United States in 2016.[1] Most of cancers are known as uncontrolled growth of abnormal cells due to the dysfunction of essential enzymes and other proteins controlling cell division and proliferation.[2, 3] Although several therapeutic regimes have been successfully approached for fighting cancer nowadays, cancer is still the second leading cause of deaths worldwide.[4] Until now, chemotherapy remains the principal strategy for the treatment of cancer diseases.[5] Accordingly, the discovery of new anticancer agents with promising bioactivity and high therapeutic index is still an urgent need.

Natural products and its metabolites are important sources of novel antitumor agents. Finding lead compounds from natural products is an effective way to develop novel antitumor agents. This is due to the natural products' intrinsic and well defined biological activity, which can be potentiated or modulated by structural optimization.

Coumarins, the most important classes of benzopyrones, are a wide group of natural compounds. They are thought to be a significant source of inspiration for new anticancer

agents.[6] They exhibit a variety of biological activities including anticancer,[7-9] anti-HIV,[10] anti-microbial,[11] anti-inflammatory activity[12] and so on. The antitumor effects, which are related to the inhibition of the cellular proliferation, through a variety of mechanisms, were extensively examined.[13-16] Due to their potential applications in cancer therapy, a lot of efforts have been performed aiming at designing properly functionalized synthetic coumarin derivatives with improved anticancer activity.[17, 18] Among them, coupling coumarin with different bioactive molecules is one of the effective ways. Adopting this approach, some 4-substituted coumarins have exhibited significant antitumor activities. For example, 4-aryl coumarin derivative (**1**, Fig. 1), was reported to show potent inhibition of tubulin polymerization activity and cytotoxicity against HBL100 Cells with IC_{50} value of 88 nM.[19] Stilbene-coumarin hybrid (**2**, Fig. 1), initially identified by Federica Belluti as a proapoptotic agent, exerted an anti-proliferative effect in H460 cells by inducing apoptosis.[20] Triphenylethylene-coumarin hybrid (**3**, Fig. 1) containing two amino side chains showed a broad-spectrum and excellent antitumor activity by acting on DNA.[21] (1, 2, 3-triazol)-coumarin hybrid (**4**, Fig. 1) with IC_{50} value of 0.52 μM against A549 cells induces G2/M-phase cell cycle arrest.[22]

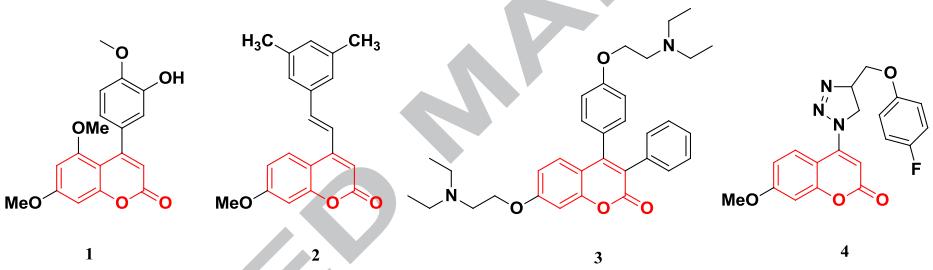


Figure 1. Structures of known coumarin derivatives

Similarly, considerable attention has been also focused on aniline moieties which have been widely used as functional groups in medicinal chemistry as exemplified in Figure 2. 4-anilino-2-phenylquinazoline (**5**, Fig. 2) was reported by Michael Wiese and coworkers as a selective inhibitor of Breast Cancer Resistance Protein ABCG2.[23] 4-biarylamino-quinazoline (**6**, Fig. 2) was found to inhibit kinase activities of EGFR, FGFR-1 and PDGFRβ at submicromolar concentrations and also showed EC_{50} values against several cancer cell lines in nanomolar range.[24] Additionally, Kuo-Hsiung Lee et al. reported 4-(3-methylphenylamino)-2H-benzo[h]chromen-2-one (**7**, Fig. 2) endowed with potent cytotoxic effect with ED_{50} value of 0.14 μM against A549 cells, which was a coumarin-containing compound.[25] So far few 4-anilino-coumarins have been investigated as apoptosis inducers. In 2016, Lijuan Chen and his coworkers reported compound **8** and its analogues, which showed potent and broad-spectrum *in vitro* and *in vivo* cytotoxic activities.[26] Therefore, more efforts are expected to discover novel 4-anilino-coumarin derivatives possessing improved activities.

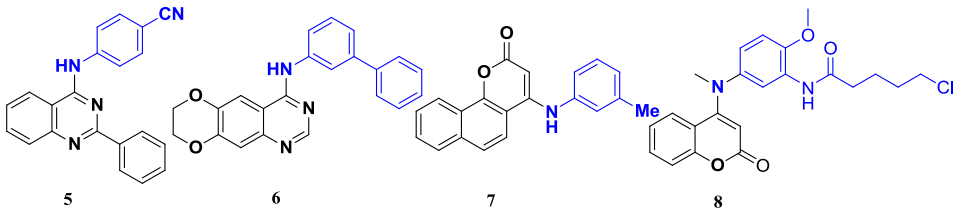


Figure 2. Structures of known compounds bearing aniline moieties

Inspired by the promising research results mentioned above, we set out to design and synthesize a number of novel heterocyclic compounds bearing coumarin scaffold and aniline moieties (Fig. 3). In order to extend investigation to the coumarin derivatives, aromatic groups such as 4-methoxyphenyl and aliphatic groups such as trifluoroacetyl were introduced to the C-3 position of coumarin nucleus (Fig. 3). Furthermore, the effect of different substituent groups (H, OMe or OH) at ring A on proliferative activity was also evaluated. Herein, we described in this paper the synthesis of twenty-seven compounds of 4-anilino-coumarins with 3-(4-methoxyphenyl) group and nine compounds with 3-trifluoroacetylgroup. Cytotoxic activity *in vitro* was examined against MCF-7 (breast carcinoma), HepG2 (liver carcinoma), HCT116 (colon cancer), Panc-1 (pancreatic carcinoma) and HUVEC (human umbilical vein endothelial cells). The effect of the most active compound **33d** on triggering cell death events was also assessed by flow cytometry assays. *In silico* ADME analysis was also performed to predict important pharmacokinetic parameters of **33d** and **33e**.

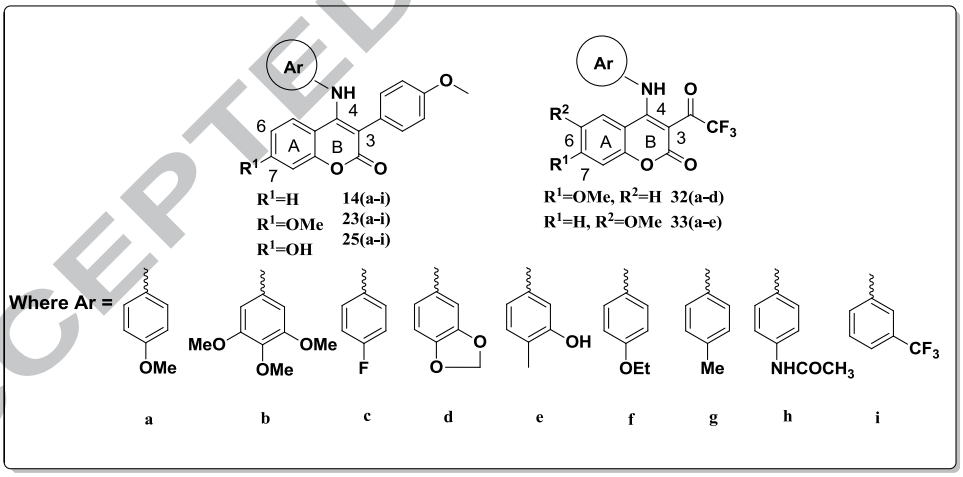
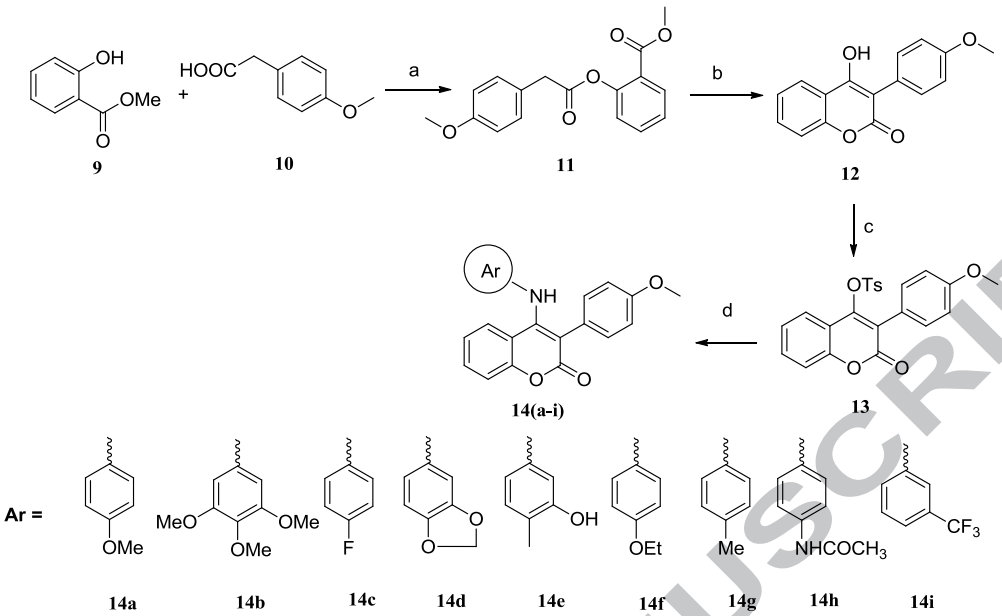


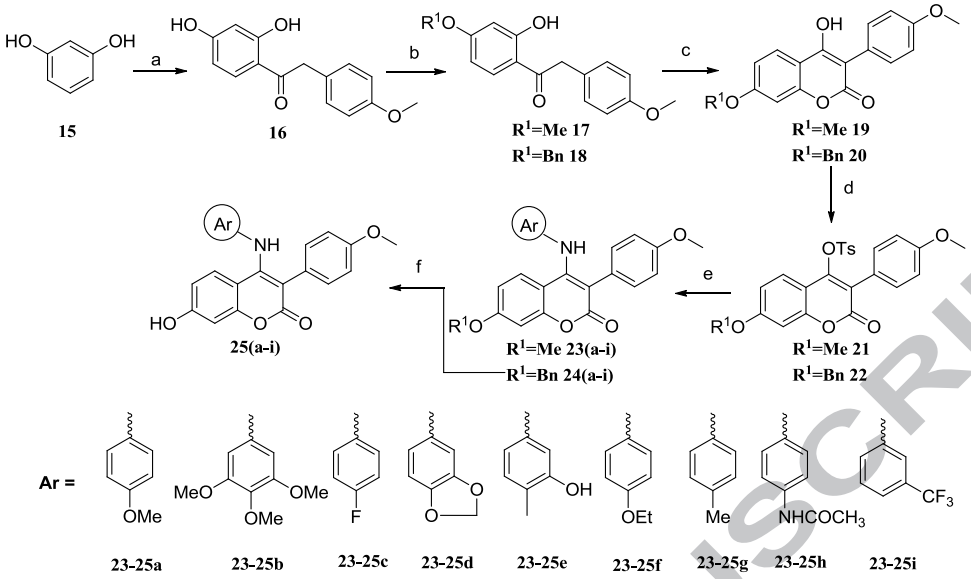
Figure 3. Structures of proposed 3-substitued 4-anilino-coumarin derivatives

The synthetic routes of compounds **14(a-i)** were shown in Scheme 1. Intermediate **11** was easily obtained from methyl salicylate **9** and 4-methoxyphenylacetic acid **10** through the synthetic strategy depicted in literature.[27] The 3-(4-methoxyphenyl)-4-hydroxy coumarin **12** was prepared from intermediate **11** via intramolecular Claisen condensation.[28] Further treatments with TsCl in the presence of TEA afforded the 3-(4-methoxyphenyl)-4-tosyloxy coumarin **13**. The target compounds **14(a-i)** were generated by nucleophilic substitution of **13** with nine kinds of anilines.



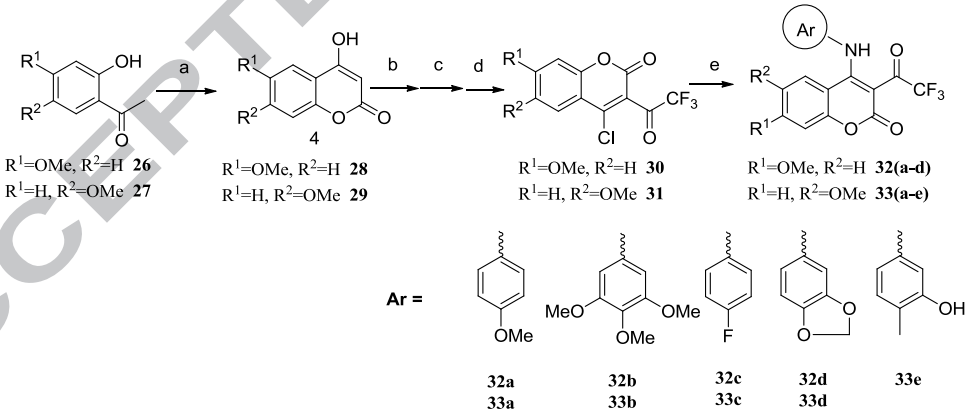
Scheme 1. Reagents and conditions: (a) $POCl_3$, pyridine, 0-10 °C, 3 h; (b) KOH, pyridine, rt, 6 h; (c) TsCl, Et_3N, dry DCM, rt, 0.5 h; (d) aromatic amines, K_2CO_3, EtOH, 75 ^oC, 2 h.

The synthesis of the target compounds **23(a-i)** and compounds **25(a-i)** were achieved by employing the corresponding 4-methoxy deoxybenzoin **17** and 4-benzyloxy deoxybenzoin **18** as key intermediates (Scheme 2) which were prepared from commercially available resorcinol and phenylacetic acids as starting materials. Briefly, resorcinol reacted with 4-hydroxybenzoic acid to yield benzophenone **16** under Fries reaction conditions. Compound **16** was then converted to the corresponding 4-substituted deoxybenzoin **17 or 18** through selective alkylation of one hydroxyl with methanol or benzyl alcohol via Mitsunobu reaction in 88% and 85% yield respectively. Further treatment of **17** and **18** with diethyl carbonate (DEC)/NaH in reflux toluene gave compound **19** and **20**, which were then converted to the corresponding compounds **21** and **22** by reacting with TsCl and TEA in DCM. Similarly, the target compounds **23(a-i)** and intermediates **24(a-i)** were prepared by nucleophilic substitution with nine kinds of anilines. Finally, the final compounds **25(a-i)** were achieved via Bn-deprotection with Pd-C in THF under the condition of H_2.



Scheme 2. Reagents and conditions: (a) BF_3-OEt_2, 80 °C, 3 h; (b) MeOH for 17 or BnOH for 18, PPh_3, DIAD, THF, 0 °C-rt, 0.5 h; (c) NaH, DEC, 120 °C, 3 h; (d) TsCl, Et_3N, dry DCM, 0.5 h; (e) aromatic amines, K_2CO_3, EtOH, 75 °C, 2 h; (f) Pd-C, H_2, THF, rt, 12 h.

The derivatives **32(a-d)** and **33(a-e)** were synthesized following the general procedures as detailed in Scheme 3. Compounds **28** and **29** were respectively furnished by the cyclization of **26** and **27** with diethyl carbonate (DEC) and NaH in reflux toluene. According to the reported procedure, [29] the following compound **30** and **31** were generated by a one-pot procedure containing three steps. The final compounds **32(a-d)** and **33(a-e)** were synthesized by the nucleophilic substitution with corresponding aniline groups in DMF.



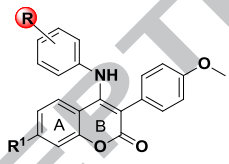
Scheme 3. Reagents and conditions: (a) NaH, DEC, 120 °C, 3 h; (b) TMSiCl, dry pyridine, dry 1,4-dioxane, rt, 1 h; (c) $(CF_3CO)_2O$, 80-90 °C, 2 h; (d) $POCl_3$, 60 °C, 2 h; (e) aromatic amines, K_2CO_3, DMF, 75 °C, 2 h.

In vitro cytotoxic activity of all the synthesized 3-substituted 4-anilino-coumarin derivatives **14(a-i)**, **23(a-i)**, **25(a-i)**, **32(a-d)** and **33(a-e)** in this paper was evaluated against four human cancer cell lines (MCF-7, HepG2, HCT116 and Panc-1) using 5-Fluorouracil (5-Fu) as the reference drug.[30] The biological results of these synthesized compounds were summarized in Table 1. From the results, it is evident that most of the tested compounds

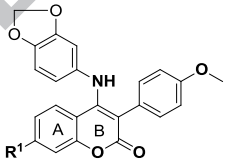
displayed moderate anti-proliferation activities with IC_{50} values less than 100 μM. Among them, fourteen compounds exhibited similar or better bioactivities *in vitro* compared to 5-Fu. On the other hand, compounds **14(a-i)**, **23(a-i)** and **25(a-i)** with 4-methoxylphenyl at C-3 and hydroxyl at C-7 position were found to be inactive except **25f**, which suggested that hydroxyl group may not be favored at C-7 position. Among those compounds with R^1 substituted by H or OMe, compounds **14e** and **23e**, comprising of hydroxyl at meta-position and methyl at para-position of substituted anilines, displayed significant cytotoxicity with IC_{50} value ranging from 9.75 to 12.34 μM and from 22.52 to 34.39 μM respectively against MCF-7, HepG2, HCT-116 and Panc-1 cell lines. An enhanced effect was observed by the replacement of 4-methoxyphenyl with trifluoroacetyl at C-3 position of coumarin (compare **23c** and **23d** with **32c** and **33d**). The compounds bearing trifluoroacetyl at C-3 position such as compounds (**32b**, **32c**, **32d**, **33b**, **33d and 33e**), showed significant anti-cancer activities with IC_{50} value of 9.74 μM for compound **32c** against MCF-7, 5.45 μM for compound **33d** against HepG2, 4.42 μM for compound **33d** against HCT116 and 5.16 μM for compound **33d** against Panc-1 respectively. The analysis of the structures of analogues **32(a-d)** and **33(a-i)** revealed that the intramolecular hydrogen bond between NH group and C=O group plays an important role in cytotoxic activity. According to previous reports,[31-33] the "endo enamine-ketone" conjugated coumarin ring might serve as a bioisosteric replacement for the olefin functionality of the cis-stilbene combretastatin structure. Due to the structural similarity with combretastatin analogues, the promising antiproliferative activities of **33d** and **33e** might be achieved through tubulin inhibition.

Table 1

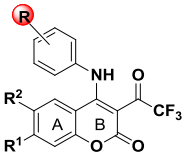
In vitro cytotoxic activities of synthesized compounds **14(a-i)**, **23(a-i)**, **25(a-i)**, **32(a-d)** and **33(a-e)**



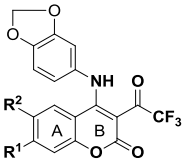
14(a-c), 14(e-i)
23(a-c), 23(e-i), 25(a-c), 25(e-i)



14d, 23d, 25d



32(a-c), 33(a-c), 33e



32d, 33d

Compound	R^1	R^2	R	Cell lines (IC_{50}, μM) [a, b]			
				MCF-7	HepG2	HCT116	Panc-1
14a	H	-	4-OMe	34.58	35.19	36.61	37.45
14b	H	-	3, 4, 5-OMe	82.34	77.26	63.54	77.59
14c	H	-	4-F	38.45	35.52	39.44	32.02
14d	H	-	-	>100	>100	N.T. [c]	N.T.
14e	H	-	3-OH, 4-Me	**12.08**	**10.16**	**12.34**	**9.75**
14f	H	-	4-OEt	**30.66**	**31.32**	**29.49**	**32.85**
14g	H	-	4-Me	65.31	59.41	64.79	66.07
14h	H	-	4-$NHCOCH_3$	>100	>100	N.T.	N.T.
14i	H	-	3-CF_3	21.62	66.43	55.73	49.08
23a	OCH_3	-	4-OMe	39.25	39.56	35.15	41.02
23b	OCH_3	-	3, 4, 5-OMe	89.45	84.26	N.T.	N.T.
23c	OCH_3	-	4-F	>100	>100	N.T.	N.T.

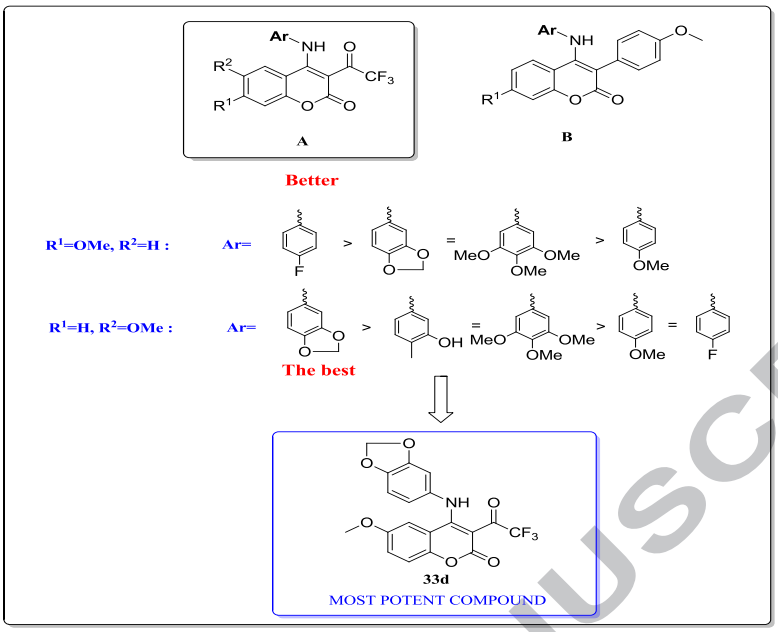
23d	OCH_3	-	-	>100	>100	N.T.	N.T.
23e	OCH_3	-	3-OH, 4-Me	**34.39**	**22.52**	**26.48**	**31.29**
23f	OCH_3	-	4-OEt	**24.31**	**31.22**	**33.03**	**36.07**
23g	OCH_3	-	4-Me	66.88	74.36	82.44	76.53
23h	OCH_3	-	4-$NHCOCH_3$	85.75	>100	N.T.	N.T.
23i	OCH_3	-	3-CF_3	36.22	57.25	41.16	39.52
25a	OH	-	4-OMe	>100	78.76	N.T.	N.T.
25b	OH	-	3, 4, 5-OMe	55.98	49.65	64.24	63.91
25c	OH	-	4-F	71.46	65.63	66.12	75.05
25d	OH	-	-	89.55	79.02	41.21	48.25
25e	OH	-	3-OH, 4-Me	>100	>75.32	N.T.	N.T.
25f	OH	-	4-OEt	32.46	36.52	46.10	43.62
25g	OH	-	4-Me	85.47	88.34	N.T.	N.T.
25h	OH	-	4-$NHCOCH_3$	>100	>100	N.T.	N.T.
25i	OH	-	3-CF_3	49.09	53.56	55.88	67.21
32a	OCH_3	H	4-OMe	55.73	38.38	61.24	47.49
32b	OCH_3	H	3, 4, 5-OMe	**19.21**	**26.43**	**16.98**	**24.11**
32c	OCH_3	H	4-F	**9.74**	**8.26**	**11.74**	**12.23**
32d	OCH_3	H	-	37.40	10.45	12.49	15.76
33a	H	OCH_3	4-OMe	>100	>100	N.T.	N.T.
33b	H	OCH_3	3, 4, 5-OMe	15.04	12.28	19.46	23.83
33c	H	OCH_3	4-F	>100	>100	N.T.	N.T.
33d	H	OCH_3	-	**16.57**	**5.45**	**4.42**	**5.16**
33e	H	OCH_3	3-OH, 4-Me	**20.14**	**6.71**	**4.62**	**5.62**
5-Fu	-	-	-	26.14	49.02	38.30	47.83

[a] Cytotoxicity as IC_{50} values for each cell line, the concentration of compound that inhibit 50% of the cell growth after 48 h of drug exposure measured by MTT assay.

[b] Each value was reproduced in triplicate.

[c] Not test.

The results indicated that the designed 3-substitued 4-anilino-coumarin derivatives exhibited potent anticancer activities. Based on the preliminary cytotoxicity results, we could discuss a clear tendency of structure–activity relationship: (i) for C-3 position substituted coumarin derivatives, placement of a trifluoroacetyl in place of the 4-methoxyphenyl remarkably enhanced the cytotoxic potential; (ii) the coumarin analogs bearing trifluoroacetyl at C-3 position and methoxy at C-7 position such as **32c** with a 4-fluoroanilino group showed much higher activity than the 4-methoxyanilino, 3, 4, 5-trimethoxyanilino and 3, 4-methylenedioxyanilino derivatives (**32a**, **32b** and **32d**); (iii) concerning analogs bearing trifluoroacetyl at C-3 position and methoxy at C-6 position of coumarin, **33d** with a 3, 4-methylenedioxyanilino group showed much higher activity than the 4-methoxyanilino, 3, 4, 5-trimethoxyanilino, 4-fluoroanilino and 3-hydroxy-4-methylanilino derivatives (**33a**, **33b**, **33c** and **33e**). Especially, among all the designed compounds, **33d** was found to be the most potent compound against 4 human tumor cell lines. The structure–activity relationship (SAR) results were summarized in Scheme 4.



Scheme 4. Structure–activity relationship of designed compounds

Many cytotoxic compounds exerted their anti-proliferative effect by either arresting the cell cycle at a particular checkpoint of cell cycle or induction of apoptosis, or by a combined effect.[34] Regulation of the cell cycle and apoptosis are considered to be effective cancer therapeutic methods. Now that compound **33d** showed the highest anti-proliferative activity among all the synthesized compounds, it was selected to assess the effect on cell-cycle arrest of MCF-7 by flow cytometry assay using propidium iodide (PI) staining. As is seen from Figure 4, compound **33d** concentration-dependently arrested cell cycle at G2/M phase, accompanied with decrease of cells at G0/G1 phase. Specifically, the percentage of cells at G2/M phase for the high concentration group (15 μM) was 63.06±2.24%, about 39% higher than that of the control group (***P < 0.001).

In order to identify whether **33d** induced cell death through apoptosis, a morphological observation study was used to evaluate its influence on cell skeleton and Hoechst 33258 staining was taken to evaluate nuclei condensation. Under the inverted light microscope (x200), incubation of 5 μM, 10 μM and 15 μM of **33d** for 24 h resulted in phenotypic changes of MCF-7 cells, such as distortion, membrane blebbing, shrinkage and rounding up (arrow pointing), while cells in untreated group grew well (Figure 5).These results were also confirmed on MCF-7 cells using Annexin V-PI staining by flow cytometry. It was observed that **33d** induced apoptosis against MCF-7 cells in a dose-dependent manner as shown in Figure 6. Results indicated that there was increased apoptotic activity in MCF-7 cells treated with **33d** at increasing concentrations (5, 10 and 15 μM) for 48 h. Specifically, when treated with **33d** at high concentration (15 μM), around 32.1% of early apoptosis rate were observed.

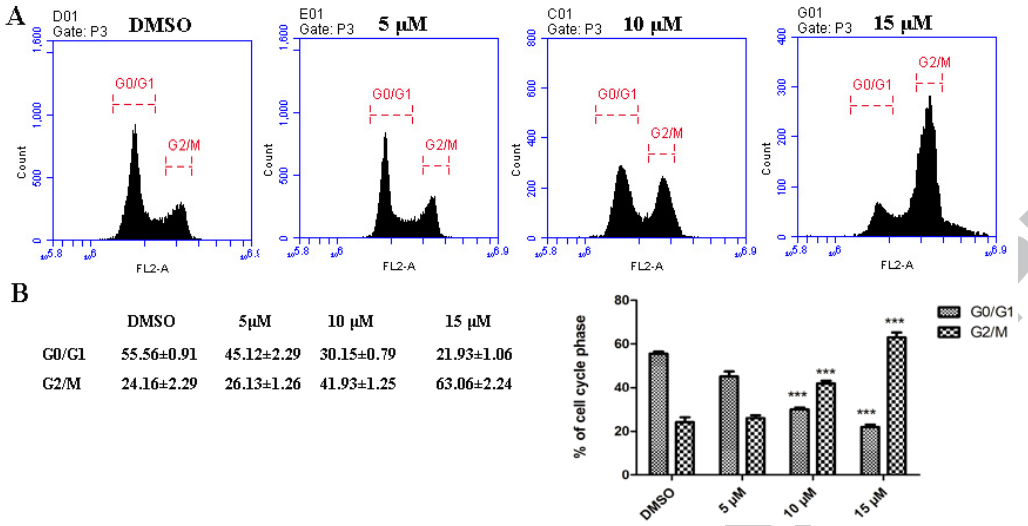


Figure 4. (A) Cell cycle analysis of MCF-7 cells treatment of compound **33d** (5, 10, 15 µM) and no treatment (DMSO) as reference control for 48 h. (B) Histogram provides percentage of cells in G0/G1 and G2/M phases of the cycle calculated by flow cytometer. Results are expressed as mean ± SEM of three independent experiments. ***P < 0.001 indicates significant statistical difference from untreated control cells.

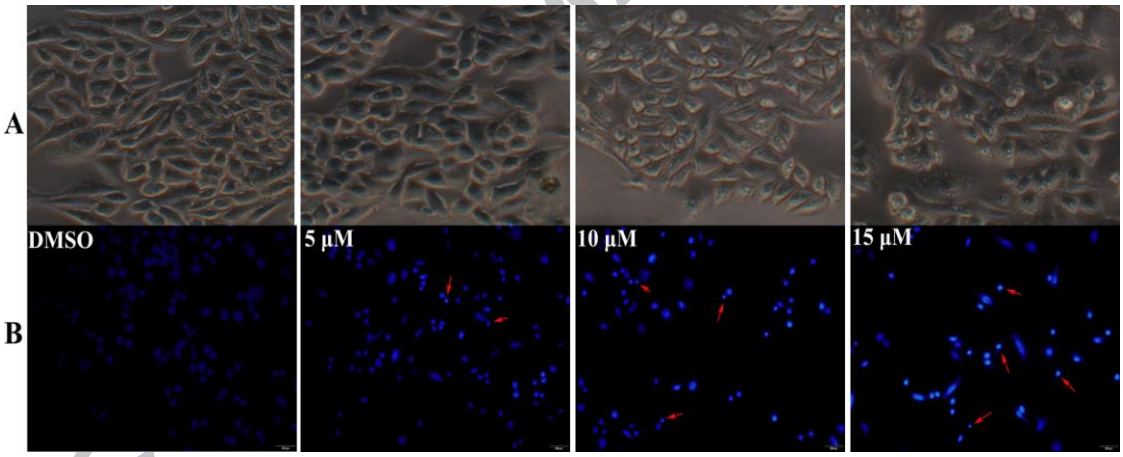


Figure 5. Morphological changes (A) and nuclear degradation (B) of MCF-7 cells induced by **33d**. The arrow pointed to apoptotic bodies.

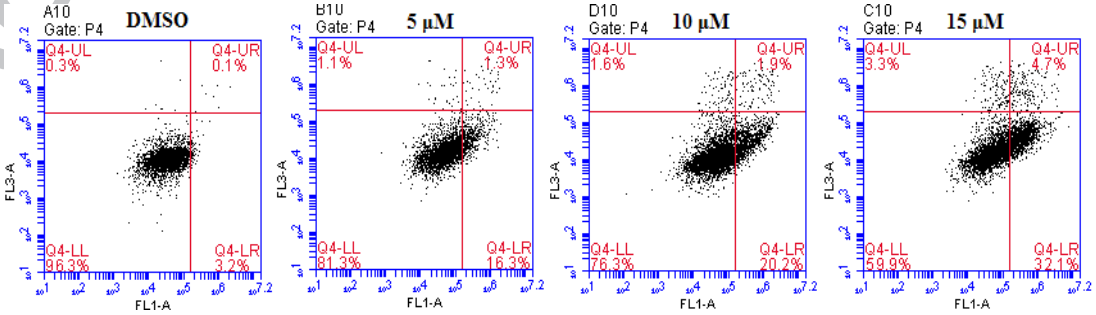


Figure 6. Flow cytometric apoptosis staining analysis of MCF-7 cells after 48 h treatment with **33d** (5, 10, 15 µM) and no treatment (DMSO): viable (lower left), early apoptotic (lower right), late apoptotic (upper right), and necrotic cells (upper left).

One of the major hindrances for druggability of compounds with effective antitumor activity is their toxicity to normal cells.[35] Thus, it is necessary to evaluate cytotoxicity on normal cells in the anticancer drug study. The promising compounds **33d** and **33e** were chosen for selectivity test on normal human umbilical vein endothelial cells (HUVEC) using the MTT assay. The selectivity indexes (SI) were calculated through IC_{50} values in cancer cells divided by IC_{50} values in normal cells. The results revealed that the two tested compounds were less sensitive to normal cells over cancer cells with SI > 4 (Table 2). The low toxicity and selectivity warrant their further development for identifying more potent anticancer agents.

Table 2

In vitro antiproliferative activities of compounds **33d** and **33e** against normal cell line (HUVEC)

Compound	Cell line (IC_{50}, μM) [a, b]	Selectivity index [c]			
	HUVEC	MCF-7	HepG2	HCT116	Panc-1
33d	26.27	1.59	4.82	5.94	5.09
33e	29.43	1.46	4.39	6.37	5.23

[a] Antiproliferative effect (IC_{50}) of HUVEC cell line, the concentration of compound that inhibit 50% of the cell growth after 48 h of drug exposure measured by MTT assay.

[b] Each value was reproduced in triplicate.

[c] Selectivity index representing IC_{50} for normal cell line/IC_{50} for cancerous cell lines.

Employing Schrödinger software, compounds **33d** and **33e** were subjected to the analyses of several physiochemical properties (ADME) related to pharmacokinetics[36]. Both the compounds follow Lipinski's rule of five for good bioavailability. With the calculated LogP values less than 5, these two compounds have good hydrophilicity with moderate lipophilicity and hence should be able to gain access to membrane surfaces. The aqueous solubility (LogS) of a compound is a major driving force that leads to good absorption and distribution characteristics. The calculated LogS values of **33d** and **33e** were within the acceptable range. Other calculations related to solubility, serum protein binding, blood–brain barrier (LogBB and apparent MDCK cell permeability), gut–blood barrier (Caco-2 cell permeability), number of likely metabolic reactions, skin permeability (Kp), and human oral absorption in the gastrointestinal tract showed these values for both **33d** and **33e** within the standard ranges for good bioavailable drugs (Table 3).

Table 3

Various physicochemical (ADME) parameters calculated for **33d** and **33e**

Parameter	Compound			Stand. range*
	33d	33e	5-Fu	
LogS for aqueous solubility	-2.832	-4.244	-1.073	(-6.5/0.5)
LogP	2.563	3.056	-0.885	(-2.0/6.5)
LogK hsa for serum protein binding	-0.402	0.115	-0.739	(-1.5/1.5)
LogBB for brain/ blood	-0.191	-0.642	-0.955	(-3.0/1.2)
No. of metabolic reactions	3	4	0	(1.0/8.0)

Apparent Caco-2 permeability (nm/s)	1584	691	120	(<25 Poor, >500 great)
Apparent MDCK permeability (nm/s)	2314	937	87	(<25 Poor, >500 great)
LogKp for skin permeability	-1.976	-2.629	-5.053	(-8.0 to -1.0, Kp in cm/h)
% Human oral absorption in GI (±20%)	100	96	59	(<25% is poor)
Qual. Model for human oral absorption	HIGH	HIGH	Medium	(>80% is high)

* Note: for 95% of known drugs based on Schrödinger, QikProp v4.4 (2015) software results.

In summary, a series of 3-substitued 4-anilino-coumarin derivatives were synthesized, characterized and evaluated for their *in vitro* cytotoxic effect on MCF-7, HepG2, HCT116 and Panc-1 cell lines. Some compounds exhibited better antiproliferative activities against the tested cells than positive control (5-Fluorouracil). Among them, compound **33d** bearing trifluoroacetyl at C-3 position and 3, 4-methylenedioxyphenylamino at C-4 position of coumarin scaffold showed broad-spectrum and excellent anti-proliferative activities with IC_{50} in low 4 μM range. The further mechanism study demonstrated that compound **33d** could obviously induce cell cycle arrest and apoptosis in MCF-7 breast carcinoma cells. Moreover, compounds **33d** and **33e** exhibited low toxicity against HUVECs, suggesting their acceptable safety profiles in normal cells. *In silico* studies showed that both **33d** and **33e** possessed good physiochemical properties (ADME) related to pharmacokinetics. Therefore, these two compounds could serve as promising lead candidates for further study.

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Supplementary data

Supplementary data (experimental procedures and characterization data) associated with this article can be found, in the online version, at xxx.

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