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Article

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Design, Synthesis, and Evaluation of *In Vitro* and *In Vivo* Anticancer Activity of 4-substituted Coumarins: A Novel Class of Potent Tubulin Polymerization Inhibitors

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Abstract

In this paper, a series of novel 4-substituted coumarin derivatives were synthesized. Among these compounds **34**, **39**, **40**, **43**, **62**, **65** and **67** exhibited significant antiproliferative activity toward a panel of tumor cell lines at subnanomolar IC_{50} values. Compound **65** showed potent antiproliferative ability (IC_{50} values = 7−47 nM) and retained full activity in multidrug resistant cancer cells. Compound **65** caused G2/M phase arrest and interacted with the colchicine-binding site in tubulin, as confirmed by immune-fluorescence staining, microtubule dynamics assays and competition assays with *N,N′*-ethylene-bis(iodoacetamide). Compound **65** reduced the cell migration and disrupted capillary like tube formation in HUVEC cells. Importantly, compound **65** significantly and dose-dependently reduced tumor growth in four xenografts models including paclitaxel sensitive and resistant ovarian tumors (A2780s and A2780/T), adrmicycin sensitive and resistant breast tumors (MCF-7 and MCF-7/ADR), suggesting that compound **65** is a promising novel antimitotic compound for the potential treatment of cancer.

Introduction

Microtubules (MTs) are mainly composed of *α, β*-tubulin heterodimers.[1-5] Microtubule-targeting agents can be classified into two classes: microtubule stabilizing agents (MSAs) and microtubule destabilizing agents (MDAs).[6-8] MSAs promote MT assembly by preventing microtubule degradation such as taxanes and epothilones. MDAs block MT assembly by inducing microtubule destabilization such as vinca alkaloids and colchicines. [9, 10]

Colchicine (**1**)[11] and its analogues are known as targeting colchicine-binding site agents. This binding site is found primarily on the *β*-tubulin subunit, at its interface with *α*-tubulin. In contrast to the taxane or vinca alkaloid binding sites, targeting the colchicine binding site may provide a promise opportunity for overcoming the ABC-transporter mediated drug-resistance.[12-16] Recently, several agents targeting the colchicine binding site have been reported and most of them have high potency, selective toxicity toward tumor vasculature, and show promising ability to overcome Pglycoprotein (P-gp) efflux pump mediated multidrug resistance.[17, 18] CA4P [19-21] (**3**), a water-soluble phosphate prodrug of combretastatin A-4 [21, 22] (**2**), has been approved as orphan drug for the treatment of anaplastic thyroid cancer by both the FDA and European Medicines Agency. The related amino acid amide of **2**, named AVE-8062 [22] (**4**), has been investigated in phase III for the treatment of advanced soft-tissue sarcomas. MPC-6827 [23-26] (**5**) and its analogues (**6**, **7**) show potent and broad-spectrum *in vitro* and *in vivo* cytotoxic activities. Compound **5** has reached phase II for the treatment of recurrent glioblastoma.[26] These work urged us for the

prompt development of novel microtubule agents targeting the colchicine-binding site for cancer treatment. (Figure 1)

Coumarin[27-29] has been reported to exhibit a wide range of pharmacological activities includes antibacterial,[30, 31] anticoagulant,[32] and anticancer activity,[33] et al. The promising biological profile and easy synthetic modification have stimulated the design and development of coumarin-based derivatives or analogues as potential antitumor agents. Recently, a lot of studies have systematically investigated the anticancer potential of derivatives or analogues of coumarin based on the mechanism of action and SAR studies.[34-38] Compound **8** [35], a 4-arylcoumarin analogue of **2**, was reported as tubulin inhibitor by binding to the colchicine site. 7-Diethylamino-3(2'-benzoxazolyl)-coumarin (**9**)[36] was also reported as a novel microtubule inhibitor with antimitotic activity in multidrug resistant cancer cells. (Figure 1)

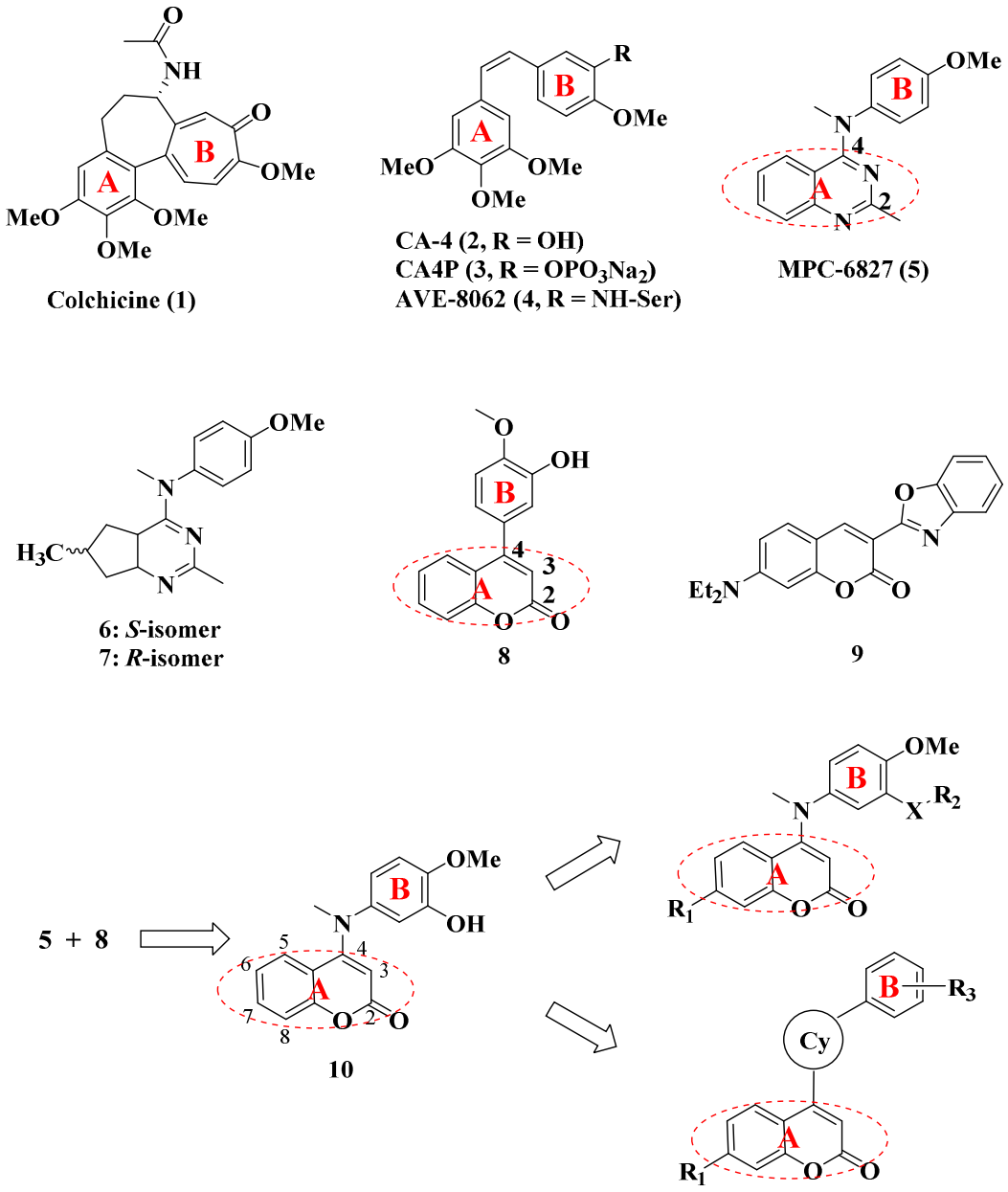


Figure 1. Some reported microtubule-targeting agents. Hybrid 4-anilinocoumarin general structure **10** derived from a combination of 4-anilinoquinazoline (**5**) and 4-arylcoumarin (**8**) templates.

In our process research of coumarin derivatives as tubulin inhibitors, we found that the coumarin shared similar basic skeleton to **5**, in which 2-methyl-quinazoline (A-

ring) of compound **5** could be replaced by coumarin ring. After molecular docking analysis (Figure S1, S2 and S3 in Support Information), we assumed that it is possible to combine the hybrid fragments of **5** and **8** and design a series of novel tubulin inhibitors by binding to colchicine site. In our design, coumarin represented as A-ring and 4-methoxy- phenyl group represented as B-ring. The 2-carbonyl of the A-ring of compound **10** is just swing to the position of the 2-methyl group of the A-ring in **5**. Further modification will focus on the substituents of B-ring and linkers between A-ring and B-ring to obtain diverse derivatives (Figure 1).

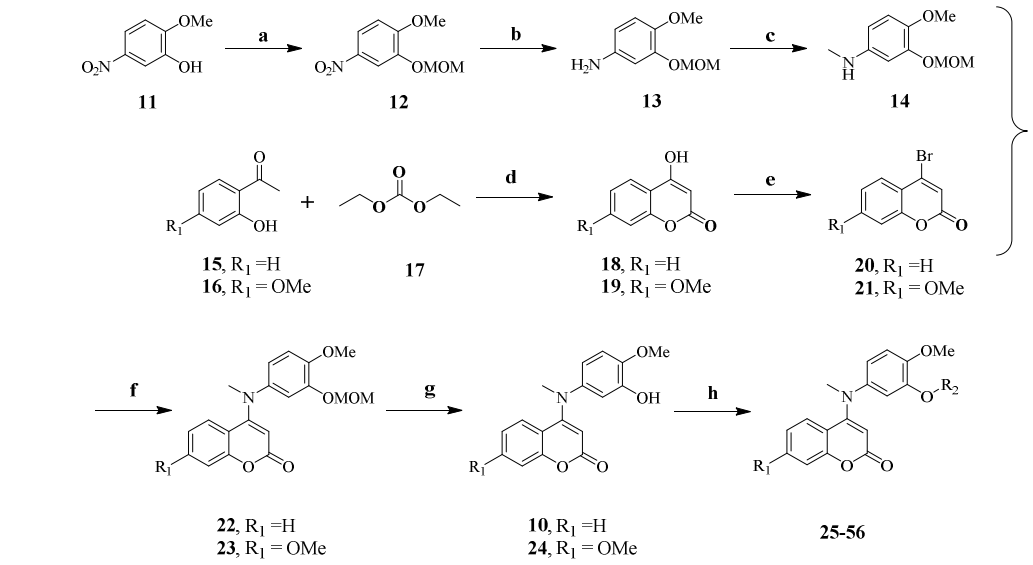
RESULTS AND DISCUSSION

Chemistry

To accomplish our task, we first began to synthesize compound **10** to confirm our assumption and then further modified the 3-hydroxyl group on B-ring to obtain diverse derivatives **25-70**.[39] To verify whether the linker group of *N*-methyl linker was the critical choice, we replaced the *N*-methyl with a five-membered heterocyclic which mimic the derivatives of **2**.[40] Compounds **77-89** were obtained by inserting a thiophene group between the basic skeleton coumarin and B-ring.

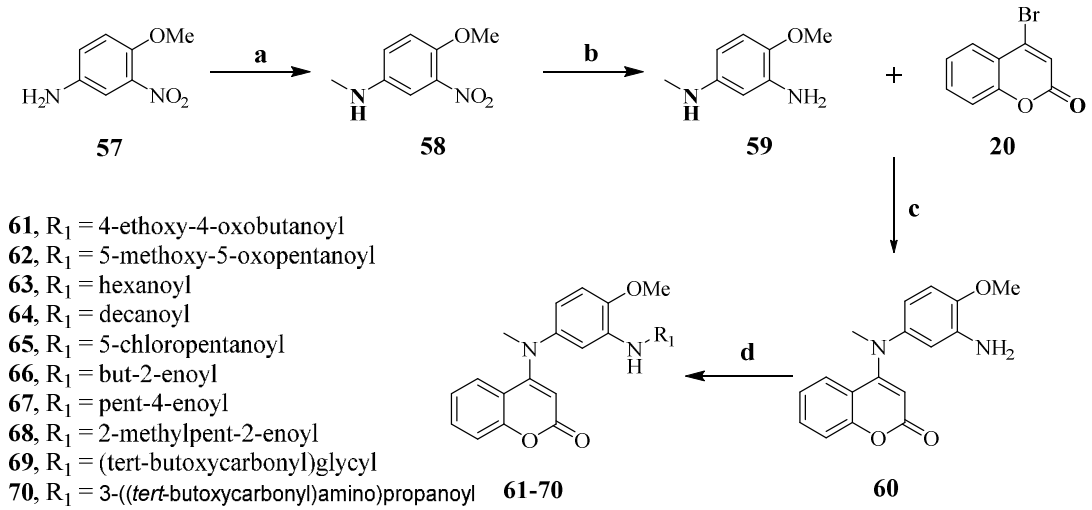
The synthetic route to compounds **10**, **24**−**56** is shown in Scheme 1.[39] Commercially available 2-methoxy-5-nitrophenol (**11**) was the starting material for the synthesis. The phenolic hydroxyl group was first protected by chloromethyl methylether (MOMCl) to give **12**. Then reduction of **12** led to **13**, which was further *N*-methylated by reacting with paraformaldehyde in MeONa−MeOH solution and reduction by $NaBH_4$ sequentially to give **14**. The cyclization of the corresponding

2-hydroxy acetophenones (**15**-**16**) with diethylcarbonate (**17**) to prepare 4-hydroxy-coumarins (**18**-**19**), and brominated with Bu_4NBr. The resulting compounds **20**-**21** were coupled with aniline **14**, giving the intermediate compounds **22**-**23**, which further removed hydroxyl-protected group to give **10** and **24**. Then we used the appropriate various acyl chlorides, carboxylic acids or halides to react with **10** to obtain compounds **25-56**.



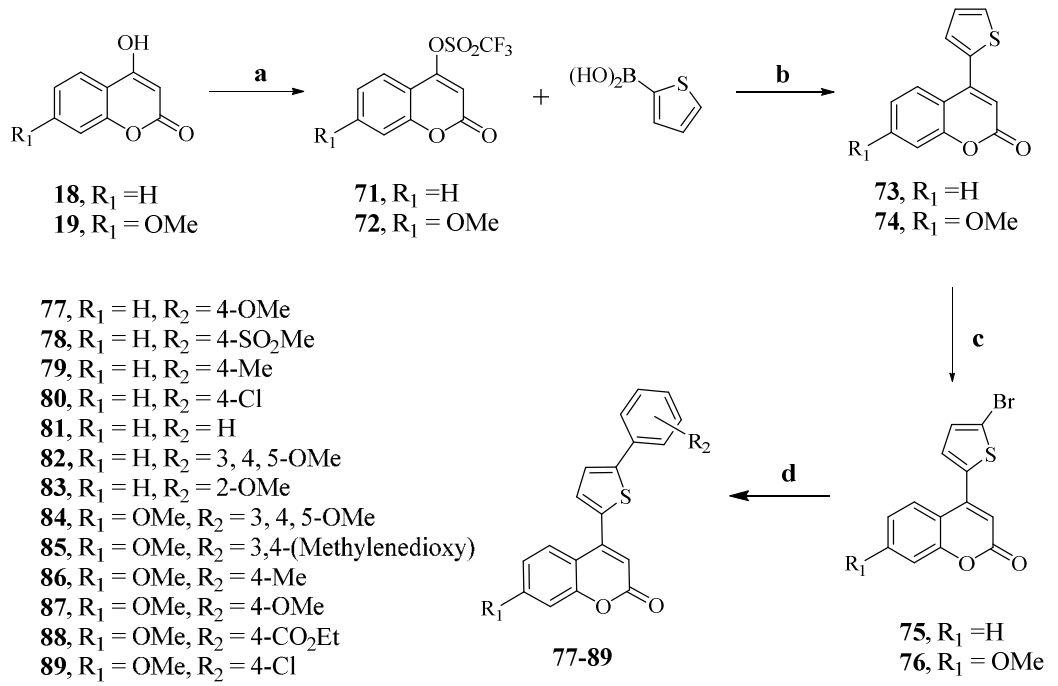
25, R_1 =H, R_2 = acetyl
26, R_1 =H, R_2 = acryloyl
27, R_1 =H, R_2 = pivaloyl
28, R_1 =H, R_2 = 2-chloroacetyl
29, R_1 =H, R_2 = 2-bromoacetyl
30, R_1 =H, R_2 = propionyl
31, R_1 =H, R_2 = pentanoyl
32, R_1 =H, R_2 = methacryloyl
33, R_1 =H, R_2 = 3-methylbut-2-enoyl
34, R_1 =H, R_2 = but-2-enoyl
35, R_1 =H, R_2 = cyclopropanecarbonyl
36, R_1 =H, R_2 = 4-ethoxy-4-oxobutanoyl
37, R_1 =H, R_2 = hexanoyl
38, R_1 =H, R_2 = decanoyl
39, R_1 =H, R_2 = 5-methoxy-5-oxopentanoyl
40, R_1 =H, R_2 = 5-chloropentanoyl
41, R_1 =H, R_2 = 3,3-dimethylbutanoyl
42, R_1 =H, R_2 = pent-4-enoyl
43, R_1 =H, R_2 = 2-methylpent-2-enoyl
44, R_1 =H, R_2 = hex-2-enoyl
45, R_1 =H, R_2 = (tert-butoxycarbonyl)glycyl
46, R_1 =H, R_2 = (tert-butoxycarbonyl)alanyl
47, R_1 =H, R_2 = (tert-butoxycarbonyl)leucyl
48, R_1 =H, R_2 = furan-2-carbonyl
49, R_1 =H, R_2 = thiophene-2-carbonyl
50, R_1 =H, R_2 = 2-methylbenzoyl
51, R_1 =H, R_2 = 3-chlorobenzoyl
52, R_1 =H, R_2 = 4-methoxybenzoyl
53, R_1 =H, R_2 = methylsulfonyl
54, R_1 =H, R_2 = (4-fluorophenyl)sulfonyl
55, R_1 =H, R_2 = (4-ethylphenyl)sulfonyl
56, R_1 =H, R_2 = 2-morpholinoethoxy

Scheme 1. Reagents and conditions: (a) MOMCl, NaH, DMF, 0 °C, 2 h; (b)10% Pd/C, H_2, EtOH, r.t., 12 h; (c) (i) $(CH_2O)_n$, MeONa, MeOH, rt; (ii) $NaBH_4$,reflux; (d) NaH, 0 °C, 30 min; 100 °C, 3 h; (e) Bu_4NBr, P_2O_5, PhMe, 120°C, 3 h; (f) DIEA, DMF, 110 °C, 24 h; (g) Ethyl acetate, HCl, 0 °C, 1h; 25 °C, 2h; (h) method A for **25-41** and **48-52**, RCO_2Cl, Et_3N, CH_2Cl_2, rt, 6 h; method B for **42−47**, RCOOH, EDCI, DMAP, CH_2Cl_2, rt, 12 h; method C for **53−55**, RSO_2Cl, Et_3N, CH_2Cl_2, rt, 6 h; method D for **56**, Cs_2CO_3, CH_3CN, 4-(2-chloroethyl)morpholine, 80 °C, 2h.



Scheme 2. (a) K_2CO_3, CH_3CN, MeI, 60 °C, 6h; (b) 10% Pd/C, H_2, EtOH, r.t., 12 h; (c) DIEA, DMF, 110 °C, 24 h; (d) method A for **61-65**, RCO_2Cl, Et_3N, CH_2Cl_2, rt, 6 h; method B for **66−70**, RCOOH, EDCI, DMAP, CH_2Cl_2, rt, 12 h.

As shown in **Scheme 2**, 4-methoxy-3-nitroaniline (**57**) was used as the starting material, and the following reactions were similar to Scheme 1. Eventually, we got the compounds **61-70**.



Scheme 3. (a) $(CF_3SO_2)_2O$, Et_3N, CH_2Cl_2; (b) $Pd(PPh_3)_4$, CuI,Na_2CO_3, PhMe, EtOH; (c) NBS, DMF, rt, 12 h. (d) $ArB(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3, PhMe, EtOH, H_2O, 80 °C, 2 h.

As shown in **Scheme 3**,[36] the activated 4-trifluorosulfonyloxy coumarins (**71-72**) were easily prepared in a high yield by treatment of the appropriate 4-hydroxycoumarins (**18-19**) with triflic anhydride as previously described.[36] Use of thiophen-2-ylboronic acid in coupling reaction led to the **73-74**, and brominated with NBS. The resulting compounds **75-76** were coupled with appropriate phenylboronic acids, giving the compounds **77-89**.

Antiproliferative Activities

The synthesized derivatives **10** and **24-56** were first evaluated for their antiproliferative activity against SKOV3 cells, HCT116 cells and HepG2 cells using MTT assay. Colchicine and compound **5** were used as the reference compounds. (Table 1) As expected, **10** showed similar antiproliferative activity to compound **5** but 10-fold increase in antiproliferative activity (IC_{50} = 4.3, 3.6, and 3.0 nM against SKOV3 cells, HCT116 cells and HepG2 cells, respectively) in compared with colchicine. However, compound **24**, which was added the methoxy group at the C-7 position of compound **10**, exhibited more than 100-fold reduced inhibitory effect (IC_{50}= 675.8, 303.5, and 392.9nM against SKOV3 cells, HCT116 cells and HepG2 cells, respectively). Inspired by **4**, we introduced a series of substituted groups at the 3-hydroxyl on B-ring of **10** to obtain compounds **25-56** (Scheme 1). The esterifiable products were distinctly more potent than ether derivatives. Compounds **25-37**, **39-44** displayed potent antiproliferative activities against tumor cell lines, the IC_{50} values varied from 3.2 nM to 336.6 nM. However, the activities of product **38** which contained long chain (ten carbon atoms) in the substitutional group were decreased (IC_{50} = 227.8, 336.6, and 244.5 nM against SKOV3 cells, HCT116 cells and HepG2 cells, respectively). (Table 1) In addition, the boc-protected amino acid derivatives **45-47** and the products **48-52** (contain a benzene ring in the substitutional group) caused significant loss of the antiproliferative activities (IC_{50} > 5000 nM against SKOV3 cells, HCT116 cells and HepG2 cells). The sulfonic acid ester derivatives

53-55 (regardless of long chains or short chains) and the ether derivative **56** showed no activity (IC_{50} > 5000 nM against the cells mentioned above).

Table 1. The antiproliferative activities of compounds **10** and **24-56**.

Compds	IC_{50}[a](nM)			Solubilities[b]
	SKOV3	HCT116	HepG2	
Colchicine	51.9 ± 3.2	27.8 ± 1.2	42.8 ± 0.8	ND
5	3.2 ± 0.1	4.3 ± 0.1	4.6 ± 0.2	ND
10	4.3 ± 0.3	3.6 ± 0.1	3.0 ± 0.2	-
24	675.8 ± 46.7	303.5 ± 26.2	392.9 ± 54.3	-
25	5.6 ± 0.8	25.6 ± 2.8	14.7 ± 3.1	-
26	249.6 ± 64.8	30.1 ± 1.6	31.3 ± 1.9	-
27	33.7 ± 1.9	15.4 ± 0.6	6.8 ± 0.8	-
28	17.5 ± 0.7	25.4 ± 7.1	7.6 ± 1.2	-
29	5.6 ± 0.9	27.2 ± 5.2	10.9 ± 1.0	-
30	3.2 ± 0.2	12.3 ± 3.6	12.9 ± 0.5	-
31	29.7 ± 0.2	67.2 ± 1.2	35.0 ± 1.0	-
32	14.0 ± 2.7	44.4 ± 5.7	60.1 ± 6.7	-
33	30.7 ± 3.1	29.5 ± 2.0	21.1 ± 2.3	-
34	43.7 ± 2.7	29.7 ± 2.3	14.3± 0.1	+
35	9.3 ± 0.4	17.5 ± 2.3	39.2 ± 6.2	-
36	43.7 ± 0.1	64.1 ± 2.4	67.9 ± 1.0	-
37	39.9 ± 0.3	70.9 ± 1.9	81.8 ± 3.4	-
38	227.8 ± 26.5	336.6 ± 80.31	244.5 ± 94.2	-
39	21.4 ± 0.4	9.0 ± 0.2	27.2 ± 6.7	+

40	35.5 ± 5.1	24.1 ± 0.9	36.1 ± 0.3	+
41	60.1 ± 2.3	44.4 ± 8.7	72.4 ±7.6	-
42	3.2 ± 0.2	14.8 ± 3.2	23.5 ± 4.6	-
43	32.7 ± 9.3	13.3 ± 1.1	8.8 ± 0.2	+
44	30.5 ±7.2	45.6 ± 2.6	10.2 ± 0.8	-

[a]IC_{50} = compound concentration required to inhibit tumor cell proliferation by 50%. Data are expressed as the mean ± SD from the dose response curves of at least three independent experiments.[b] The solubilities were measured by a method as following: 1.0 mg of the appropriate compound dissolved in 1.0 mL physiological saline(containing 2.5% ethanol and 2.5% Tween-80); +, completely dissolved; -,not all dissolved; ND, not determined.

Subsequently, we decided to replace the 3-hydroxyl group on B-ring of **10** with an amino group to obtain **60**. Compound **60** showed potent antiproliferative activity (IC_{50}= 23.4, 2.9, and 3.3nM against SKOV3 cells, HCT116 cells and HepG2 cells, respectively). Diverse substituents were introduced into the 3-amino group of the B ring for structure and activity relationship analysis, giving the amides **61-70**. Compounds **61-68** slightly decreased the antiproliferative activities in comparison to compound **60**, the IC_{50} values varied from 3.5 nM to 585.0 nM. Similar to the compounds **45-47**, the boc-protected amino acid derivatives **69-70** caused significant loss of the antiproliferative activities (IC_{50}> 5000 nM). (Table 2)

Table 2. The antiproliferative activities of compounds **60-70**.

Compds	IC_{50}[a](nM)			Solubilities[b]
	SKOV3	HCT116	HepG2	
60	23.4 ± 2.1	2.9 ± 0.5	3.3 ± 0.1	-
61	23.4 ± 4.1	113.9 ± 10.1	29.6 ± 7.3	-
62	29.9 ±1.0	49.6 ± 17.3	9.6 ± 5.3	+
63	27.3 ± 7.0	398.2 ± 39.2	48.1 ± 2.5	-
64	40.1 ± 11.7	293.3 ± 16.6	286.7 ± 14.1	-
65	3.5 ± 0.1	31.9 ± 13.4	27.3 ± 11.0	+
66	33.6 ± 12.3	146.7 ± 13.1	21.1 ± 2.3	-
67	56.8 ± 3.9	36.4 ± 3.6	26.2 ± 4.3	+
68	129.9 ± 1.0	318.9 ± 58.1	585.0 ± 65.4	-
69	>5000	>5000	>5000	ND
70	>5000	>5000	>5000	ND

To verify whether the *N*-methyl linker group is the critical choice, we replaced the *N*-methyl with a five-membered heterocyclic ring.[40] Compounds **77-89** were obtained by inserting a thiophene group between the basic skeleton coumarin A-ring and B-ring, All these compounds **77-89** showed low antiproliferative activity (IC_{50} > 5000 nM against SKOV3 cells, HCT116 cells and HepG2 cells), suggesting that *N*-methyl linker group plays a crucial role in maintaining the antitumor activity.

Low aqueous solubility or poor bioavailability is a major obstacle for the use of antimitotic agents. Although several compounds showed potent antiproliferative activity with low sub nanmolar IC_{50}values, these compounds were also faced with the challenge of low solubility, which limited our further in vivo test. In order to compare

the relative solubility, we determined the solubility of selected compounds in suitable solution for further *in vivo* test. 1.0 mg of the appropriate compound was respectively dissolved in 1.0 mL physiological saline containing 2.5% ethanol and 2.5% Tween-80. As shown in Table 1 and Table 2, most compounds had low solubility and difficult for further *in vivo* test, however, compounds **34**, **39**, **40**, **43**, **62**, **65**, **67** were relatively soluble under the same conditions. Considering the *in vitro* IC_{50} values and relative better solubility, we selected seven compounds **34**, **39**, **40**, **43**, **62**, **65** and **67** for further *in vitro* and *in vivo* antitumor activity evaluation.

Subsequently, we focused on the evaluation of the antiproliferative activities of **34**, **39**, **40**, **43**, **62**, **65** and **67** across various tumor cells, with colchicine as the positive control. These tumor cells were evaluated including cancer cell lines coming from colon (C26, HCT-8), lung (NHI-H358, H460), liver (SK-KEP-1, BEL-7402), breast (MCF-7, ZR-75-1), ovarian (ES-2, A2780s) and prostate (DU1450) cancers. As summarized in Table S1 in Support Information, compounds **34**, **39**, **40**, **43**, **62**, **65** and **67** possessed potent inhibitory activity, with IC_{50} values ranged from 7 to 83 nM against all the tested tumor cell lines. It was worth noting that all the compounds **34**, **39**, **40**, **43**, **62**, **65** and **67** were more potent than colchicine against C26 and H460 cells.

Evaluation on cellular microtubule networks using immunofluorescence

We further examined the effect of compounds **34**, **39**, **40**, **43**, **62**, **65** and **67** on cellular microtubule networks by using immunofluorescence techniques (Figure S4). The microtubule network exhibited normal arrangement and organization in HepG2 cells in the absence of drug treatment. However, treatment with compounds **34**, **39**, **40**,

43, **62**, **65** and **67** at respective 100 nM led to dramatic changes in microtubule configuration in HepG2 cells, the microtubule network in cytosol was disrupted and chromosome was also abnormally aggregated after 24 h treatment. Similar pattern was observed when the cells were treated with colchicine. These results suggested that compounds **34**, **39**, **40**, **43**, **62**, **65** and **67** inhibited microtubule formations through direct binding with tubulin components.

Initial *in vivo* evaluation in C26 and H460 xenograft models

To determine whether the antitumor potency of compounds **34**, **39**, **40**, **43**, **62**, **65** and **67** could also be reflected *in vivo* tumor bearing mice model, we established two xenograft models including a mice colon line (C26), and a lung cancer line (H460). The administration, dosing schedules and results were presented in Table S2 in Support Information.

As shown in Table S2, compounds **34**, **39**, **40**, **43** and **62** at respective 5mg/kg *iv* injection exhibited relative weak inhibitions on tumor growth, with the tumor mass change (percent of tumor mass change [TGI] values) less than 40% in C26 tumor bearing mice model. Compounds **65** and **67** at respective 5mg/kg *iv* injection showed remarkable reduction of tumor growth, and the TGI values were respective 60.3% and 45.9%. **65** was identified the most antitumor one among these selected compounds

In H460 tumor bearing mice models, similar results were also observed in selected compounds. Compound **65** showed the best antitumor activity among them. The TGI of **65-**treated group was 79.1% at 10 mg/kg *iv* injection in H460 model. In addition, no serious side-effects such as weight loss, abnormal behaviors were observed in all the treated groups. Notably, compound **5** showed serious side effects at both 2.5

mg/kg and 5mg/kg *iv* treatments in both tumor bearing micel models, all mice died during treatment due to significant loss of body weight. Hence, **65** was identified the most antitumor one for further *in vitro* and *in vivo* investigation.

Effect of 65 on Multidrug Resistant Cells

Drug resistance, notably multidrug resistance (MDR), is another major obstacle for the first-line chemotherapy.[14-16] A recent study indicated that overexpression of class III β-tubulin[18], as well as changes in microtubule regulation, is also associated with MDR to antimicrotubule agents that interact with the taxane or vinca site. However, colchicine-binding site agents were generally active in cells overexpressing class III β-tubulin. Hence, we compared the activities of **65** in these resistant and their related sensitive cancer cells, and paclitaxel, colchicine, vinblastine and adriamycin as reference compounds. As shown in Table 3, **65** exhibited potent cytotoxic activity toward sensitive cells and resistant cells, the IC_{50} values of **65** against A2780/T (taxol resistant ovarian cancer cell line, β-tubulin III overexpressed), HCT-8/V (vinblastine resistant colon cancer lines, P-gp overexpressed) and MCF-7/ADR (adriamycin resistant human breast cancer lines, P-gp overexpressed) were 57.3 and 78.9 and 144.6 nM, respectively. In comparison, although paclitaxel, vinblastine, adramycin and colchicines showed high activity in all parental cancer cell lines, all of them were no sensitive to the tested drug resistant cancer cell lines. Compounds **65** showed much lower drug resistant indexes (DRIs) (1.7 for A2780/T, 4.4 for HCT-8/V, and 1.6 for MCF-7/ADR, respectively) than paclitaxel (964.0 for A2780/T), vinblastine (352.4 for HCT-8/V), and adriamycin (367.3 for MCF-7/ADR). These results indicated that

the P-gp and β-tubulin III gene have no influence on the antiproliferative activity of **65**, suggesting that **65** might be useful in the treatment of drug refractory tumors, especially those with resistance to other anti-tubulin drugs.

Table 3. Drug Tolerances of Selected Compounds against Different Drug Resistant Cancer Cells

	IC_{50}[a] mean±SE (nM)				
Cell lines	**Paclitaxel**	**Colchicine**	**Vinblastine**	**Adriamycin**	**65**
A2780S	58.1±24.1	30.8±9.8	22.5±2.7	ND[c]	33.7±12.4
A2780/T	56600±56198	8671±3100	6899±1396	ND	57.3±15.9
DRI[b]	964.0	281.4	307.3	ND	1.7
HCT-8	36.9±23.8	29.7±9.3	14.77±3.15	ND	32.8±11.7
HCT-8/V	189.4±52.5	2433±573	5205±13	ND	144.6±37.9
DRI	5.1	81.9	352.4	ND	4.4
MCF-7	32.3±19.5	64.1±9.1	54.7±527.6	22.2±19.4	54.6±3.9
MCF-7/ADR	9153±561	6005±3100	5846±1396	8153±561	88.6±14.8
DRI	283.6	93.6	106.8	367.3	1.6

[a]IC_{50} = compound concentration required to inhibit tumor cell proliferation by 50%. Data are expressed as the mean ± SD from the dose response curves of at least three independent experiments. [b]Drug resistant index: (IC_{50} of drug resistant cancer cell) / (IC_{50} of parental cancercell). [c]ND: no detection.

Compound 65 Induces G2/M Arrest and Apoptosis of A2780S and A2780T cells

To explore whether the cytotoxicity of **65** was due to the cell cycle arrest, we first examined the effect on cell cycle progression using propidiumiodide (PI) staining by flow cytometry analysis both in taxol sensitive ovarian A2780s and taxol-resistant ovarian A2780T cells.[41] As depicted in Figure 2A, treatment with **65** for 48h cause a remarkable and concentration-dependently G_2/M arrest both in A2780S and A2780T. Compound **65** induced obvious G2/M arrest at 30 nM, the percentages of A2780S and A2780T cells in G_2/M phase were respective 37.07% and 29.94%, when cells were exposed to 300 nM of **65**, the percentage of G2/M phase were increased to 90.23% in A2780T and 76.9% in A2780S, respectively (Figures 2B and 2C).

To assess whether compound **65** would induce cell apoptosis, **65** treated A2780S and A2780T cells were stained with annexin V-FITC and propidium iodide (PI) and analyzed by flow cytometry (Figure 2D).This dual staining for annexin-V and with PI can provide discrimination between live cells (annexin-V^-/PI^-), early apoptotic cells (annexin-V^+/PI^-), late apoptotic cells (annexin-V^+/PI^+), and necrotic cells (annexin-V^-/PI^+).As exhibited in Figure 2D and 2E, treatment with **65** showed concentration-dependent high activity on inducing both in taxol sensitive A2780s and taxol-resistant A2780T cells apoptosis. The total numbers of early apoptotic cells (annexin-V^+/PI^-) and late apoptotic cells (annexin-V^+/PI^+) were 8.2%, 37.5%, 42.0%, 40.7% and 9.1%, 20.9%, 22.0%, 25.5% after A2780s cells were exposed to 10, 30, 100, and 300 nM of **65**, respectively. In contrast, only 1.6% and 2.7% early and late apoptotic cells were found in untreated control. The similar results were observed in A2780T cells. These

results confirmed that compound **65** could induce efficient apoptosis of both A2780S and A2780T cells.

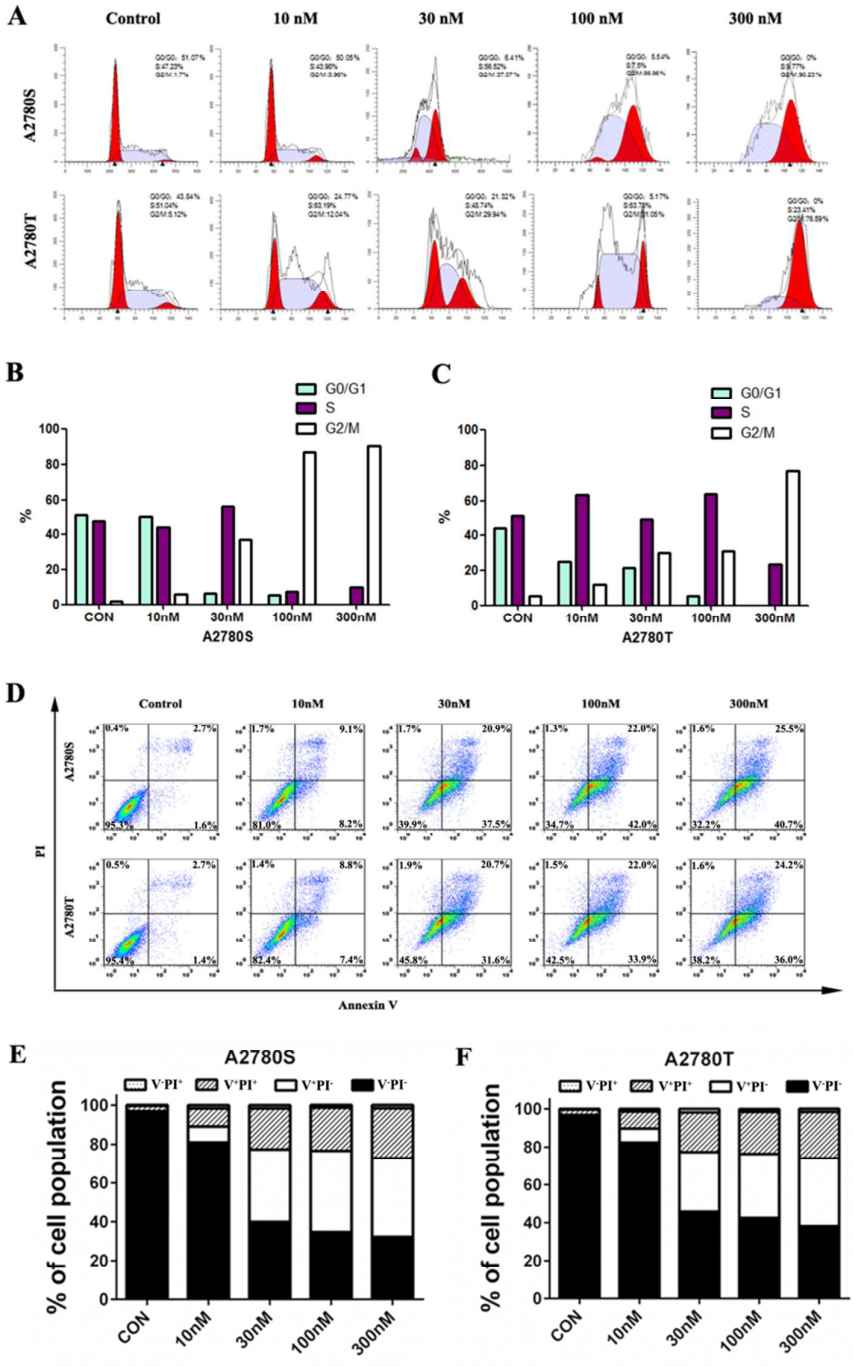


Figure 2. Cell cycle distribution and apoptosis of A2780S and A2780T with or without treatment of **65** at indicated concentration. (A) **65** caused G2/M phase arrest.

A2780S and A2780T cells were treated with **65** at 10 nM, 30 nM, 100 nM and 300 nM for 48h, and control group indicates the vehicle control. (B) Percentages of A2780S cells in the different phases of the cell cycle. (C) Percentages of A2780T cells in the different phases of the cell cycle. (D) Representative flow cytometric histograms of apoptotic A2780S and A2780T cells after 48 h treatment with **65**. The cells were harvested and labeled with annexin-V-FITC and PI and analyzed by flow cytometry. (E) Percentage of A2780S cells found in the different regions of the biparametric histograms after incubation with **65** for 48h as indicated. (F) Percentage of A2780T cells found in the different regions of the biparametric histograms after incubation with **65** for 48 h as indicated.

Immunofluorescence Staining

To investigate whether the cytotoxicity activities of **65** were derived from an interaction with tubulin, we examined the effect on the cellular microtubule network treated with different concentration of **65** for 24 h and stained for DNA (blue) and α-tubulin (green). Taxol (paclitaxel, PTX) and colchicine were used as reference compounds. The confocal images shown in Figure 3A revealed that the microtubule network in HepG2 cells exhibited normal arrangement and organization in the absence of drug treatment. Taxol treatment resulted in obvious bundling of the microtubule fibers near the nucleus. Colchicine at 200 nM almost completely depolymerizes cellular microtubules as indicated by the disruption of microtubule network in cytosol and abnormally aggregation of chromosome in HepG2 cells. Compound **65** exhibited similar effect in microtubule depolymerization to colchicine

and concentration-dependently inhibited microtubules polymerization and spindle formation. In comparison to colchicine, compound **65** at 30 nM obviously inhibited microtubules polymerization; further increase to 100 nM of **65** completely disrupted the microtubules polymerization, suggesting that compound **65** is more active than colchicine as a novel polymerization inhibitor.

Effects on Microtubule Dynamics

It is well-known that the tubulin binding agents were divided into two types: MSAs (*e.g.*, taxol) and MDAs (*e.g.*, colchicine). To further confirm which type of compound **65** related to, **65**, PTX and compound **5** were first employed at 10 μM for the effects on microtubule dynamics (Figure 3B). Similar but inferior to compound **5**, we found that **65** can inhibit tubulin polymerization. Furthermore, compound **65** was employed at respective 1, 5, and 25 μM for microtubule dynamics assays. As shown in Figure 3C, **65** inhibited tubulin polymerization in a concentration-dependent manner. These data indicated that the mechanism of **65** was in accordance with previously reported MDAs, suggesting that compound **65** is a novel MDA.

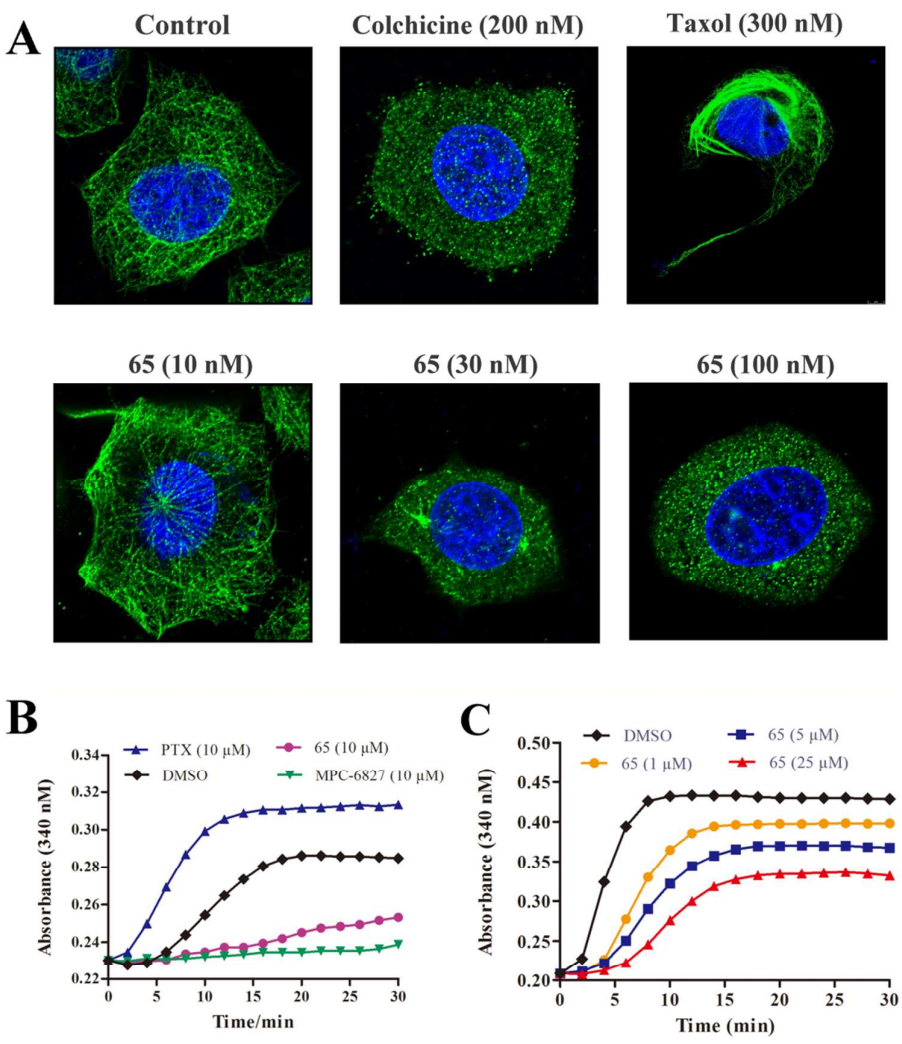


Figure 3. Effects of compounds on the organization of cellular microtubule network and microtubule dynamics. (A) Effect of **65**, taxol and colchicine on the organization of cellular microtubule network. HepG2cells were untreated (Control) and treated with **65** (10, 30 and 100 nM), taxol (300 nM) or colchicine (200 nM) at appropriate concentration for 24 h. Microtubules and unassembled tubulin are shown in green and the Nuclei in blue. (B) Effect of **65**, PTX and colchicine on tubulin polymerization. Tubulin had been pre incubated for 5 min with **65**, PTX or colchicine at 10 μM, or vehicle DMSO at room temperature before GTP was added to start the tubulin

polymerization reactions. The reaction was monitored at OD340 nm at 37°C. (C) Effect of **65** on tubulin polymerization. Tubulin had been pre incubated for 5 min with **65** at 1, 5 and 25 μM, or vehicle DMSO at room temperature before GTP was added to start the tubulin polymerization reactions. The reaction was monitored at OD340 nm at 37°C.

EBI competition assay

In order to evaluate whether compound **65** directly binds to tubulin at the colchicine-binding site, we carried out a recently described competition assay wit*h N,N*'- ethylene-bis-(iodoacetamide) (EBI) in HepG2 cells. EBI is an alkylating agent that has the property to specifically crosslink the Cys-239 and the Cys-354 residues of β-tubulin involved in the colchicine-binding site.[42] The β-tubulin adduct formed by EBI is easily detectable by western blot as a second immunoreactive β-tubulin band that migrates faster than β-tubulin itself. The occupancy of colchicine-binding site by antimitotic drugs could inhibit the formation of the EBI: β-tubulin adduct, resulting in a convenient method to evaluate the ability of a molecule to bind to the colchicine site.[42] As shown in Figure 4, EBI caused the appearance of a second band below the β-tubulin band. Pre-incubation of compound **65** (0.2 1, 5 and 25 μM) dose-dependently prevented the formation of the EBI, resulting in the disappearance of the adduct band, which was consistent with the effect of colchicine (5 μM). Vincristine, a drug which has a distinct and non-overlapping binding site from colchicine, was utilized as a negative control. It was illustrated that no false results were obtained.

Thus, these data indicate that compound **65** induce the antimitotic effect by directly binding to the colchicine-binding site in *β*-tubulin.

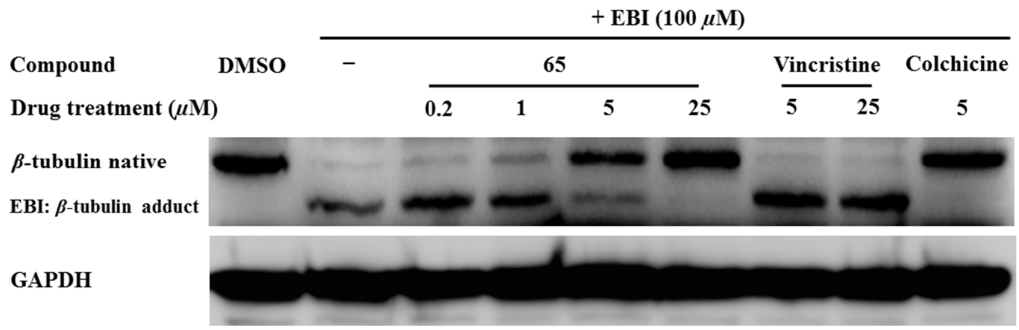


Figure 4. EBI assay: competition assay of compound **65** (0.2, 1, 5, 25 μM), vincristine (5, 25 μM) and colchicine (5 μM) with EBI (100 μM) on HepG2 cells. EBI could form a *β*-tubulin adduct, which could be detected via western blot as it migrates faster. Compounds that pre-occupied the colchicine-binding site in tubulin could inhibit the formation of the *β*-tubulin adduct.

Molecular Modeling

To rationalize our experimental findings, a series of molecular docking simulations of **65** on tubulin were performed using a procedure reported previously.[39] The overview of the binding site of **65** is shown in Figure 5. Due to the flexibility of Lys352*β* in different crystal structures of tubulin complex, we set the residue of Lys352*β* as flexible residue to perform the accurate docking of **65**. The coumarin ring of **65** forms hydrophobic interactions with the residues of Ala354*β*, Leu254*β*, Ala250*β* and Leu255*β*. 4-Methoxyphenyl group of **65** forms hydrophobic interactions with Ala316 and the aliphatic chain of Lys352*β*. And the tail alkyl chain forms a hydrophobic interaction with Val781. (Figure 5B)

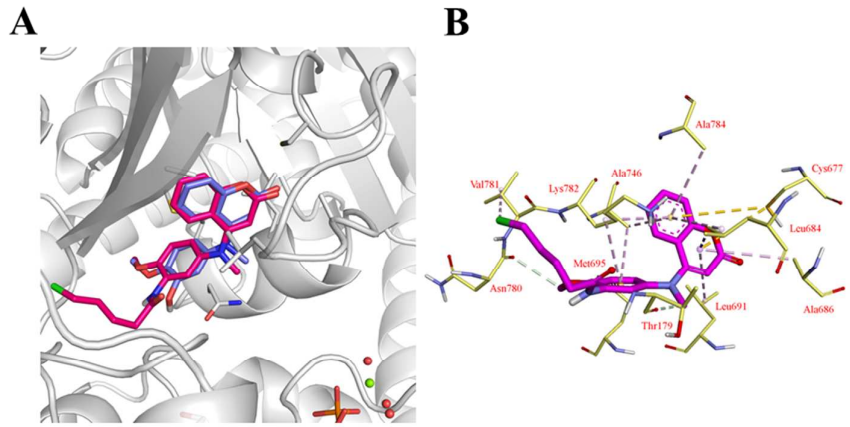


Figure 5. (A) Proposed binding model of **10** (blue) and **65** (red) within the colchicine-binding site. (PDB code 4YJ3). (B) Hydrophobic interactions between **65** and tubulin.

In Vitro Evaluation of Antivascular Activity

Most microtubule binding drugs possess vascular disrupting activity, which are thought to disrupt microtubule dynamics to induce endothelial cell shape change. We used HUVEC cells culture assay to test the ability of **65** to induce rapid endothelial cell shape changes. As the migration of ECs is the key step to generate new blood vessels, wound-healing migration assay was applied to assess the HUVECs migration. As shown in Figure 6A, the untreated cells migrated to fill the area that was initially scraped after 24 h, in contrast, compound **65** significantly inhibited the HUVEC migration in a concentration-dependent manner. (Figure 6C)

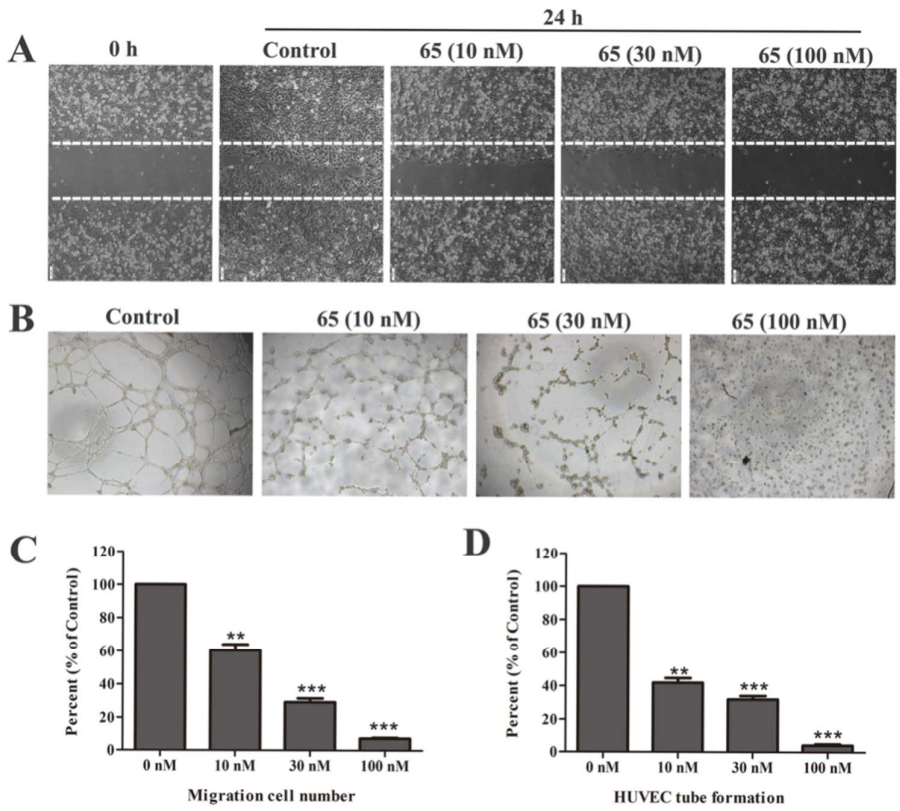


Figure 6. Effects on the HUVECs migration and tube formation. (A) HUVECs suspended in serum-free Dulbecco's Modified Eagle Medium (DMEM) containing **65** (10, 30, and 100 nM) for 20 h were photographed under a phase contrast microscopy (magnification: 100). Control was treated with serum-free DMEM. (B) HUVECs (1×10^4 cells) suspended in DMEM containing **65** (10, 30, and 100 nM) were added to the Matrigel. Control was treated with DMEM alone. After incubation for 6 h at 37°C, capillary networks were photographed and quantified (magnification: 100×). (C) Statistical data of cell migration assay. (D) Statistical data of tube formation assay. **, $P< 0.01$; ***, $P< 0.001$, significantly different compared with control by *t*-test.

Then we also evaluated the ability of compound **65** in a tube formation assay. After being seeded on Matrigel, HUVECs form the capillary-like tubules with multi centric

junctions. After 6 h treatment in different concentrations (10−100 nM) of compound **65**, the capillary-like tubes were interrupted in different levels. At the two higher concentrations of **65**, most cells were spherical and aggregated in small clumps (Figure 6B). Quantitative image analysis showed that **65** markedly decreased the capillary-like tubes in a concentration-dependent manner (Figure 6D).

To evaluate whether the inhibition of cell migration and tube formation was due to a cytotoxic action of **65**, we analyzed cell proliferation of the HUVECs by the MTT assay to determine the IC_{50} value of compound **65** at 48 h. The calculated IC_{50} of compound **65** was 0.26 ± 0.06μM, which is much higher than the concentration of 10 nM required for the inhibition of cell migration and tube formation. This result indicates that the activity of **65** on HUVECs cell migration and tube formation is not due to a cytotoxic action at indicated concentration.

Antitumor Activity in A2780s, A2780/T, MCF-7 and MCF-7/ADR Xenograft Models

To determine whether the antitumor potency of compound **65** could also be reflected in drug resistant tumors xenografts models *in vivo,* we established four xenograft models including sensitive and drug resistant ovarian cancer (A2780s, A2780/T), and sensitive and drug resistant human breast cancer (MCF-7, MCF-7/ ADR). The administration, dosing schedules of **65** and results are presented in Table S3 in Support Information.

We first investigated the administration frequency of **65** on antitumor effect in A2780s tumor bearing mice models. Compound **65** was *iv* administrated either once a

week with 20 mg/kg or once other day with 2.5, 5 and 10mg/kg, respectively. PTX was *ip* administrated once a week with 30mg/kg or once other day with 10mg/kg for comparison. As shown in Figures 7A and 7B, **65** showed remarkable reduction of tumor growth in two dose schedule, the tumor inhibitory effects were superior to positive drug PTX. The significant antitumor activities were observed by *iv* administration of **65** at 2.5, 5 and 10 mg/kg every 2 day*s* (Figure 7A) or 20 mg/kg every 7 days (Figure 7B). The average TGIs of A2780sxenografts were 76.5%, 85.1%, 88.8% (2.5, 5 and 10 mg/kg, Q2D), and 85.9% (20 mg/kg, Q7D) (Table S3), respectively. In comparison, PTX caused the TGI of 85.0% (10 mg/kg, Q2D) and 82.9% (30 mg/kg, Q7D).

The similar results were also observed in the PTX resistant A2780/T xenografts model, and the tumor growth was suppressed by 59.2%, 78.0% and 82.7% (2.5, 5 and 10 mg/kg of **65**, respectively, Q2D). In contrast, PTX (30 mg/kg) only showed 37.7 % tumor growth inhibition, demonstrating that PTX has developed resistant to A2780/T. Importantly, in all **65**-treatment groups, no serious side-effects such as weight loss, and abnormal behavior were observed. On the contrary, PTX-treated group showed serious toxicity as indicated by significantly loss of body weight and abnormal behavior either once a week or once other day administration.

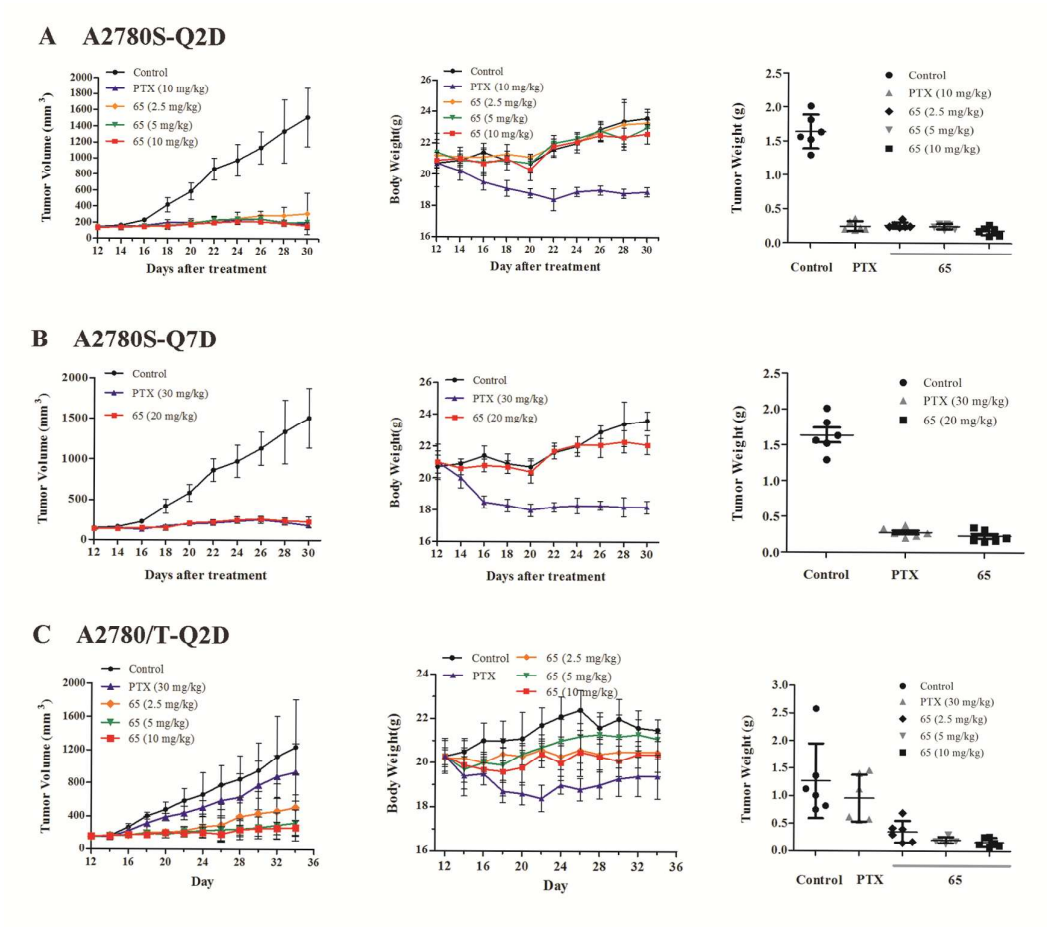


Figure 7. Antitumor effect of **65** and PTX on the A2780s and A2780/T xenograft models. (A) **65** inhibited tumor growth on the A2780s xenograft model, dosing schedules of Q2D (every 2 days). (B) **65** inhibited tumor growth on the A2780s xenograft model, dosing schedules of Q7D (every 7 days). (C) **65** inhibited tumor growth on the A2780/T xenograft model, dosing schedules of Q2D (every 2 days).

Subsequently, we further established a couple of human breast cancer (MCF-7, MCF-7/ADR) xenograft models to evaluate the antitumor activities of compound **65**, with PTX and adriamycin (ADM) administrated for comparison. The administration, dosing schedules were also presented in Table S3 in Support Information. As displayed in Figure 8, **65** showed remarkable reduction of tumor growth in both the

MCF-7 and MCF/ADR xenografts model, and the tumor inhibitory effects were superior to positive drug PTX and ADM. In the MCF-7 tumor xenografts model, **65** dose-dependently inhibited tumor growths, the TGI were 81.3 and 91.47 % at 5, 10 mg/kg (Q2D) *iv* administration, respectively. In contrast, PTX at the same dose (5 mg/kg, Q2D) only showed 59.2%, which was much inferior to the **65**-treatment group at the same dose administration.

In the MCF-7/ADR tumor xenografts model, **65** also dose-dependently inhibited tumor growths, the TGI were respectively 71.2, 79.87 and 85.7% at 2.5, 5, 10 mg/kg iv administration. It was worth noting that reference drug ADM had only 33.3% tumor growth inhibition, suggesting that ADM had developed resistant to MCF-7/ADM cell lines. (Figure 8)

In addition, we observed obviously loss of body weight in ADM-treated and PTX-treated groups. However, all **65**-treated groups have no obviously loss of body weight and abnormal behavior. These results above proved that **65** were effective in both the drug sensitive and drug resistant tumors without obvious toxicity. Taken together, our *in vitro* and *in vivo* results demonstrated that compound **65** represented a new scaffold targeting tubulin and mitosis for novel antitumor drug discovery.

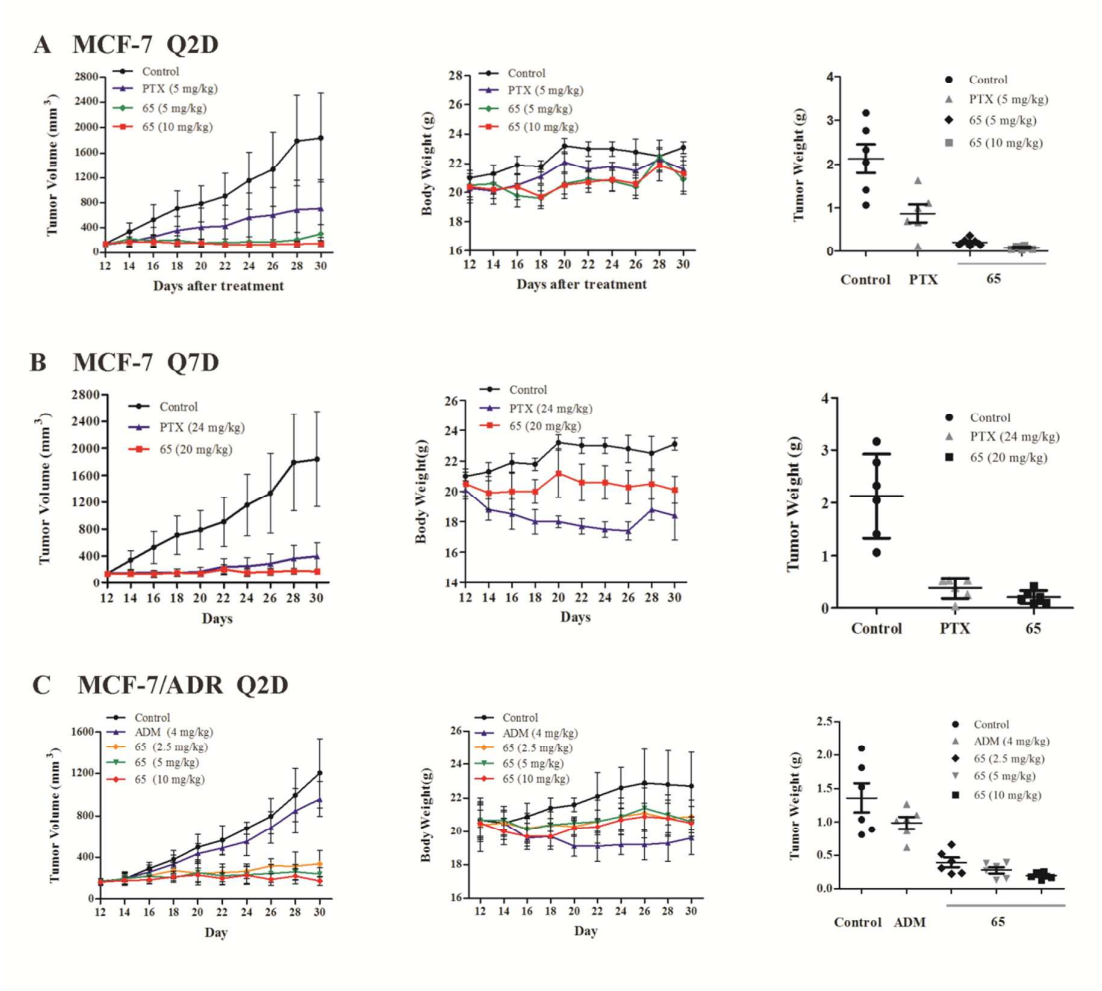


Figure 8. Antitumor effect of **65**, ADM and PTX on the MCF-7 and MCF-7/ADR xenograft models. (A) **65** inhibited tumor growth on the MCF-7 xenograft model, dosing schedules of G2D (every 2 days). (B) **65** inhibited tumor growth on the MCF-7 xenograft model, dosing schedules of Q7D (every 7 days). (C) **65** inhibited tumor growth on the MCF-7/ADR xenograft model, dosing schedules of Q2D (every 2 days).

CONCLUSIONS

Fifty-eight novel 4-substituted coumarin derivatives were synthesis and evaluated the anti-tumor activities. Among them, compounds **34**, **39**, **40**, **43**, **62**, **65** and **67** possessed potent antiproliferative abilities, with IC_{50} values ranged from 7 to 83 nM against all the tested tumor cell lines. Preliminary mode of action studies demonstrated that **65** caused accumulation of cells in the G2/M phase of the cell cycle and induced apoptosis in HepG2 cells. The antimitotic ability on multidrug resistant cells was also tested and the results demonstrated that **65** were not a substrate for P-glycoprotein drug pump and not affected by the *β*-tubulin III gene. The immunofluorescence assays, microtubule dynamics experiment, EBI assays and molecular modeling studies identified that compound **65** was a novel polymerization inhibitor probably binding to the colchicine site. Then we evaluated the *in vivo* activity on six xenografts models including two couples of sensitive and drug resistant tumors (C26, H460, A2780s, A2780/T, MCF-7 and MCF-7/ADR). Our results demonstrated that compound **65** had a potent effect on inhibiting the tumor growth without significant body weight loss or behavior disorders. Therefore, **65** probably represent a novel class of microtubule-stabilizing agent with clinical potential for the treatment of cancer.

EXPERIMENTAL SECTION

Experimental apparatus and reagents

All chemicals were used without purification as commercially available unless otherwise noted. Column chromatography was performed on silica gel (300-400 mesh) eluting with ethyl acetate and petroleum ether. TLC was performed on 0.20 mm Silica Gel 60 F_{254} plates (Qingdao Ocean Chemical Factory, Shandong, China). UV light and I_2 were used to visualize products. NMR datas were obtained for ^1H at 400 MHz, and for ^{13}C at 100 MHz, on a Bruker Avance400 spectrometer (Bruker Company, Germany) or Varian spectrometer (Varian, Palo Alto, CA) , using TMS as an internal standard. Chemical shifts were given in ppm (parts per million). Low-resolution and high-resolution mass spectral (MS) data were acquired on an Agilent 1100 series LC−MS instrument with UV detection at 254 nm in low-resonance electrospray mode (ESI). The purity of each compound (>95%) was determined on an Waters e2695 series LC system (column, Xtimate C18, 4.6 mm×150 mm, 5μm; mobile phase, methanol (60%)/H_2O (40%); low rate, 1.0 mL/min; UV wavelength, 254−400 nm; temperature, 25℃; injection volume, 10 μL). The melting point of each compound was determined on a SGWX-4A melting point instrument (±1℃, <200°C; ±2°C, 200～300°C. Shanghai Precision and Scientific Instrument Corporation, Shanghai, China).

***Preparation of* 4-*((3-hydroxy-4-methoxyphenyl)(methyl)amino)-2H-chromen-2-one* (10).** To a solution of 4-((4-methoxy-3-(methoxymethoxy)phenyl)(methyl)-amino)-*2H*- chromen-2-one **22** (5 mmol) in 30 mL of anhydrous ethyl acetate cooled

at 0°C was added 3 mL concentrated hydrochloric acid. The mixture was stirred at 0°C for 1 h, then allowed to warm to room temperature and stirred for 2 h. The solution was washed with saturated $NaHCO_3$ (2x), water (2x), brine (1x) and dried over $MgSO_4$, filtered, and concentrated. The residue was purified by chromatography (30% ethyl acetate/hexanes) to give **10**. 63% yield as a light yellow solid; mp 187.6-189.4 °C; ^{1}H NMR (400 MHz, DMSO) δ 9.27 (s, 1H), 7.47 – 7.40 (m, 1H), 7.31 (d, J = 8.2 Hz, 1H), 7.05-6.95 (m, 2H), 6.88 (d, J = 8.5 Hz, 1H), 6.63 (d, J = 2.5 Hz, 1H), 6.58 (dd, J = 8.5, 2.5 Hz, 1H), 5.79 (s, 1H), 3.75 (s, 3H), 3.28 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.9, 157.0, 154.1, 145.6, 143.8, 141.5, 130.7, 126.9, 122.7, 117.4, 116.2, 114.9, 111.6, 110.8, 95.0, 55.6, 44.1. MS (ESI, m/z): 298.3 $[M + H]^+$. HPLC purity: 98.9%.

Preparation of 4-((3-hydroxy-4-methoxyphenyl)(methyl)amino)-7-methoxy-2H-chromen-2-one **(24).** To a solution of **23** (5 mmol) in 30 mL of anhydrous ethyl acetate cooled at 0°C was added 3 mL concentrated hydrochloric acid. The mixture was stirred at 0°C for 1 h, then allowed to warm to room temperature and stirred for 2 h. The solution was washed with saturated $NaHCO_3$ (2x), water (2x), brine (1x) and dried over $MgSO_4$, filtered, and concentrated. The residue was purified by chromatography (30% ethyl acetate/hexanes) to give **24**. 61% yield as a light yellow solid; mp 190.6-192.6 °C; ^{1}H NMR (400 MHz, DMSO) δ 9.24 (s, 1H), 6.91-6.85 (m, 3H), 6.62 – 6.55 (m, 3H), 5.63 (s, 1H), 3.90 (s, 3H), 3.79 (s, 3H), 3.26 (s, 3H); ^{13}C NMR (101 MHz, DMSO) δ 161.9, 161.7, 157.1, 155.9, 147.8, 146.5, 141.9, 127.9,

116.3, 113.2, 113.1, 111.3, 109.3, 101.4, 92.7, 56.2, 56.2, 44.3. ESI HRMS: calcd. for $C_{18}H_{17}NO_5$+H^+ 328.1179, found 328.1156. HPLC purity: 98.0%.

General Procedures of Method A for the Synthesis of 25−41, 48-52. To a solution of **10** (365 mg, 1 mmol) in CH_2Cl_2 (5 mL) was added RCO_2Cl (2 equiv) and Et_3N (2 equiv) and stirred at room temperature for 6 h. When completed, the solvent was removed under vacuum and the residue was purified by flash chromatograph using petroleum ether/ethyl acetate (5:1) as eluent to give the title compounds.

2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl acetate **(25). 25** was obtained from **10** and acetyl chloride as described for method A: 69% yield as a light yellow solid; mp 202.2-204.6 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (t, *J* = 7.6 Hz, 1H), 7.28 (d, *J* = 8.5 Hz, 1H), 7.01 (d, *J* = 8.2 Hz, 1H), 6.95 – 6.86 (m, 4H), 5.86 (s, 1H), 3.84 (s, 3H), 3.36 (s, 3H), 2.30 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.5, 162.6, 156.8, 154.2, 149.5, 141.2, 140.4, 131.0, 126.6, 123.2, 122.9, 120.1, 117.6, 115.8, 113.0, 96.7, 56.1, 44.0, 20.6; ESI HRMS: calcd. for $C_{19}H_{17}NO_5$+H^+ 340.1179, found 340.1156. HPLC purity: 97.1%.

2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl acrylate **(26). 26** was obtained from **10** and acryloyl chloride as described for method A: 59% yield as a light yellow solid; mp 182.2-184.6 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (t, *J* = 7.6 Hz, 1H), 7.29 (d, *J* = 8.2 Hz, 1H), 7.02 (t, *J* = 8.8 Hz, 1H), 6.93 (d, *J* = 10.8 Hz, 4H), 6.61 (d, *J* = 17.3 Hz, 1H), 6.32 (dd, *J* = 17.3, 10.5 Hz, 1H), 6.03 (d, *J* = 10.5 Hz, 1H), 5.86 (s, 1H), 3.83 (s, 3H), 3.37 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.8, 162.7, 160.6, 156.9, 154.2, 149.8, 141.1, 140.4, 130.9, 126.7, 123.0, 123.0, 120.4, 117.5,

115.9, 114.5, 112.9, 96.5, 56.1, 44.0. ESI HRMS: calcd. for $C_{20}H_{17}NO_5$+H^+ 352.1179, found 352.1173. HPLC purity: 97.7%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl pivalate* (27).** **27** was obtained from **10** and pivaloyl chloride as described for method A: 62% yield as a light yellow solid; mp 153.8-155.7 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (t, *J* = 7.6 Hz, 1H), 7.29 (d, *J* = 8.7 Hz, 1H), 7.03 (d, *J* = 8.0 Hz, 1H), 6.95 – 6.88 (m, 2H), 6.89-6.80 (m, 2H), 5.85 (s, 1H), 3.81 (s, 3H), 3.35 (s, 3H), 1.36 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.1, 162.6, 156.9, 154.2, 149.7, 141.2, 140.6, 130.9, 126.7, 123.0, 122.9, 120.1, 117.5, 115.9, 112.9, 96.6, 56.1, 44.1, 39.1, 27.3, 27.3, 27.3. MS (ESI, m/z): 382.4 $[M + H]^+$. HPLC purity: 98.2%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl 2-chloroacetate* (28).** **28** was obtained from **10** and 2-chloroacetyl chloride as described for method A: 53% yield as a light yellow solid; mp 125.1-127.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 (t, *J* = 7.7 Hz, 1H), 7.29 (d, *J* = 8.2 Hz, 1H), 7.00 (d, *J* = 8.1 Hz, 1H), 6.97 – 6.88 (m, 4H), 5.88 (s, 1H), 4.32 (s, 2H), 3.85 (s, 3H), 3.35 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.5, 162.6, 156.8, 154.2, 149.5, 141.2, 140.4, 131.0, 126.6, 123.2, 122.9, 120.1, 117.5, 115.9, 113.0, 96.8, 56.1, 44.0, 40.8. MS (ESI, m/z): 396.4 $[M + Na]^+$. HPLC purity: 97.2%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl 2-bromoacetate* (29).** **29** was obtained from **10** and 2-bromoacetyl bromide as described for method A: 59% yield as a yellow solid; mp 154.6-155.9 °C; ^{1}H NMR (400 MHz, DMSO) δ 7.50 – 7.42 (m, 1H), 7.33 (d, *J* = 8.3 Hz, 1H), 7.19-7.10 (m, 2H), 7.08 (s, 1H), 7.03-6.95 (m,

2H), 5.89 (s, 1H), 4.41 (s, 2H), 3.78 (s, 3H), 3.32 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.6, 162.7, 157.0, 154.1, 145.6, 141.6, 130.7, 128.9, 126.8, 122.7, 120.0, 117.5, 116.5, 116.1, 110.2, 96.2, 55.9, 36.9, 25.8. MS (ESI, m/z): 440.3, 442.3 $[M + Na]^+$. HPLC purity: 97.0%.

2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl propionate **(30). 30** was obtained from **10** and propionyl chloride as described for method A: 55% yield as a light yellow solid; mp 121.3-125.6 °C; ^{1}H NMR (400 MHz, DMSO) δ 7.48 – 7.42 (m, 1H), 7.33 (d, *J* = 8.2 Hz, 1H), 7.12 – 7.04 (m, 3H), 7.02 – 6.94 (m, 2H), 5.87 (s, 1H), 3.76 (s, 3H), 3.32 (s, 3H), 2.54 (d, *J* = 7.6 Hz, 2H), 1.10 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.1, 162.6, 156.8, 154.2, 149.6, 141.2, 140.6, 131.0, 126.7, 123.1, 122.9, 120.2, 117.6, 115.9, 112.9, 96.7, 56.1, 44.0, 27.3, 9.1. MS (ESI, m/z): 376.2 $[M + Na]^+$. HPLC purity: 98.2%.

2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl pentanoate **(31). 31** was obtained from **10** and pentanoyl chloride as described for method A: 56% yield as a light yellow solid; mp 91.3-95.6 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (t, *J* = 7.6 Hz, 1H), 7.28 (d, *J* = 9.3 Hz, 1H), 7.01 (d, *J* = 8.2 Hz, 1H), 6.94 – 6.86 (m, 4H), 5.85 (s, 1H), 3.83 (s, 3H), 3.36 (s, 3H), 2.56 (t, *J* = 7.5 Hz, 2H), 1.78 – 1.70 (m, 2H), 1.49-1.40 (m, 2H), 0.96 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.1, 162.6, 156.8, 154.2, 149.6, 141.2, 140.6, 131.0, 126.7, 123.1, 122.9, 120.2, 117.6, 115.9, 112.9, 96.7, 56.1, 44.0, 33.2, 27.2, 21.4, 13.1. MS (ESI, m/z): 404.6 $[M + Na]^+$. HPLC purity: 97.7%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl methacrylate* (32).** **32** was obtained from **10** and methacryloyl chloride as described for method A: 58% yield as a light yellow solid; mp 99.7-104.0 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (t, *J* = 8.4 Hz, 1H), 7.28 (d, *J* = 9.2 Hz, 1H), 7.09 – 6.85 (m, 5H), 6.35 (s, 1H), 5.86 (s, 1H), 5.77 (s, 1H), 3.81 (s, 3H), 3.36 (s, 3H), 2.06 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.8, 162.6, 156.8, 154.1, 149.8, 141.1, 140.4, 135.3, 130.9, 128.0, 126.7, 123.0, 123.0, 120.4, 117.5, 115.9, 112.9, 96.5, 56.1, 44.0, 17.7. MS (ESI, m/z): 388.5 $[M + Na]^+$. HPLC purity: 98.2%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl 3-methylbut-2-enoate* (33). 33** was obtained from **10** and 3-methylbut-2-enoyl chloride as described for method A: 67% yield as a yellow solid; mp 142.5-143.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (t, *J* = 8.0 Hz, 1H), 7.28 (d, *J* = 8.0 Hz, 1H), 7.02 (t, *J* = 8.9 Hz, 1H), 6.98-6.82 (m, 4H), 5.93 (s, 1H), 5.85 (s, 1H), 3.83 (s, 3H), 3.36 (s, 3H), 2.22 (s, 3H), 1.99 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.8, 162.6, 160.6, 156.8, 154.1, 149.8, 141.1, 140.4, 130.9, 126.7, 123.0, 123.0, 120.4, 117.5, 115.9, 114.5, 112.9, 96.5, 56.1, 44.0, 27.6, 20.5. MS (ESI, m/z): 402.8 $[M + Na]^+$. HPLC purity: 98.1%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl-(E)-but-2-enoate* (34). 34** was obtained from **10** and (*E*)-but-2-enoyl chloride as described for method A: 62% yield as a light yellow solid; mp 146.1-148.3 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (t, *J* = 8.0 Hz, 1H), 7.28 (d, *J* = 9.2 Hz, 1H), 7.19 (dq, *J* = 13.9, 6.9 Hz, 1H), 7.03 (d, *J* = 8.2 Hz, 1H), 6.99-6.87 (m, 4H), 6.05 (dd, *J* = 15.5, 1.4 Hz, 1H), 5.85 (s, 1H), 3.82 (s, 3H), 3.36 (s, 3H), 1.97 (dd, *J* = 6.9, 1.2 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$)

δ 163.8, 162.7, 160.6, 156.9, 154.2, 149.8, 141.1, 140.4, 130.9, 126.7, 123.0, 123.0, 120.4, 117.5, 115.9, 114.5, 112.9, 96.5, 56.1, 44.0, 20.5. MS (ESI, m/z): 388.5 [M + Na]$^+$. HPLC purity: 98.8%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl cyclopropane-carboxylate* (35). 35** was obtained from **10** and cyclopropanecarbonyl chloride as described for method A: 52% yield as a light yellow solid; mp 150.8-152.7 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.27 (m, 2H), 7.01 (d, *J* = 8.1 Hz, 1H), 6.95-6.85 (m, 4H), 5.85 (s, 1H), 3.83 (s, 3H), 3.35 (s, 3H), 1.90 – 1.78 (m, 1H), 1.22 – 1.14 (m, 2H), 1.09 – 0.98 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.6, 162.6, 156.8, 154.1, 149.6, 141.1, 140.5, 130.9, 126.6, 123.1, 123.1，120.2, 117.5, 115.8, 112.9, 96.6, 56.1, 44.0, 12.7, 9.3, 9.3. MS (ESI, m/z): 388.5 [M + Na]$^+$. HPLC purity: 98.1%.

***Ethyl (2-methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl) succinate* (36). 36** was obtained from **10** and 4-ethoxy-4-oxobutanoyl chloride as described for method A: 58% yield as a light yellow solid; mp 74.8-76.5 °C; ^{1}H NMR (400 MHz, DMSO) δ 7.48-7.40 (m, 1H), 7.33 (d, *J* = 8.4 Hz, 1H), 7.16 – 7.05 (m, 2H), 7.02-6.95 (m, 3H), 5.87 (s, 1H), 4.13 – 3.96 (m, 2H), 3.75 (s, 3H), 3.33 (s, 3H), 2.79 (t, *J* = 6.5 Hz, 2H), 2.61 (t, *J* = 6.5 Hz, 2H), 1.17 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.9, 170.1, 162.6, 156.8, 154.2, 149.5, 141.2, 140.4, 131.0, 126.7, 123.3, 123.0, 120.1, 117.5, 115.9, 113.0, 96.7, 60.8, 56.1, 44.0, 29.1, 28.9, 14.2. MS (ESI, m/z): 448.5 [M + Na]$^+$. HPLC purity: 98.1%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl hexanoate* (37). 37** was obtained from **10** and hexanoyl chloride as described for method A: 59% yield as

a light yellow solid; mp 79.2-81.7 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.33 (m, 1H), 7.28 (d, *J* = 9.2 Hz, 1H), 7.04 – 6.99 (m, 1H), 6.95 – 6.85 (m, 4H), 5.85 (s, 1H), 3.82 (s, 3H), 3.36 (s, 3H), 2.55 (t, *J* = 7.5 Hz, 2H), 1.80 – 1.70 (m, 2H), 1.44 – 1.33 (m, 4H), 0.92 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.4, 162.6, 156.8, 154.2, 149.6, 141.2, 140.6, 130.9, 126.7, 123.0, 122.9, 120.1, 117.5, 115.9, 112.9, 96.7, 56.1, 44.0, 33.9, 31.2, 24.6, 22.3, 13.9. MS (ESI, m/z): 418.5 $[M + Na]^+$. HPLC purity: 98.1%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl decanoate* (38). 38** was obtained from **10** and decanoyl chloride as described for method A: 51% yield as a light yellow solid; mp 75.2-76.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (t, *J* = 7.4 Hz, 1H), 7.28 (d, *J* = 7.8 Hz, 1H), 7.01 (d, *J* = 8.1 Hz, 1H), 6.95 – 6.85 (m, 4H), 5.85 (s, 1H), 3.82 (s, 3H), 3.36 (s, 3H), 2.55 (t, *J* = 7.4 Hz, 2H), 1.80 – 1.68 (m, 2H), 1.32-1.19 (m, 12H), 0.88 (t, *J* = 6.5 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.4, 162.6, 156.8, 154.1, 149.5, 141.1, 140.5, 130.9, 126.6, 123.1, 122.9, 120.1, 117.5, 115.8, 112.9, 96.6, 56.0, 44.0, 33.9, 31.8, 29.4, 29.3, 29.3, 29.0, 24.9, 22.6, 14.1. MS (ESI, m/z): 474.5 $[M + Na]^+$. HPLC purity: 97.2%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl methyl glutarate* (39). 39** was obtained from **10** and 5-methoxy-5-oxopentanoyl chloride as described for method A: 65% yield as a light yellow solid; mp 119.4-121.1 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (t, *J* = 7.6 Hz, 1H), 7.28 (d, *J* = 8.8 Hz, 1H), 7.01 (d, *J* = 8.1 Hz, 1H), 6.96 – 6.85 (m, 4H), 5.86 (s, 1H), 3.82 (s, 3H), 3.69 (s, 3H), 3.36 (s, 3H), 2.64 (t, *J* = 7.2 Hz, 2H), 2.47 (q, *J* = 7.2 Hz, 2H), 2.11 – 2.03 (m, 2H); ^{13}C NMR (101 MHz,

$CDCl_3$) δ 173.3, 170.5, 162.6, 156.8, 154.1, 149.4, 141.1, 140.3, 131.0, 126.6, 123.2, 122.9, 120.0, 117.5, 115.8, 112.9, 96.7, 56.0, 51.6, 44.0, 32.9, 32.9, 20.1. MS (ESI, m/z): 448.7 $[M + Na]^+$. HPLC purity: 98.4%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl 5-chloropentanoate* (40). 40** was obtained from **10** and 5-chloropentanoyl chloride as described for method A: 62% yield as a light yellow solid; mp 83.7-85.9 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.33 (m, 1H), 7.28 (dd, *J* = 8.2, 1.2 Hz, 1H), 7.01 (dd, *J* = 8.2, 1.2 Hz, 1H), 6.95 – 6.85 (m, 4H), 5.86 (s, 1H), 3.83 (s, 3H), 3.64 – 3.53 (m, 2H), 3.36 (s, 3H), 2.67-2.57 (m, 2H), 1.98 – 1.84 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.7, 162.8, 157.1, 154.0, 145.6, 141.7, 130.8, 128.9, 126.8, 122.7, 120.1, 117.5, 116.6, 116.2, 110.2, 96.2, 55.9, 44.5, 43.9, 36.9, 31.1, 22.5. MS (ESI, m/z): 438.3 $[M + Na]^+$. HPLC purity: 98.4%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl 3,3-dimethyl-butanoate* (41). 41** was obtained from **10** and 3,3-dimethylbutanoyl chloride as described for method A: 58% yield as a light yellow solid; mp 98.5-100.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.32 (m, 1H), 7.28 (dd, *J* = 9.1, 1.8 Hz, 1H), 7.02 (dd, *J* = 8.3, 1.3 Hz, 1H), 6.95 – 6.85 (m, 4H), 5.85 (s, 1H), 3.82 (s, 3H), 3.36 (s, 3H), 2.44 (s, 2H), 1.13 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.7, 162.6, 156.8, 154.2, 149.6, 141.1, 140.5, 130.9, 126.6, 123.0, 122.9, 120.2, 117.5, 115.8, 112.9, 96.6, 55.9, 47.5, 44.0, 31.0, 29.5, 29.5, 29.5. MS (ESI, m/z): 418.5 $[M + Na]^+$. HPLC purity: 98.1%.

General Procedures of Method B for the Synthesis of 42−47. RCOOH (1.5 mmol) was added to a stirred mixture of compound **10** (1 mmol), EDCI (288 mg, 1.5 mmol), and DMAP (61 mg, 0.5 mmol) in anhydrous CH_2Cl_2 (5 mL). The mixture was stirred at room temperature for 12 h. On completion, the slurry was partitioned between water (20 mL) and CH_2Cl_2 (20 mL), and the water was extracted with CH_2Cl_2 (3×10 mL). The organic solvents were combined and removed under reduced pressure to yield a yellow solid. Chromatographic separation (petroleum ether−ethyl acetate, 5:1) gave the title compounds.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl pent-4-enoate* (42).** **42** was obtained from **10** and pent-4-enoic acid as described for method B: 63% yield as a yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 – 7.32 (m, 1H), 7.28 (d, *J* = 7.9 Hz, 1H), 7.01 (d, *J* = 8.2 Hz, 1H), 6.96-6.85 (m, 4H), 5.95-5.85 (m, 1H), 5.85 (s, 1H), 5.19 – 5.08 (m, 1H), 5.06 (d, *J* = 10.2 Hz, 1H), 3.82 (s, 3H), 3.35 (s, 3H), 2.67 (t, *J* = 7.4 Hz, 2H), 2.50 (dd, *J* = 13.8, 6.7 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.6, 162.8, 157.1, 154.2, 145.7, 141.6, 136.7, 130.7, 129.0, 126.8, 122.7, 120.0, 117.5, 116.6, 116.2, 115.9, 110.2, 96.1, 55.9, 43.9, 37.0, 29.2. MS (ESI, m/z): 380.2 $[M + H]^+$. HPLC purity: 97.9%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl (E)-2-methylpent-2-enoate* (43). 43** was obtained from **10** and (*E*)-2-methylpent-2-enoic acid as described for method B: 61% yield as a light yellow solid; mp 140.9-142.1 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.33 (m, 1H), 7.30 – 7.27 (m, 1H), 7.05 (dd, *J* = 8.2, 1.2 Hz, 1H), 7.04-6.98 (m, 1H), 6.96 – 6.86 (m, 4H), 5.85 (s, 1H), 3.82 (s, 3H), 3.36

(s, 3H), 2.27 (p, *J* = 7.4 Hz, 2H), 1.94 (s, 3H), 1.10 (t, *J* = 7.6 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.8, 162.6, 156.8, 154.1, 149.8, 146.6, 141.1, 140.9, 130.9, 126.7, 126.1, 123.1, 123.1, 120.3, 117.5, 115.9, 112.9, 96.5, 56.1, 44.0, 22.3, 12.9, 12.4. MS (ESI, m/z): 394.2 $[M + H]^+$. HPLC purity: 98.9%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl (E)-hex-2-enoate* (44). 44** was obtained from **10** and (*E*)-hex-2-enoic acid as described for method B: 57% yield as a light yellow solid; mp 172.2-174.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.32 (m, 1H), 7.28 (dd, *J* = 8.5, 1.1 Hz, 1H), 7.18 (dt, *J* = 15.6, 6.9 Hz, 1H), 7.03 (dd, *J* = 8.2, 1.2 Hz, 1H), 6.96-6.87 (m, 4H), 6.02 (dt, *J* = 15.6, 1.5 Hz, 1H), 5.85 (s, 1H), 3.83 (s, 3H), 3.36 (s, 3H), 2.32 – 2.21 (m, 2H), 1.54 (d, *J* = 7.4 Hz, 2H), 0.97 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.1, 162.6, 156.8, 154.2, 152.3, 149.7, 141.1, 140.5, 130.9, 126.7, 123.0, 122.9, 120.2, 119.9, 117.5, 115.9, 113.0, 96.6, 56.1, 44.0, 34.5, 21.1, 13.7. MS (ESI, m/z): 394.2 $[M + H]^+$. HPLC purity: 98.6%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl (tert-butoxy-carbonyl) -glycinate* (45). 45** was obtained from **10** and (tert-butoxycarbonyl)glycine as described for method B: 59% yield as a light yellow solid; mp 172.2-174.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.33 (m, 1H), 7.29 (dd, *J* = 8.3, 1.0 Hz, 1H), 7.00 (dd, *J* = 8.2, 1.2 Hz, 1H), 6.92 (d, *J* = 8.5 Hz, 4H), 5.87 (s, 1H), 5.04 (s, 1H), 4.19 (d, *J* = 5.4 Hz, 2H), 3.82 (s, 3H), 3.35 (s, 3H), 1.46 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.3, 162.6, 156.8, 155.6, 154.2, 149.3, 141.2, 139.9, 131.0, 126.6, 123.5, 123.0, 119.9, 117.6, 115.8, 113.1, 96.9, 80.2, 56.1, 44.0, 29.7, 28.3, 28.3, 28.3. MS (ESI, m/z): 455.2 $[M + H]^+$. HPLC purity: 98.2%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl (tert-butoxy-carbonyl) -alaninate* (46). 46** was obtained from **10** and (tert-butoxycarbonyl)alanine as described for method B: 56% yield as a light yellow solid; mp 172.2-174.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.33 (m, 1H), 7.29 (dd, *J* = 8.3, 1.1 Hz, 1H), 7.01 (dd, *J* = 8.3, 1.4 Hz, 1H), 6.95 – 6.86 (m, 4H), 5.85 (s, 1H), 5.19 (s, 1H), 3.87 (s, 3H), 3.56-3.50 (m, 1H), 3.35 (s, 3H), 1.58 (s, 3H), 1.45 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.9, 162.5, 156.8, 155.8, 154.1, 149.1, 141.2, 140.1, 131.0, 126.6, 123.4, 123.0, 120.0, 117.5, 115.8, 112.9, 96.8, 79.4, 56.1, 43.9, 36.2, 34.8, 28.4, 28.4, 28.4. MS (ESI, m/z): 469.2 $[M + H]^+$. HPLC purity: 98.0%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl (tert-butoxy-carbonyl) -leucinate* (47). 47** was obtained from **10** and (tert-butoxycarbonyl)leucine as described for method B: 56% yield as a light yellow solid; mp 172.2-174.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.32 (m, 1H), 7.31 – 7.27 (m, 1H), 7.01 (dd, *J* = 8.3, 1.3 Hz, 1H), 6.97 – 6.83 (m, 4H), 5.86 (s, 1H), 4.92 (d, *J* = 8.6 Hz, 1H), 4.54 (s, 1H), 3.80 (s, 3H), 3.35 (s, 3H), 1.90 – 1.76 (m, 2H), 1.71 – 1.56 (m, 1H), 1.44 (s, 9H), 1.00 (d, *J* = 6.3 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.3, 162.6, 156.8, 155.4, 154.2, 149.4, 141.2, 140.3, 131.0, 126.6, 123.4, 123.0, 120.0, 117.6, 115.9, 113.0, 96.9, 80.1, 56.1, 52.3, 44.0, 41.6, 28.3, 28.3 28.3, 24.8, 23.0, 21.9. MS (ESI, m/z): 511.2 $[M + H]^+$. HPLC purity: 98.2%.

***4- (1-(3-(2-(Cyclopenta-1,3-dien-1-yl)allyl)- 4- ethylphenyl)ethyl)-2-methylene -1,2- dihydronaphthalene* (48). 48** was obtained from **10** and furan-2-carbonyl chloride as described for method A: 56% yield as a light yellow solid; mp

148.4-150.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 (s, 1H), 7.39 (d, *J* = 3.4 Hz, 1H), 7.36 (d, *J* = 7.6 Hz, 1H), 7.29 (d, *J* = 8.4 Hz, 1H), 7.07 – 7.01 (m, 2H), 6.99-6.85 (m, 3H), 6.60 (d, *J* = 1.7 Hz, 1H), 5.87 (s, 1H), 3.83 (s, 3H), 3.38 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.6, 156.8, 155.9, 154.2, 149.7, 147.3, 143.5, 141.3, 139.8, 131.0, 126.6, 123.6, 123.0, 120.2, 119.8, 117.6, 115.9, 113.2, 112.3, 96.9, 56.2, 44.0. MS (ESI, m/z): 414.2 $[M + Na]^+$. HPLC purity: 98.6%.

***2- Methoxy- 5- (methyl (2- oxo- 2H- chromen- 4 -yl) amino) phenyl thiophene-2 -carboxylate* (49). 49** was obtained from **10** and thiophene-2-carbonyl chloride as described for method A: 55% yield as a light yellow solid; mp 137.5-139.3 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 (d, *J* = 4.9 Hz, 1H), 7.45 (d, *J* = 3.5 Hz, 1H), 7.40 (t, *J* = 7.4 Hz, 1H), 7.32 (d, *J* = 8.2 Hz, 1H), 7.07 – 7.02 (m, 1H), 7.02-6.92 (m, 3H), 6.89-6.81 (m, 2H), 5.85 (s, 1H), 3.70 (s, 3H), 3.32 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.38 (s), 156.74 (s), 155.4, 154.26 (s), 150.41 (s), 141.10 (s), 138.85 (s), 135.36 (s), 135.14 (s), 134.56 (s), 131.16 (s), 127.40 (s), 126.58 (s), 124.71 (s), 123.09 (s), 120.8, 117.6, 115.9, 113.5, 97.5, 56.1, 43.9. MS (ESI, m/z): 408.1 $[M + H]^+$. HPLC purity: 97.9%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl 2-methylbenzoate* (50). 50** was obtained from **10** and 2-methylbenzoyl chloride as described for method A: 58% yield as a light yellow solid; mp 142.3-145.1 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (d, *J* = 7.8 Hz, 1H), 7.48 (t, *J* = 7.5 Hz, 1H), 7.37 (t, *J* = 7.7 Hz, 1H), 7.35-7.25 (m, 3H), 7.08 (d, *J* = 8.1 Hz, 1H), 7.02 (s, 1H), 6.98 – 6.91 (m, 3H), 5.88 (s, 1H), 3.84 (s, 3H), 3.39 (s, 3H), 2.66 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.9, 162.6, 156.8,

154.2, 149.7, 141.2, 141.1, 140.7, 132.8, 131.8, 131.2, 130.9, 128.1, 126.7, 125.9, 123.2, 123.0, 120.3, 117.6, 115.9, 113.0, 96.7, 56.1, 44.0, 21.7. MS (ESI, m/z): 438.1 $[M + Na]^+$. HPLC purity: 98.0%.

***2-methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl 3-chlorobenzoate* (51). 51** was obtained from **10** and 3-chlorobenzoyl chloride as described for method A: 53% yield as a light yellow solid; mp 190.2-192.5°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.17 (s, 1H), 8.07 (d, *J* = 7.6 Hz, 1H), 7.61 (d, *J* = 8.2 Hz, 1H), 7.46 (t, *J* = 7.9 Hz, 1H), 7.38 (t, *J* = 7.5 Hz, 1H), 7.30 (d, *J* = 8.4 Hz, 1H), 7.07 (d, *J* = 8.2 Hz, 1H), 7.01 (s, 1H), 7.00 – 6.91 (m, 3H), 5.88 (s, 1H), 3.83 (s, 3H), 3.39 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.2, 162.6, 156.9, 154.2, 149.6, 141.3, 140.4, 134.8, 133.7, 131.0, 130.7, 130.3, 129.9, 128.4, 126.6), 123.5, 123.0, 120.0, 117.6, 115.9, 113.1, 96.9, 56.2, 44.1. MS (ESI, m/z): 458.5 $[M + Na]^+$. HPLC purity: 97.9%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl 4-methoxybenzoate* (52). 52** was obtained from **10** and 4-methoxybenzoyl chloride as described for method A: 53% yield as a light yellow solid; mp 136.1-138.6°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (d, *J* = 8.8 Hz, 2H), 7.37 (t, *J* = 7.6 Hz, 1H), 7.29 (d, *J* = 8.2 Hz, 1H), 7.11 – 6.89 (m, 7H), 5.87 (s, 1H), 3.90 (s, 3H), 3.82 (s, 3H), 3.38 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.1, 164.0, 162.7, 156.9, 154.2, 149.9, 141.2, 140.8), 132.5, 132.5, 131.0, 126.7, 123.2, 123.0, 121.2, 120.4, 117.6, 115.9, 113.9, 113.9, 113.0, 96.7, 56.2, 55.5, 44.1. MS (ESI, m/z): 454.4 $[M + Na]^+$. HPLC purity: 97.9%.

General Procedures of Method C for the Synthesis of 53−55. To a solution of **10** (365 mg, 1 mmol) in CH_2Cl_2 (5 mL) was added RSO_2Cl (2 equiv) and Et_3N (2 equiv)

and stirred at room temperature for 6 h. When completed, the solvent was removed under vacuum and the residue was purified by flash chromatograph using petroleum ether/ethyl acetate (5:1) as eluent to give the title compounds.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl methanesulfonate* (53). 53** was obtained from **10** and methylsulfonyl chloride as described for method C: 63% yield as a light yellow solid; mp 185.4-186.9 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 (t, *J* = 7.3 Hz, 1H), 7.30 (d, *J* = 8.2 Hz, 1H), 7.16 (d, *J* = 2.1 Hz, 1H), 7.02 – 6.87 (m, 4H), 5.90 (s, 1H), 3.90 (s, 3H), 3.37 (s, 3H), 3.19 (s, 3H); ^{13}C NMR (101 MHz, DMSO) δ 157.7, 152.0, 149.5, 144.9, 136.7, 133.9, 126.4, 121.7, 119.6, 118.3, 116.8, 112.9, 111.0, 108.8, 93.0, 51.6, 39.1, 33.9. ESI HRMS: calcd. for $C_{18}H_{17}NO_6S+H^+$ 376.0849, found 376.1383. HPLC purity: 97.3%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl 4-fluorobenzene-sulfonate* (54). 54** was obtained from **10** and 4-fluorobenzenesulfonyl chloride as described for method C: 63% yield as a light yellow solid; mp 167.9-169.6 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81-7.77 (m, 2H), 7.41 (t, *J* = 7.4 Hz, 1H), 7.33 (d, *J* = 8.2 Hz, 1H), 7.16 (t, *J* = 8.4 Hz, 2H), 7.01 – 6.90 (m, 4H), 6.81 (d, *J* = 8.6 Hz, 1H), 5.87 (s, 1H), 3.62 (s, 3H), 3.34 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.3, 164.7, 162.4, 156.7, 154.3, 149.9, 141.2, 138.6, 131.4, 131.3, 131.2, 126.5, 124.6, 122.9, 121.1, 117.7, 116.3, 116.1, 115.8, 113.4, 97.6, 55.9, 43.8. ESI HRMS: calcd. for $C_{23}H_{18}FNO_6S+H^+$ 456.0912, found 456.1704. HPLC purity: 98.3%.

***2- Methoxy- 5- (methyl (2-oxo-2H-chromen-4-yl) amino) phenyl 4- ethylbenzene-sulfonate* (55). 55** was obtained from **10** and 4-ethylbenzenesulfonyl chloride as

described for method C: 62% yield as a light yellow solid; mp 180.1-181.3 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (d, *J* = 7.9 Hz, 2H), 7.38-7.30 (m, 4H), 7.08 (d, *J* = 8.2 Hz, 1H), 7.03 (s, 1H), 6.99 – 6.90 (m, 3H), 5.87 (s, 1H), 3.81 (s, 3H), 3.38 (s, 3H), 2.74 (q, *J* = 7.6 Hz, 2H), 1.28 (t, *J* = 7.6 Hz, 3H); ^{13}C NMR (101 MHz, DMSO) δ 158.1, 152.3, 149.4, 148.1, 141.9, 140.3, 137.4, 132.5, 129.1, 129.1, 126.7, 126.7, 126.1, 121.9, 118.1, 112.8, 112.1, 111.4, 107.1, 106.4, 91.3, 51.4, 39.3, 28.2, 14.5. HPLC purity: 98.3%.

***Preparation of 4-((4-methoxy-3-(2-morpholinoethoxy)phenyl)(methyl)amino)-2H-chromen-2-one* (56).** Compound **10** (1 mmol, 1 equiv) and Cs_2CO_3 (652 mg, 2 mmol, 2 equiv) were added into 5 mL of CH_3CN, and the resulting mixture was heated at reflux for 0.5 h. Then the 4-(2-chloroethyl)morpholine (2 mmol, 2 equiv) was added. After 2 h, the solvent was filtered through Celite. Thefiltrate was concentrated in vacuo to yield a yellow oily product. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (t, *J* = 7.6 Hz, 1H), 7.29 (s, 1H), 6.98 (d, *J* = 8.2 Hz, 1H), 6.87 (t, *J* = 7.7 Hz, 1H), 6.81 (d, *J* = 8.5 Hz, 1H), 6.74 – 6.64 (m, 2H), 5.83 (s, 1H), 4.16-4.06 (m, 2H), 3.86 (s, 3H), 3.82-3.70 (m, 4H), 3.35 (s, 3H), 2.89-2.40 (m, 2H), 2.66-2.56 (m, 4H). ESI HRMS: calcd. for $C_{23}H_{26}N_2O_5$+H^+ 411.1914, found 411.1912. HPLC purity: 98.3%.

***Preparation of 4-((3-amino-4-methoxyphenyl)(methyl)amino)-2H-chromen-2-one* (60).** A mixture of 4-methoxy-3-(methoxymethoxy)-*N*-methylaniline **59** (1.52 g, 10 mmol), 4-bromo-*2H*-chromen-2-one **20** (10 mmol), and DIEA (15 mmol) in 50 mL DMF was stirred at 100°C for 24 h. The reaction mixture was diluted with 500 mL of ethyl acetate, and it was washed with water (2x), brine (1x) and dried over $MgSO_4$,

filtered, and concentrated. The crude product was purified by chromatography (40% ethyl acetate/hexanes) on silica gel to give **60**. 62% yield as a light yellow solid; mp 168.5-170.2 °C; ^{1}H NMR (400 MHz, DMSO) δ 7.47 – 7.39 (m, 1H), 7.30 (dd, *J* = 8.2, 0.8 Hz, 1H), 7.10 (dd, *J* = 8.2, 1.3 Hz, 1H), 7.01 – 6.95 (m, 1H), 6.77 (d, *J* = 8.5 Hz, 1H), 6.45 (d, *J* = 2.6 Hz, 1H), 6.37 (dd, *J* = 8.4, 2.6 Hz, 1H), 5.75 (s, 1H), 4.90 (s, 2H), 3.76 (s, 3H), 3.28 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.9, 157.0, 154.1, 145.6, 141.8, 137.5, 130.7, 126.9, 122.7, 117.4, 116.2, 114.9, 111.6, 110.8, 95.0, 55.7, 44.2. MS (ESI, *m/z*): 297.2 $[M + H]^+$. HPLC purity: 98.5%.

General Procedures of Method A for the Synthesis of 61-65. To a solution of **60** (365 mg, 1 mmol) in CH_2Cl_2 (5 mL) was added RCO_2Cl (2 equiv) and Et_3N (2 equiv) and stirred at room temperature for 6 h. When completed, the solvent was removed under vacuum and the residue was purified by flash chromatograph using petroleum ether/ethyl acetate (5:1) as eluent to give the title compounds.

***Ethyl 4-((2-methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl)amino)-4-oxobutanoate* (61). 61** was obtained from **60** and 4-ethoxy-4-oxobutanoyl chloride as described for method A: 63% yield as a light yellow solid; mp 117.0-118.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.46 (d, *J* = 2.3 Hz, 1H), 8.02 (s, 1H), 7.36 – 7.30 (m, 1H), 7.30-7.24 (m, 1H), 7.03 (dd, *J* = 8.2, 1.1 Hz, 1H), 6.92 – 6.82 (m, 1H), 6.71 (d, *J* = 8.6 Hz, 1H), 6.56 (dd, *J* = 8.6, 2.6 Hz, 1H), 5.83 (s, 1H), 4.17 (q, *J* = 7.3 Hz, 2H), 3.88 (s, 3H), 3.35 (s, 3H), 2.78-2.70 (m, 4H), 1.28 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.8, 169.8, 162.8, 157.0, 154.2, 145.8, 141.6, 130.7, 129.0, 126.8, 122.7,

120.1, 117.5, 116.6, 116.2, 110.3, 96.1, 60.9, 56.0, 44.0, 32.3, 29.2, 14.2. MS (ESI, *m/z*): 447.1 $[M + Na]^+$. HPLC purity: 98.0%.

***Methyl 5-((2-methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl)amino)-5-oxopentanoate* (62). 62** was obtained from **60** and 5-methoxy-5-oxopentanoyl chloride as described for method A: 62% yield as a light yellow solid; mp 118.7-120.2 °C; ^{1}H NMR (400 MHz, CDCl3) δ 8.48 (s, 1H), 7.87 (s, 1H), 7.38 – 7.29 (m, 1H), 7.27 (d, *J* = 4.3 Hz, 1H), 7.03 (t, *J* = 10.1 Hz, 1H), 6.86 (t, *J* = 7.5 Hz, 1H), 6.71 (d, *J* = 8.7 Hz, 1H), 6.56 (dd, *J* = 8.6, 2.6 Hz, 1H), 5.83 (s, 1H), 3.88 (s, 3H), 3.70 (s, 3H), 3.36 (s, 3H), 2.53-2.47 (m, 4H), 2.11 – 2.03 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.3, 170.5, 162.6, 156.8, 154.1, 149.4, 141.1, 140.3, 131.0, 126.6, 123.2, 122.9, 120.0, 117.5, 115.8, 112.9, 96.7, 56.0, 51.6, 44.0, 32.9, 32.9, 20.1. MS (ESI, *m/z*): 447.1 $[M + Na]^+$. HPLC purity: 98.1%.

***N-(2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl)hexanamide* (63). 63** was obtained from **60** and hexanoyl chloride as described for method A: 62% yield as a light yellow solid; mp 146.8-149.1 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 (d, *J* = 2.3 Hz, 1H), 7.83 (s, 1H), 7.33 (t, *J* = 7.6 Hz, 1H), 7.30-7.25 (m, 1H), 7.05 (d, *J* = 7.4 Hz, 1H), 6.86 (t, *J* = 7.1 Hz, 1H), 6.71 (d, *J* = 8.6 Hz, 1H), 6.55 (dd, *J* = 8.6, 2.5 Hz, 1H), 5.83 (s, 1H), 3.88 (s, 3H), 3.38 (s, 3H), 2.40 (t, *J* = 7.6 Hz, 2H), 1.80 – 1.69 (m, 2H), 1.42 – 1.33 (m, 4H), 0.93 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.6, 162.8, 157.1, 154.2, 145.7, 141.7, 130.7, 129.1, 126.8, 122.7, 119.9, 117.5, 116.5, 116.2, 110.2, 96.1, 55.9, 43.9, 38.0, 31.4, 25.1, 22.4, 13.9. MS (ESI, *m/z*): 417.4 $[M + Na]^+$. HPLC purity: 98.1%.

***N-(2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl)decanamide* (64).** **64** was obtained from **60** and decanoyl chloride as described for method A: 54% yield as a yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 (d, *J* = 2.5 Hz, 1H), 7.83 (s, 1H), 7.36 – 7.30 (m, 1H), 7.29 – 7.27 (m, 1H), 7.05 (dd, *J* = 8.2, 1.2 Hz, 1H), 6.91 – 6.81 (m, 1H), 6.71 (d, *J* = 8.6 Hz, 1H), 6.55 (dd, *J* = 8.6, 2.6 Hz, 1H), 5.83 (s, 1H), 3.88 (s, 3H), 3.36 (s, 3H), 2.42 (t, *J* = 7.6 Hz, 2H), 1.78-1.92 (m, 2H), 1.64 – 1.00 (m, 12H), 0.88 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.6, 162.8, 157.1, 154.2, 145.7, 141.6, 130.7, 129.1, 126.8, 122.7, 119.9, 117.5, 116.5, 116.2, 110.2, 96.1, 56.0, 43.90, 38.00, 31.9, 29.4, 29.4, 29.3, 29.3, 25.4, 22.7, 14.1. MS (ESI, *m/z*): 473.4 [M + Na]$^+$. HPLC purity: 98.1%.

***5-Chloro-N-(2-methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl)pentan amide* (65). 65** was obtained from **60** and 5-chloropentanoyl chloride as described for method A: 62% yield as a light yellow solid; mp 154.8-156.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.48 (d, *J* = 2.4 Hz, 1H), 7.83 (s, 1H), 7.33 (t, *J* = 8.4 Hz, 1H), 7.30-7.25 (m, 1H), 7.07 – 7.01 (m, 1H), 6.91 – 6.81 (m, 1H), 6.72 (d, *J* = 8.6 Hz, 1H), 6.57 (dd, *J* = 8.6, 2.6 Hz, 1H), 5.83 (s, 1H), 3.88 (s, 3H), 3.64 – 3.55 (m, 2H), 3.36 (s, 3H), 2.46 (d, *J* = 6.6 Hz, 2H), 1.95 – 1.83 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.7, 162.8, 157.1, 154.2, 145.7, 141.7, 130.8, 128.9, 126.8, 122.7, 120.1, 117.5, 116.6, 116.2, 110.2, 96.2, 55.9, 44.5, 43.9, 36.9, 31.9, 22.7. MS (ESI, *m/z*): 437.5 [M + Na]$^+$. HPLC purity: 99.1%.

General Procedures of Method B for the Synthesis of 66-70. RCOOH (1.5 mmol) was added to a stirred mixture of compound **10** (1 mmol), EDCI (288 mg, 1.5 mmol),

and DMAP (61 mg, 0.5 mmol) in anhydrous CH_2Cl_2 (5 mL). The mixture was stirred at room temperature for 12 h. On completion, the slurry was partitioned between water (20 mL) and CH_2Cl_2 (20 mL), and the water was extracted with CH_2Cl_2 (3×10 mL). The organic solvents were combined and removed under reduced pressure to yield a yellow solid. Chromatographic separation (petroleum ether−ethyl acetate, 5:1) gave the title compounds.

***(E)-N-(2-methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl)but-2-enamide* (66). 66** was obtained from **60** and (*E*)-but-2-enoic acid as described for method B: 62% yield as a light yellow solid; mp 178.2-179.1 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.58 (s, 1H), 7.83 (s, 1H), 7.36 – 7.29 (m, 1H), 7.27 (d, *J* = 2.7 Hz, 1H), 7.08 – 6.95 (m, 2H), 6.85 (t, *J* = 7.0 Hz, 1H), 6.72 (d, *J* = 8.6 Hz, 1H), 6.57 (dd, *J* = 8.6, 2.5 Hz, 1H), 6.01 (d, *J* = 14.9 Hz, 1H), 5.84 (s, 1H), 3.88 (s, 3H), 3.37 (s, 3H), 1.94 (d, *J* = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.8, 161.7, 159.6, 156.9, 154.2, 149.8, 141.1, 140.4, 130.9, 126.7, 123.0, 123.0, 120.4, 117.5, 115.9, 114.5, 112.9, 96.5, 56.1, 44.0, 20.5. MS (ESI, *m/z*): 387.5 $[M + Na]^+$. HPLC purity: 98.3%.

***N-(2-methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl)pent-4-enamide* (67). 67** was obtained from **60** and pent-4-enoic acid as described for method B: 62% yield as a light yellow solid; mp 127.6-129.4 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50 (d, *J* = 2.1 Hz, 1H), 7.86 (s, 1H), 7.32 (t, *J* = 7.2 Hz, 1H), 7.27 (d, *J* = 8.4 Hz, 1H), 7.05 (d, *J* = 8.4 Hz, 1H), 6.86 (t, *J* = 7.6 Hz, 1H), 6.71 (d, *J* = 8.6 Hz, 1H), 6.56 (dd, *J* = 8.6, 2.4 Hz, 1H), 5.93-5.89 (m, 1H), 5.84 (s, 1H), 5.14 (d, *J* = 16.6 Hz, 1H), 5.07 (d, *J* = 10.3 Hz, 1H), 3.88 (s, 2H), 3.36 (s, 3H), 2.56-2.50 (m, 4H); ^{13}C NMR (101 MHz,

$CDCl_3$) δ 170.6, 162.8, 157.1, 154.2, 145.7, 141.6, 136.7, 130.7, 129.0, 126.8, 122.7, 120.0, 117.5, 116.6, 116.2, 115.9, 110.2, 96.1, 55.9, 43.9, 37.0, 29.2. MS (ESI, *m/z*): 379.3 $[M + H]^+$. HPLC purity: 98.9%.

***(E)-N-(2-methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl)-2-methylpent-2-enamide* (68). 68** was obtained from **60** and (*E*)-2-methylpent-2-enoic acid as described for method B: 62% yield as a yellow oil; ^{1}H NMR (400 MHz, DMSO) δ 8.67 (s, 1H), 7.91 (d, *J* = 2.4 Hz, 1H), 7.43 (t, *J* = 7.1 Hz, 1H), 7.32 (d, *J* = 8.1 Hz, 1H), 7.08 – 6.84 (m, 4H), 6.42 (t, *J* = 6.9 Hz, 1H), 5.84 (d, *J* = 6.3 Hz, 1H), 3.85 (s, 3H), 3.32 (s, 3H), 2.16 (p, *J* = 7.2 Hz, 2H), 1.83 (s, 3H), 1.00 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR (101 MHz, DMSO) δ 167.3, 161.6, 156.9, 154.1, 148.3, 141.3, 138.8, 131.6, 130.8, 128.8, 126.8, 123.4, 121.5, 118.9, 117.6, 116.2, 112.2, 96.0, 56.6, 44.5, 21.7, 13.6, 12.8. MS (ESI, *m/z*): 415.2 $[M + Na]^+$. HPLC purity: 97.9%.

***Tert-butyl-(2-((2-methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl)-amino)-2-oxoethyl)carbamate* (69). 69** was obtained from **60** and (tert-butoxycarbonyl)-glycine as described for method B: 62% yield as a light yellow solid; mp 127.6-129.4 °C; H19 : ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 (s, 1H), 8.45 (d, *J* = 2.4 Hz, 1H), 7.36 – 7.31 (m, 1H), 7.31-7.25 (m, 1H), 7.03 (dd, *J* = 8.2, 1.2 Hz, 1H), 6.93 – 6.82 (m, 1H), 6.72 (d, *J* = 8.6 Hz, 1H), 6.59 (dd, *J* = 8.6, 2.3 Hz, 1H), 5.84 (s, 1H), 5.20 (s, 1H), 3.97 (d, *J* = 3.9 Hz, 2H), 3.86 (s, 3H), 3.36 (s, 3H), 1.50 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.8, 162.8, 157.0, 156.2, 154.1, 146.0, 141.6, 130.7, 128.6, 126.8, 122.7, 120.4, 117.5, 116.6, 116.0, 110.4, 96.0, 80.6, 56.0, 45.5, 43.9, 28.3, 28.3, 28.3. MS (ESI, *m/z*): 476.2 $[M + Na]^+$. HPLC purity: 98.0%.

***2-Methoxy-5-(methyl(2-oxo-2H-chromen-4-yl)amino)phenyl 3-((tert-butoxy-carbonyl)amino)propanoate* (70). 70** was obtained from **60** and (tert-butoxy-carbonyl)leucine as described for method B: 62% yield as a light yellow solid; mp 89.5-92.7 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (d, *J* = 2.3 Hz, 1H), 7.86 (s, 1H), 7.33 (t, *J* = 7.6 Hz, 1H), 7.30 – 7.27 (m, 1H), 7.03 (t, *J* = 8.0 Hz, 1H), 6.87 (t, *J* = 7.6 Hz, 1H), 6.73 (d, *J* = 8.7 Hz, 1H), 6.59 (dd, *J* = 8.6, 2.5 Hz, 1H), 5.84 (s, 1H), 5.13 (s, 1H), 3.87 (s, 3H), 3.53 – 3.46 (m, 2H), 3.36 (s, 3H), 2.65 (t, *J* = 5.8 Hz, 2H), 1.45 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.9, 162.7, 157.0, 156.0, 154.2, 145.7, 141.6, 130.7, 128.7, 126.7, 122.7, 120.3, 117.5, 116.6, 116.1, 110.3, 96.2, 79.4, 55.9, 44.0, 37.5, 36.5, 28.4, 28.4, 28.4. MS (ESI, m/z): 490.3 $[M + Na]^+$. HPLC purity: 98.3%.

General Procedures for the Synthesis of Compounds 77-89. To a solution of **75** (177 mg, 0.5 mmol) in EtOH (3 mL), PhMe (3 mL) and H_2O (1 mL) were added Na_2CO_3 (159 mg, 1.5 mmol), the appropriate boronic acid (0.6 mmol), and $Pd(PPh_3)_4$ (0.1 mol). The resulting mixture was stirred for 2 h at 80 °C and then filtered. The catalyst was washed with H_2O (3 mL) and CH_2Cl_2 (5 mL). The aqueous phase was extracted twice with CH_2Cl_2. The collected organic extracts were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by flash chromatography to afford the expected compound.

***4-(5-(4-Methoxyphenyl)thiophen-2-yl)-2H-chromen-2-one* (77). 77** was obtained from **75** and (4-methoxyphenyl)boronic acid as described for general procedures: 52% yield as a light yellow solid; mp 164.0-165.5 °C; ^{1}H NMR (400 MHz, DMSO) δ 7.68-7.63 (m, 2H), 7.51 (d, *J* = 8.0 Hz, 1H), 7.38-7.34 (m, 2H), 7.18 – 7.13 (m, 2H),

7.13 – 7.08 (m, 2H), 6.81 (d, *J* = 8.7 Hz, 2H), 3.72 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.1, 159.3, 152.9, 144.4, 134.7, 131.5, 131.5, 131.4, 129.9, 128.3, 127.9, 127.5, 126.9, 126.2, 124.3, 120.6, 116.8, 113.4, 113.4, 55.2. MS (ESI, m/z): 357.1 [M + Na]$^+$. HPLC purity: 98.0%.

4-(5-(4-(Methylsulfonyl)phenyl)thiophen-2-yl)-2H-chromen-2-one **(78). 78** was obtained from **75** and (4-(methylsulfonyl)phenyl)boronic acid as described for general procedures: 52% yield as a light yellow solid; mp 174.5-176.8 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (d, *J* = 8.4 Hz, 2H), 7.61 (t, *J* = 7.8 Hz, 1H), 7.59 – 7.54 (m, 1H), 7.50 – 7.39 (m, 4H), 7.29 (t, *J* = 7.7 Hz, 1H), 7.03 (dd, *J* = 5.0, 3.7 Hz, 1H), 6.99 – 6.95 (m, 1H), 3.04 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.3, 153.2, 146.4, 140.1, 139.7, 133.3, 132.5, 131.4, 131.4, 130.4, 128.8, 127.9, 127.4, 127.1, 127.1, 126.6, 124.7, 120.1, 116.9, 44.5. MS (ESI, m/z): 405.0 [M + Na]$^+$. HPLC purity: 98.0%.

4-(5-(p-Tolyl)thiophen-2-yl)-2H-chromen-2-one **(79). 79** was obtained from **75** and *p*-tolylboronic acid as described for general procedures: 52% yield as a light yellow solid; mp 165.5-167.8 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 – 7.50 (m, 2H), 7.42 (d, *J* = 8.2 Hz, 1H), 7.37 (d, *J* = 5.2 Hz, 1H), 7.23 (d, *J* = 7.1 Hz, 1H), 7.11-7.05 (m, 4H), 7.05 – 7.01 (m, 1H), 7.01 – 6.97 (m, 1H), 2.30 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.0, 153, 144.5, 137.9, 134.6, 131.5, 131.0, 130.0, 130.0, 129.9, 128.7, 128.7, 128.7, 127.9, 127.5, 126.9, 124.3, 120.6, 116.8, 21.4. MS (ESI, m/z): 341.1 [M + Na]$^+$. HPLC purity: 97.8%.

4-(5-(4-Chlorophenyl)thiophen-2-yl)-2H-chromen-2-one **(80). 80** was obtained from **75** and (4-chlorophenyl)boronic acid as described for general procedures: 48%

yield as a light yellow solid; mp 179.2-181.5 °C; ^{1}H NMR (400 MHz, DMSO) δ 7.72 – 7.64 (m, 2H), 7.53 (d, *J* = 8.2 Hz, 1H), 7.40 (d, *J* = 8.7 Hz, 1H), 7.37 – 7.30 (m, 3H), 7.25 (d, *J* = 8.5 Hz, 2H), 7.17 – 7.14 (m, 1H), 7.13 – 7.09 (m, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.6, 153.0, 145.3, 134.1, 134.0, 132.5, 131.9, 131.7, 131.7, 130.1, 128.3, 128.2, 128.2, 127.7, 127.4, 127.1, 124.5, 120.3, 116.9. MS (ESI, m/z): 361.0 $[M + Na]^+$. HPLC purity: 98.2%.

4-(5-phenylthiophen-2-yl)-2H-chromen-2-one **(81). 81** was obtained from **75** and phenylboronic acid as described for general procedures: 49% yield as a light yellow solid; mp 206.8-208.6 °C; ^{1}H NMR (400 MHz, DMSO) δ 7.68 (d, *J* = 7.0 Hz, 1H), 7.65 (dd, *J* = 5.0, 1.0 Hz, 1H), 7.52 (d, *J* = 8.2 Hz, 1H), 7.43 – 7.39 (m, 1H), 7.35 (t, *J* = 7.5 Hz, 1H), 7.29 – 7.19 (m, 5H), 7.15 – 7.12 (m, 1H), 7.08 (dd, *J* = 4.9, 3.6 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.8, 153.0, 144.9, 134.4, 134.0, 131.7, 130.2, 130.2, 130.0, 128.6, 128.0, 128.0, 127.9, 127.9, 127.6, 126.9, 124.3, 120.5, 116.8. MS (ESI, m/z): 327.1 $[M + Na]^+$. HPLC purity: 97.6%.

4-(5-(3,4,5-Trimethoxyphenyl)thiophen-2-yl)-2H-chromen-2-one **(82). 82** was obtained from **75** and (3,4,5-trimethoxyphenyl)boronic acid as described for general procedures: 51% yield as a light yellow solid; mp 163.5-165.8 °C; ^{1}H NMR (400 MHz, DMSO) δ 7.70 (d, *J* = 4.9 Hz, 1H), 7.68 – 7.64 (m, 1H), 7.52 (d, *J* = 7.9 Hz, 1H), 7.42 (d, *J* = 6.8 Hz, 1H), 7.36 (t, *J* = 7.3 Hz, 1H), 7.17 (d, *J* = 2.4 Hz, 1H), 7.15 – 7.11 (m, 1H), 6.55 (s, 2H), 6.05 (s, 1H), 3.64 (s, 3H), 3.61 (s, 5H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.7, 152.9, 152.8, 145.0, 137.8, 134.7, 131.7, 129.8, 129.2, 128.2,

127.6, 127.0, 124.4, 120.4, 116.8, 107.8, 107.8, 104.7, 92.9, 60.8, 56.1, 56.1. MS (ESI, m/z): 417.1 $[M + Na]^+$. HPLC purity: 98.5%.

***4-(5-(2-methoxyphenyl)thiophen-2-yl)-2H-chromen-2-one* (83). 83** was obtained from **75** and (2-methoxyphenyl)boronic acid as described for general procedures: 50% yield as a light yellow solid; mp 202.4-204.8 °C; ^{1}H NMR (400 MHz, DMSO) δ 7.73 – 7.65 (m, 1H), 7.63 (dd, *J* = 4.9, 1.2 Hz, 1H), 7.52 (d, *J* = 8.3 Hz, 1H), 7.44 – 7.33 (m, 2H), 7.29 – 7.23 (m, 1H), 7.11 – 7.01 (m, 3H), 6.96 (d, *J* = 8.3 Hz, 1H), 6.83 (t, *J* = 7.4 Hz, 1H), 3.68 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.5, 157.7, 153.4, 145.8, 134.5, 131.6, 131.2, 130.0, 129.3, 127.7, 127.6, 126.8, 126.6, 124.2, 123.7, 120.6, 120.4, 117.0, 110.9, 55.6. MS (ESI, m/z): 357.1 $[M + Na]^+$. HPLC purity: 98.1%.

***7-Methoxy-4-(5-(3,4,5-trimethoxyphenyl)thiophen-2-yl)-2H-chromen-2-one* (84). 84** was obtained from **76** and (3,4,5-trimethoxyphenyl)boronic acid as described for general procedures: 51% yield as a light yellow solid; mp 172.0-175.5 °C; ^{1}H NMR (400 MHz, DMSO) δ 7.68 (d, *J* = 4.9 Hz, 1H), 7.32 (d, *J* = 8.9 Hz, 1H), 7.18 – 7.09 (m, 3H), 6.96 (dd, *J* = 8.9, 2.4 Hz, 1H), 6.51 (s, 2H), 3.89 (s, 3H), 3.62 (s, 9H); ^{13}C NMR (101 MHz, DMSO) δ 162.7, 161.2), 154.7, 152.8, 152.8, 145.2, 137.7, 135.1, 129.6, 129.5, 128.7, 128.1, 127.0, 125.1, 114.0, 112.5, 108.0, 108.0, 100.7, 60.9, 56.1, 56.1, 55.9. MS (ESI, m/z): 447.1 $[M + Na]^+$. HPLC purity: 98.0%.

***4-(5-(Benzo[d][1,3]dioxol-5-yl)thiophen-2-yl)-7-methoxy-2H-chromen-2-one* (85). 85** was obtained from **76** and benzo[*d*][1,3]dioxol-5-ylboronic acid as described for general procedures: 47% yield as a light yellow solid; mp 175.8-179.5 °C; ^{1}H NMR (400 MHz, DMSO) δ 7.72 – 7.65 (m, 1H), 7.29 (d, *J* = 8.9 Hz, 1H), 7.17 – 7.13 (m,

1H), 7.11 (d, *J* = 4.8 Hz, 2H), 6.95 (dd, *J* = 8.9, 2.3 Hz, 1H), 6.81 – 6.74 (m, 2H), 6.62 (dd, *J* = 8.0, 1.3 Hz, 1H), 5.99 (s, 2H), 3.88 (s, 3H); ^{13}C NMR (101 MHz, DMSO) δ 162.7, 161.4, 154.7, 147.3, 147.3, 145.1, 134.9, 129.8, 128.7, 127.9, 127.8, 126.9, 124.9, 124.4, 113.9, 112.5, 110.9, 108.1, 101.1, 100.7, 55.9. MS (ESI, m/z): 401.1 $[M + Na]^+$. HPLC purity: 97.8%.

7-Methoxy-4-(5-(p-tolyl)thiophen-2-yl)-2H-chromen-2-one **(86). 86** was obtained from **76** and *p*-tolylboronic acid as described for general procedures: 54% yield as a light yellow solid; mp 164.5-168.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 (d, *J* = 4.4 Hz, 1H), 7.28 (d, *J* = 8.9 Hz, 1H), 7.15 – 7.01 (m, 7H), 6.95 (dd, *J* = 8.9, 2.0 Hz, 1H), 3.88 (s, 3H), 2.50 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.5, 160.3, 153.6, 143.7, 136.5, 133.9, 130.2, 129.2, 129.2, 128.7, 127.6, 127.6, 127.5, 126.7, 125.8, 124.3, 113.0, 111.3, 99.6, 54.8, 20.3. MS (ESI, m/z): 371.1 $[M + Na]^+$. HPLC purity: 97.8%.

7-Methoxy-4-(5-(4-methoxyphenyl)thiophen-2-yl)-2H-chromen-2-one **(87). 87** was obtained from **76** and (4-methoxyphenyl)boronic acid as described for general procedures: 53% yield as a light yellow solid; mp 164.5-168.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (dd, *J* = 3.7, 2.3 Hz, 1H), 7.31 (d, *J* = 8.9 Hz, 1H), 7.25 (t, *J* = 7.8 Hz, 1H), 7.12 (d, *J* = 2.1 Hz, 1H), 7.05 (d, *J* = 2.3 Hz, 2H), 7.01 (d, *J* = 7.4 Hz, 1H), 6.95 (dd, *J* = 8.3, 3.3 Hz, 2H), 6.81 (t, *J* = 7.4 Hz, 1H), 3.89 (s, 3H), 3.67 (s, 3H); ^{13}C NMR (101 MHz, DMSO) δ 162.5, 160.8, 157.7, 154.9, 145.9, 134.8, 131.4, 129.7, 129.1, 128.5, 127.5, 126.6, 123.7, 123.0, 120.3, 114.0, 112.2, 110.8, 100.7, 55.8, 55.5. MS (ESI, m/z): 387.1 $[M + Na]^+$. HPLC purity: 97.6%.

***Ethyl 4-(5-(7-methoxy-2-oxo-2H-chromen-4-yl)thiophen-2-yl)benzoate* (88). 88** was obtained from **76** and (4-(ethoxycarbonyl)phenyl)boronic acid as described for general procedures: 51% yield as a light yellow solid; mp 122.8-124.5 °C; ^{1}H NMR (400 MHz, DMSO) δ 7.82 (d, *J* = 7.1 Hz, 2H), 7.65 (d, *J* = 4.5 Hz, 1H), 7.45 (d, *J* = 7.7 Hz, 1H), 7.40 (d, *J* = 7.8 Hz, 1H), 7.32 (d, *J* = 9.0 Hz, 1H), 7.15-7.11 (m, 2H), 7.10 – 7.05 (m, 1H), 6.97 (dd, *J* = 8.9, 2.3 Hz, 1H), 4.28 (q, *J* = 7.1 Hz, 2H), 3.90 (s, 3H), 1.30 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.2, 162.9, 161.0, 154.8, 145.8, 134.6, 134.6, 134.3, 131.7, 130.2, 130.0, 129.0, 128.8, 128.0, 128.0, 127.0, 124.3, 113.9, 112.6, 100.7, 61.0, 55.9, 14.3. MS (ESI, m/z): 429.1 $[M + Na]^+$. HPLC purity: 98.0%.

***4-(5-(4-Chlorophenyl)thiophen-2-yl)-7-methoxy-2H-chromen-2-one* (89). 89** was obtained from **76** and (4-chlorophenyl)boronic acid as described for general procedures: 51% yield as a light yellow solid; mp 178.3-182.2 °C; ^{1}H NMR (400 MHz, DMSO) δ 7.70 – 7.66 (m, 1H), 7.31 (d, *J* = 8.2 Hz, 3H), 7.21 (d, *J* = 8.5 Hz, 2H), 7.15 – 7.08 (m, 3H), 6.96 (dd, *J* = 8.9, 2.4 Hz, 1H), 3.89 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.0, 161.1, 154.9, 145.6, 134.6, 134.0, 132.9, 131.9, 131.9, 130.1, 128.9, 128.3, 128.3, 128.2, 127.2, 124.2, 114.0, 112.7, 100.8, 56.1. MS (ESI, m/z): 391.1 $[M + Na]^+$. HPLC purity: 97.0%.

Cell culture and reagents. PI and caspase-3 (C9598) antibody were obtained from Sigma; PARP (AP102) antibody was purchased from Beyotime Co. (Nantong, Jiangsu, China). Cell lines were purchased from American Type Culture Collection (ATCC; Manassas, USA) and cultured in DMEM or RMPI 1640 medium following ATCC's

recommendations. The culture medium was supplemented with 10% fetal bovine serum (Gibco, Waltham, MA, USA) and 1 unit/mL penicillin-streptomycin (Gibco). The cell lines were maintained at 37 °C under 5% CO_2.

Cell proliferation assay. The antiproliferation activities of the compounds were tested in the SKOV3 cells, HCT116 cells, HepG2 cells, C26 cells, HCT-8 cells, NHI-H358 cells, H460 cells, SK-HEP-1 cells, BEL-7402 cells, MCF-7 cells, MCF-7/ADR cells, ZR-75-1 cells, ES-2 cells, A2780s cells, A2780/T cells, MCF-7 cells and DU145 cells. Cells in logarithmic phase were seeded in 96-well plates and allowed to adhere. Then the cells were incubated with indicated concentrations of the compounds for 24 h. MTT was subsequently added for an extra 2-3 h incubation. The MTT formazan precipitate was dissolved in DMSO, and the absorbance was measured at a wave length of 570 nm by a Spectramax M5 Microtiter Plate Luminometer (Molecular Devices, Sunnyvale, CA, USA).

Flow cytometry. A2780s cells were incubated with various concentrations of **65** or DMSO vehicle for 24 h at 37 °C. The cells were collected and washed by PBS, then fixed in cold 70% ethanol overnight at 4 °C. The cells were washed again by PBS, then the cell DNA was stained with 50 μg/mL PI containing 1 mg/mL of DNase-free RNaseA for a minimum of 10 min. The samples were analyzed by a flow cytometer (BD FACS Calibur, Franklin Lakes, NJ, USA).

Immunofluorescence staining. The immunofluorescence study of microtubule system was conducted generally as describe. HepG2 cells were seeded into 6-well plates, and then treated with **65**, taxol, colchicine as indicated for 16 h. The cells were

fixed with 4% paraformaldehyde and then penetrated with PBS containing 0.5% Triton X-100. After blocking for 30 min in 5% goat serum albumin at room temperature, cells were incubated with a monoclonal antibody (anti-α-tubulin) at room temperature for 1 h. Then the cells was washed three times by PBS following staining by fluorescence antibody and labeling of Nuclei by 4, 6-diamidino-2-phenylindole (DAPI). Cells were finally washed thrice and visualized using a fluorescence microscope (OLYMPUS, Tokyo, Japan).

In Vitro Tubulin Polymerization Assay. Two mg/mL tubulin (Cytoskeleton) was resuspended in PEM buffer [80 mM PIPES, (pH 6.9), 0.5 mM EGTA, 2 mM MgCl2, and 15% glycerol] and then was pre incubated with compounds or vehicle DMSO on ice. PEG containing GTP was added to the final concentration of 3 mg/ml before detecting the tubulin polymerization reaction. The reaction was monitored by a spectrophotometer in absorbance at 340 nm at 37°C every 2 min. The final concentrations of the compound were list as follow: **65** (1, 5, 25 μM).

Wound Healing Assay. Human umbilical vascular endothelial cells (HUVEC) were incubated in DMEM medium. Cells were allowed to grow into full confluence in 6-well plates and then incubated without FBS for 16 h to inactivate cells. Monolayer cells were wounded by scratching with a pipet tips and washed with PBS. Fresh DMEM containing vehicle or different concentrations of compound **65** was added to the scratched monolayers. After treating for 24 h, the images were taken byfluorescence microscope (Carl Zeiss Microimaging Inc.). The cells were quantified

by manual counting, and the percentage of migrated cells inhibited by compound **65** was expressed on the basis of vehicle wells.

Tube Formation Assay. The tube formation assay was conducted as described previously (Liang Ma et al. 2011). BD Matrigel matrix (BD Biosciences) were thawed at 4°C for overnight, and HUVECs ($2-4 \times 10^4$) suspended in DMEM were seeded in 96-well culture plates after polymerization of the Matrigel at 37°C for 30 min. They were then treated with different concentrations of compound **65** or vehicle. After 6−8 h, cells were photographed with a digital camera attached to an inverted microscope (Carl Zeiss Microimaging Inc.).

EBI competition assay. Six-well plates were seeded with HepG2 cells at 5×10^5 cells per well. Cells were first incubated with compound **65** (0.2, 1, 5 and 25 μM), vincristine (5 and 25 μM) or colchicine (5 μM) for 2 h and afterwards treated with EBI (100 μM). After 1.5 h, the cells were harvested and cell extracts were prepared for Western blot analysis. Twenty micrograms of proteins was subjected to gel electrophoresis using 10% polyacrylamide gels. The proteins were transferred onto PVDF membranes, then blocked by 5% non-fat milk for 1 h, and subsequently incubated with anti-β-tubulin antibody (Cell Signaling Technology, #2146) for 16 h at 4 °C. Next, the membranes were washed extensively. Immunoreactive proteins were finally detected by chemiluminescence (Millipore, USA). GAPDH was also examined to approve equal loading of protein.

Molecular Docking. In order to perform the molecular docking, we analysed all publicly availiable crystal structures of tubulin. We chose the following crystal

structures as docking templates: 4O2B, 3HKC and 4YJ3 (PDB code). The docking procedure was performed by employing Gold5.0 implanted in Discovery Studio3.1 package. In the process of docking, proteins were prepared by Discovery Studio modules , all water molecules were eliminated, all atoms within 10 Å of ligands were defined as binding site, and Goldscore was chosen as the fittess function.

Animal Tumor Models and Treatment. We established C26, H460, A2780s, A2780/T, MCF-7, MCF-7/ADR *in vivo* xenograft model in a method similar as described [39]. For every tumor xenograft model, the mice were randomly assigned to groups. We used 5−6-week-old female Balb/C and athymic nude mice, respectively, implanted the indicated number of cells suspended in 100 CE HBSS in the right flank of mice. When tumor volumes reached 100 mm^3, the animals were treated with vehicle (containing 2.5% Tween-80 and 2.5% ethanol), PTX (5, 10 mg/kg, every 2 days; 24, 30 mg/kg, every 7 days), adriamycin (4 mg/kg, every 7 days) or **65** (2.5, 5, 10 mg/kg, every 2 days; 20 mg/kg, every 7 days). Signs of toxicity and mortality were observed daily. Tumor volumes and body weights were measured every 2 days when administrated with a caliper (calculated volume (mm^3) = π/6×length × width × width). The antitumor activity of compound was evaluated by tumor inhibitor = (1−tumor weight of treated-group/tumor weight of control group) × 100%.

Ethics statement: Animal studies were conducted in conformity with the Institutional Guide for the Care and Use of Laboratory Animals. All mouse protocols were approved by the Animal Care and Use Committee of Sichuan University (Chengdu, Sichuan, China).

Supporting Information

Synthesis and characterization of compounds **12-14**, **18-23**, **58-59** and **71-76**; Model docking of compound **5**, **8** and **10** (Figure S1, S2 and S3); Activity of selected compounds against various tumor cell lines (Table S1); Immunofluorescence of selected compounds (Figure S4); Evaluation in C26 and H460 tumor models (Table S2); Evaluation in A2780S, A2780/T, MCF-7 and MCF-7/ADR tumor models (Table S3); The Photographs Tumor Models (Figure S3, S4, S5 and S6).

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Notes

The authors declare no competing financial interest

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ABBREVIATIONS USED

DMSO, dimethyl sulfoxide; DMF, *N*,*N*-dimethyl formamide; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; MDAs, microtubule destabilizing agents; MSAs,

microtubule stabilizing agents; CA4, combretastatin A4; PTX, paclitaxel; ADM, adriamycin; iv, intravenous; ip, intraperitoneal; EBI, *N,N′*-ethylene-bis-(iodoacet-amide).

PDB ID CODES

PDB code 4YJ3 was used for model docking of compound **5**, **8** and **10**. Authors will release the atomic coordinates and experimental data upon article publication.

REFERENCES

(1) Amos, L. A. Microtubule structure and its stabilisation. *Org. Biomol. Chem.* **2004**, *2*, 2153-2160.

(2) Risinger, A. L.; Giles, F. J.; Mooberry, S. L. Microtubule dynamics as a target in oncology. *Cancer Treat. Rev.* **2009**, *35*, 255-261.

(3) Kavallaris, M.; Verrills, N. M.; Hill, B. T. Anticancer therapy with novel tubulin-interacting drugs. *Drug Resist. Updates* **2001**, *4*, 392-401.

(4) Dumontet, C.; Jordan, M. A. Microtubule-binding agents: a dynamic field of cancer therapeutics. *Nat. Rev. Drug Discovery* **2010**, *9*, 790-803.

(5) Jordan, M. A.; Wilson, L. Microtubules as a target for anticancer drugs. *Nat. Rev. Cancer* **2004**, *4*, 253-265.

(6) Chen, S. M.; Meng, L. H.; Ding, J. New microtubule-inhibiting anticancer agents. *Expert Opin. Invest. Drugs* **2010**, *3*, 329-343.

(7) Lu, Y.; Chen, J.; Xiao, M.; Li, W.; Miller, D. D. An overview of tubulin inhibitors that interact with the colchicine binding site. *Pharm. Res.* **2012**, *29*, 2943-2971.

(8) Gigant, B.; Wang, C.; Ravelli, R. B.; Roussi, F.; Steinmetz, M. O.; Curmi, P. A.; Sobel, A.; Knossow, M. Structural basis for the regulation of tubulin by vinblastine. *Nature* **2005**, *435*, 519-522.

(9) Honore, S.; Pasquier, E.; Braguer, D. Understanding microtubule dynamics for improved cancer therapy. *Cell. Mol. Life Sci.* **2005**, *62*, 3039-3056.

(10) Amos, L. A. What tubulin drugs tell us about microtubule structure and dynamics. *Semin. Cell Dev. Biol.* **2011**, *22*, 916-926.

(11) Cosentino, L.; Redondo-Horcajo, M.; Zhao, Y.; Santos, A. R.; Chowdury, K. F.; Vinader, V.; Abdallah, Q. M.; Abdel-Rahman, H.; Fournier-Dit-Chabert, J.; Shnyder, S. D.; Loadman, P. M.; Fang, W. S.; Diaz, J. F.; Barasoain, I.; Burns, P. A.; Pors, K. Synthesis and biological evaluation of colchicine B-ring analogues tethered with halogenated benzyl moieties. *J. Med. Chem.* **2012**, *55*, 11062-11066.

(12) Eckford, P. D.; Sharom, F. J. ABC efflux pump-based resistance to chemotherapy drugs. *Chem. Rev.* **2009**, *109*, 2989-3011.

(13) Fojo, T.; Menefee, M. Mechanisms of multidrug resistance: the potential role of microtubule-stabilizing agents. *Ann. Oncol.* **2007**, *18*, 3-8.

(14) Ling, V. Multidrug resistance: Molecular mechanisms and clinical relevance. *Cancer Chemother. Pharmacol.* **1997**, *40*, 3-8.

(15) Yeh, J. J.; Hsu, W. H.; Wang, J. J.; Ho, S. T.; Kao, A. Predicting chemotherapy response to paclitaxel-based therapy in advanced nonsmall-cell lung cancer with P-glycoprotein expression. *Respiration* **2003**, *70*, 32-35.

(16) Leonard, G. D.; Fojo, T.; Bates, S. E. The role of ABC transporters in clinical practice. *Oncologist* **2003**, *8*, 411-424.

(17) Kavellaris, M. Microtubules and resistance to tubulin-binding agents. *Nat. Rev. Cancer* **2010**, *10*, 194-204.

(18) Stengel, C.; Newman, S. P.; Leese, M. P.; Potter, B. V.; Reed, M. J.; Purohit, A. Class III beta-tubulin expression and in vitro resistance to microtubule targeting agents. *Br. J. Cancer* **2010**, *102*, 316-324.

(19) Pettit, G. R.; Temple, C., Jr.; Narayanan, V. L.; Varma, R.; Boyd, M. R.; Rener, G. A.; Bansal, N. Antineoplastic agents 322. synthesis of combretastatin A-4 prodrugs. *Anti-Cancer Drug Des.* **1995**, *10*, 299-309.

(20) Rustin, G. J.; Shreeves, G.; Nathan, P. D.; Gaya, A.; Ganesan, T. S.; Wang, D.; Boxall, J.; Poupard, L.; Chaplin, D. J.; Stratford, M. R. L.; Balkissoon, J.; Zweifel, M. A. Phase Ib trial of CA-4P (combretastatin A-4 phosphate), carboplatin, and paclitaxel in patients with advanced cancer. *Br. J. Cancer* **2010**, *102*, 1355-1360.

(21) Zweifel, M.; Jayson, G. C.; Reed, N. S.; Osborne, R.; Hassan, B.; Ledermann, J.; Shreeves, G.; Poupard, L.; Lu, S. P.; Balkissoon, J.; Chaplin, D. J.; Rustin, G. J. S. Phase II trial of combretastatin A4 phosphate, carboplatin, and paclitaxel in patients with platinum resistant ovarian cancer. *Ann. Oncol.* **2011**, *22*, 2036-2041.

(22) Lara-Ochoa, F.; Espinosa-perez, G. A new synthesis of combretastatins A-4 and AVE-8062A. *Tetrahedron Lett.* **2007**, *48*, 7007-7010.

(23) Kasibhatla, S.; Baichwal, V.; Cai, S. X.; Roth, B.; Skvortsova, I.; Skvortsov, S.; Lukas, P.; English, N. M.; Sirisoma, N.; Drewe, J.; Pervin, A.; Tseng, B.; Carlson, R.

O.; Pleiman, C. M. MPC-6827: a small-molecule inhibitor of microtubule formation that is not a substrate for multidrug resistance pumps. *Cancer Res.* **2007**, *67*, 5865-5871.

(24) Tsimberidou, A. M.; Akerley, W.; Schabel, M. C.; Hong, D. S.; Uehara, C.; Chhabra, A.; Warren, T.; Mather, G. G.; Evans, B. A.; Woodland, D. P.; Swabb, E. A.; Kurzrock, R. Phase I clinical trial of MPC-6827 (Azixa), a microtubule destabilizing agent, in patients with advanced cancer. *Mol. Cancer Ther.* **2010**, *9*, 3410-3419.

(25) Grossmann, K. F.; Colman, H.; Akerley, W. A.; Glantz, M.; Matsuoko, Y.; Beelen, A. P.; Yu, M.; De Groot, J. F.; Aiken, R. D.; Olson, J. J.; Evans, B. A.; Jensen, R. L. Phase I trial of verubulin (MPC-6827) plus carboplatin in patients with relapsed glioblastoma multiforme. *J. Neuro-Oncol.* **2012**, *110*, 257-264.

(26) Chamberlain, M. C.; Grimm, S.; Phuphanich, S.; Recht, L.; Zhu, J. Z.; Kim, L.; Rosenfeld, S.; Fadul, C. E. A phase 2 trial of verubulin for recurrent glioblastoma: a prospective study by the brain tumor investigational consortium (BTIC). *J. Neuro-Oncol.* **2014**, *118*, 335-343.

(27) Bubols, G. B.; Vianna Dda, R.; Medina-Remon, A.; von Poser, G.; Lamuela-Raventos, R. M.; Eifler-Lima, V. L.; Garcia, S. C. The antioxidant activity of coumarins and flavonoids. *Mini-Rev. Med. Chem.* **2013**, *13*, 318-334.

(28) Zhang, L.; Jiang, G.; Yao, F.; He, Y.; Liang, G.; Zhang, Y.; Hu, B.; Wu, Y.; Li, Y.; Liu, H. Growth inhibition and apoptosis induced by osthole, a natural coumarin, in hepatocellular carcinoma. *PloS One* **2012**, *7*, e37865.

(29) Davis, R. A.; Vullo, D.; Maresca, A.; Supuran, C. T.; Poulsen, S. A. Natural product coumarins that inhibit human carbonic anhydrases. *Bioorg. Med. Chem.* **2013**, *21*, 1539-1543.

(30) Matos, M. J.; Vazquez-Rodriguez, S.; Santana, L.; Uriarte, E.; Fuentes-Edfuf, C.; Santos, Y.; Munoz-Crego, A., Looking for new targets: simple coumarins as antibacterial agents. *Med. Chem.* **2012**, *8*, 1140-1145.

(31) Shi, Y.; Zhou, C. H. Synthesis and evaluation of a class of new coumarin triazole derivatives as potential antimicrobial agents. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 956-960.

(32) Weigt, S.; Huebler, N.; Strecker, R.; Braunbeck, T.; Broschard, T. H. Developmental effects of coumarin and the anticoagulant coumarin derivative warfarin on zebrafish (Danio rerio) embryos. *Reprod. Toxicol.* **2012**, *33*, 133-141.

(33) Basanagouda, M.; Jambagi, V. B.; Barigidad, N. N.; Laxmeshwar, S. S.; Devaru, V.; Narayanachar. Synthesis, structure-activity relationship of iodinated-4-aryloxymethyl-coumarins as potential anti-cancer and anti-mycobacterial agents. *Eur. J. Med. Chem.* **2014**, *74*, 225-233.

(34) Thakur, A.; Singla, R.; Jaitak, V., Coumarins as anticancer agents: A review on synthetic strategies, mechanism of action and SAR studies. *E Eur. J. Med. Chem.* **2015**, *101*, 476-495.

(35) Combes, S.; Barbier, P.; Douillard, S.; McLeer-Florin, A.; Bourgarel-Rey, V.; Pierson, J. T.; Fedorov, A. Y.; Finet, J. P.; Boutonnat, J.; Peyrot, V., Synthesis and

biological evaluation of 4-arylcoumarin analogues of combretastatins. part 2. *J. Med. Chem.* **2011**, *54*, 3153-3162.

(36) Kim, S.-N.; Kim, N. H.; Park, Y. S.; Kim, H.; Lee, S.; Wang, Q.; Kim, Y. K., 7-Diethylamino-3(2′-benzoxazolyl)-coumarin is a novel microtubule inhibitor with antimitotic activity in multidrug resistant cancer cells. *Biochem. Pharmacol.* **2009**, *77*, 1773-1779.

(37) Fortin, S.; Wei, L.; Moreau, E.; Lacroix, J.; Cote, M. F.; Petitclerc, E.; Kotra, L. P.; R, C. G., Design, synthesis, biological evaluation, and structure-activity relationships of substituted phenyl 4-(2-oxoimidazolidin-1-yl)benzenesulfonates as new tubulin inhibitors mimicking combretastatin A-4. *J. Med. Chem.* **2011**, *54*, 4559-4580.

(38) Hwang, D. J.; Wang, J.; Li, W.; Miller, D. D. Structural optimization of indole derivatives acting at colchicine binding site as potential anticancer agents. *ACS Med. Chem. Lett.* **2015**, *6*, 993-997.

(39) Yang, Z.; Wang, T.; Wang, F.; Niu, T.; Liu, Z.; Chen, X.; Long, C.; Tang, M.; Cao, D.; Wang, X.; Xiang, W.; Yi, Y.; Ma, L.; You, J.; Chen, L. Discovery of selective histone deacetylase 6 inhibitors using the quinazoline as the cap for the treatment of cancer. *J. Med. Chem.* **2015**, *59*, 1455-1470.

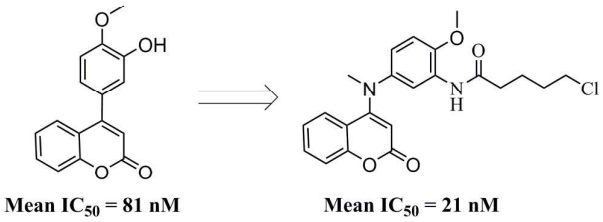
(40) Theeramunkong, S.; Caldarelli, A.; Massarotti, A.; Aprile, S.; Caprioglio, D.; Zaninetti, R.; Teruggi, A; Pirali, T; Grosa, G; Tron, G. C.; Genazzani, A. A. Regioselective Suzuki coupling of dihaloheteroaromatic compounds as a rapid

strategy to synthesize potent rigid combretastatin analogues. *J. Med. Chem.* **2011**, *54*, 4977–4986.

(41) Wang, S.; Fang, K.; Dong, G.; Chen, S.; Liu, N.; Miao, Z.; Yao, J.; Li, J.; Zhang, W.; Sheng, C. Scaffold diversity inspired by the natural product evodiamine: discovery of highly potent and multitargeting antitumor agents. *J. Med. Chem.* **2015**, *58*, 6678-6696.

(42) Fortin, S.; Lacroix, J.; Cote, M.-F.; Moreau, E.; Petitclerc, E.; Gaudreault, R. C. Quick and simple detection technique to assess the binding of antimicrotubule agents to the colchicine-binding site. *Biol. Proced. Online* **2010**, *12*, 113−117.

Table of Contents Graphic



Mean IC_{50} = 81 nM

Mean IC_{50} = 21 nM