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Novel coumarin-3-(*N*-aryl)carboxamides arrest breast cancer cell growth by inhibiting ErbB-2 and ERK1

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Abstract—A series of novel coumarin carboxamides were synthesized, and their tumor cell cytotoxic activity was investigated. These compounds specifically inhibited the growth of cancer cells that have a high level of ErbB-2 expression. Immunoprecipitation analysis of the cell lysates prepared from carboxamide treated cancer cells showed the inhibition of ErbB-2 phosphorylation suggesting the interaction of these compounds with ErbB-2 receptor. The down regulation of the kinase activity was further confirmed by performing in vitro kinase assay with recombinant ErbB-2 incubated with carboxamides. The inhibition of ErbB-2 phosphorylation correlated with down-regulation of ERK1 MAP kinase activation that is involved in proliferative signaling pathway. Furthermore, the cell-killing activity of many of these inhibitors is restricted to tumor cells with no demonstrable cytotoxicity against normal human fibroblasts suggesting that these compounds are tumor-specific.

1. Introduction

Coumarins are structurally simple compounds belonging to a large class of molecules known as benzopyrones.[1] Coumarins and related compounds have diverse biological activities, including anti-coagulant and anti-thrombotic properties.[2] Coumarin derivatives have also been shown to be novel lipid lowering agents that possess moderate triglyceride lowering activity.[3] Many coumarin derivatives have the unique ability to scavenge reactive oxygen species such as hydroxyl free radicals, superoxide radicals, or hypochlorous acid to prevent free radical injury.[4] Recently, certain coumarin derivatives have been shown to function as human immunodeficiency virus integrase inhibitors and have been evaluated in the treatment of HIV infection,[5] whereas others have been evaluated as anti-invasive compounds due to their inhibitory activity against some serine proteases and matrix metalloproteases (MMPs).[6] Recently, it has been shown that 6-nitro-7-hydroxy coumarin acts as a selective anti-proliferative agent by activating p38, stress activated protein kinase (SAPK) and the $p21^{WAF1/CIP1}$ cyclin dependent kinase inhibitor, in the human renal cell carcinoma cell line, A-498.[7,8] More recently, two new naturally occurring coumarins have been isolated and shown to inhibit the polymerization of tubulin and arrest cells in mitotic phase by inhibiting microtubule formation.[9] These coumarin were found to act synergistically with paclitaxel in inhibiting KB (human epidermoid carcinoma) cell proliferation.[9]

Recently, we have described the growth inhibitory properties of novel coumarin sulfonamides using a panel of cancer cell lines.[10] These coumarin derivatives up-regulated the activity of c-Jun NH_2 terminal kinase 1 (JNK1), which is a member of the stress activated protein kinases (SAPKs) that are involved in apoptotic signaling.[10] Based on the diverse biological activities of coumarins and their derivatives, we have designed and synthesized a series of novel coumarin 3-carboxamides and examined the cytotoxic activity of these compounds in different cancer cell lines. Our results show that coumarin 3-carboxamides selectively inhibit the tumor cells that have higher levels of epidermal growth factor receptor (EGFR or erbB1) and erbB2 (HER2) receptor expression.

Keywords: Coumarin carboxamides; ErbB-2; MAP kinase; In vitro kinase activity.
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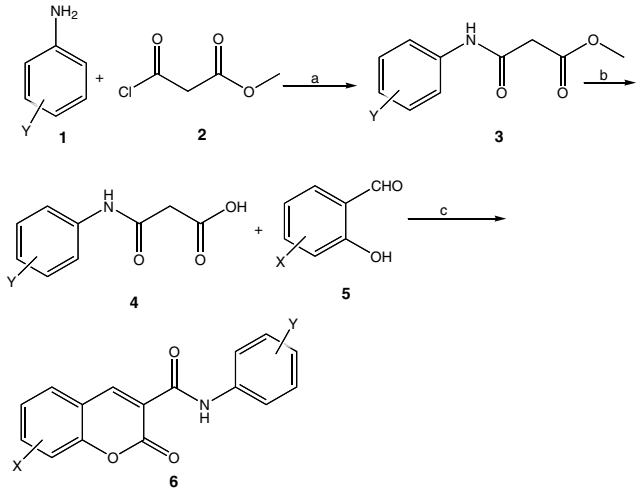
2. Chemistry

Although several substituted coumarins have been described in the literature, very little information is known about coumarin 3-carboxamides. In earlier publications,[11] these compounds were synthesized in a circuitous way by condensing malonic esters with salicylaldehydes, followed by hydrolysis, halogenation, and condensation with amines as illustrated in Scheme 1.[12] This method has a limitation due to a variety of substituents at different positions on both aromatic rings and thus may not be useful in creating a large library of coumarin 3-carboxamides. In this manuscript, we report a novel route for the synthesis of coumarin-3-carboxamides using the Knoevenagel type of condensation reaction.

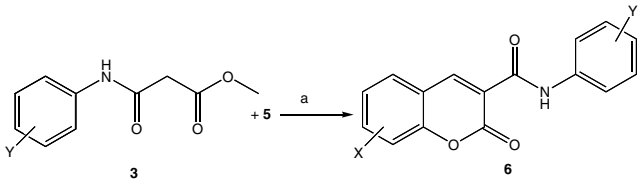
The reaction of methyl-3-chloro-3-oxopropionate **2** with aromatic amines **1** in the presence of triethylamine produced methyl 3-anilino-3-oxopropionate **3**, which on hydrolysis yielded 3-anilino-3-oxopropionic acid **4**. Condensation of 3-anilino-3-oxopropionic acid with substituted salicylaldehydes **5** in glacial acetic acid in the presence of a catalytic amount of benzylamine produced coumarin-3-carboxamides **6** in quantitative yields[13] (Scheme 2). Alternatively, coumarin-3-carboxamides **6** were also prepared by reacting methyl 3-anilino-3-oxopropionate **3** with substituted salicylaldehydes **5** in the presence of piperidine in ethanol (Scheme 3).

3. Results

To determine the cytotoxic activity of these compounds, two human breast cancer cell lines, SKBR-3, BT474 (with high expressing levels of ErbB-2 and EGFR) and HFL cells (human lung fibroblasts that do not over-express the ErbB-2 and EGFR) were incubated in the presence of all the newly synthesized coumarin-3-carboxamides and the cell viability determined by trypan blue exclusion. The results of this study are summarized in Table 1.

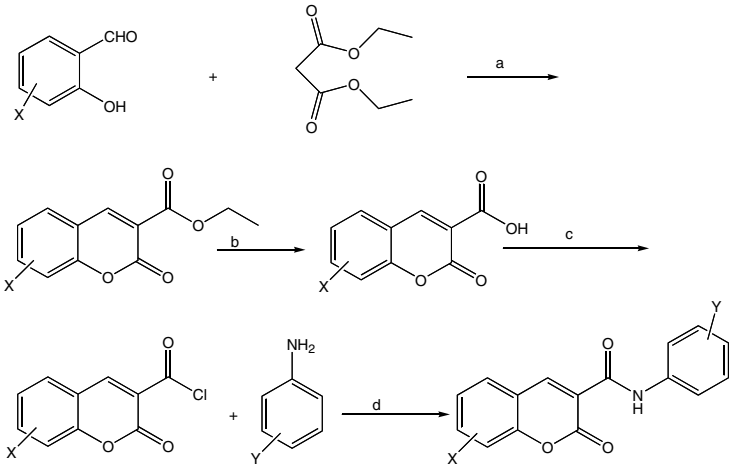


Scheme 2. Reagents and conditions: (a) Et_3N, CH_2Cl_2, 0 °C to rt; (b) 10% NaOH, HCl; (c) $PhCH_2NH_2$, HOAc, reflux.



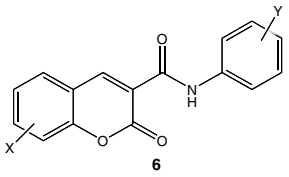
Scheme 3. Reagents and conditions: (a) piperidine, ethanol, reflux 1 h.

The cytotoxic properties of coumarin-3-carboxamides (**6a–l**) were determined using the SKBR-3 and BT474 breast cancer cell lines as well as normal HFLs. Both SKBR-3 and BT474 over-express ErbB-2 receptor at about 7×10^5 receptors per cell[14,15] where as HFL cells do not express this receptor. The results showed that coumarin carboxamides inhibited the growth of ErbB-2 over-expressing cells (SKBR-3 and BT474) more effectively when compared to HFL cells that do not express ErbB-2 receptors (Table 1). Furthermore, the carboxamides (**6c**, **6e**, **6g**, **6j**, and **6l**) exhibited more potent cytotoxic activity (Table 1) than the coumarin carboxa-



Scheme 1. Reagents and conditions: (a) piperidine/EtOH/HOAc; (b) HCl/H_2O/EtOH; (c) $SOCl_2$; (d) $PhNH_2$/pyridine/dioxane.

Table 1. Cell growth and ErbB-2 kinase inhibition by coumarin-3-carboxamides



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Compd	X	Y	Cell line (GI_{50}, μM)[a]			ErbB-2 (IC_{50}, μM)[b]
			BT474	SKBR-3	HFL	
6a	H	4-Br	120	135	>300	>10
6b	H	4-I	115	110	>300	>10
6c	8-EtO	4-Br	21.2	16.3	>300	0.316
6d	8-EtO	3-NO_2, 4-Cl	87	64	>300	0.549
6e	8-EtO	3-NH_2, 4-Cl	21	20.6	>300	0.709
6f	8-EtO	4-I	94	84	>300	3.84
6g	6-Br	4-I	21.4	21.8	>300	0.204
6h	6-Cl	4-Br	81	74	>300	>10
6i	6-Cl	4-I	72	75	>300	>10
6j	6-Cl	3-NO_2, 4-Cl	20.8	19.4	>300	0.166
6k	6-Br	4-Br	63	60.5	>300	>10
6l	6-Cl	3-NH_2, 4-Cl	20.3	19.4	>300	2.17

[a] The GI_{50} was determined by direct extrapolation from each dose response curve.
[b] The IC_{50} of ErbB-2 kinase activity was determined using an in vitro kinase assay.

mides (**6a**, **6b**, **6d–f**, **6h**, **6i**) against SKBR-3 and BT474 cell lines. These results clearly show that the substitutions on the coumarin ring and on the phenyl ring substantially affect the cell-killing ability of the molecules.

To determine the mechanism by which these compounds induce cell death, we have examined the effects of coumarin-3-carboxamides on the activation state of ErbB-2, as well as downstream proliferation and survival pathways using SKBR-3 and BT474 cells that have been treated with epidermal growth factor (EGF). In this study, the coumarin-3-carboxamides that showed higher cytotoxic activity in cell culture-based assays (**6c**, **6e**, **6g**, **6j**, and **6l**) were added to SKBR-3 and BT474 cells that have been treated with EGF. When whole cell extracts derived from these cells were subjected to immunoprecipitation using an ErbB-2 specific antibody followed by western blot analysis using a phosphotyrosine antibody, we observed a total inhibition of EGF-induced phosphorylation of ErbB-2 by these compounds (Fig. 1). To confirm that these compounds directly inhibit the kinase activity ErbB-2, we performed an in vitro kinase assay with recombinant ErbB-2 protein using polyglutamine tyrosine as a substrate. These results showed that while all of the compounds, in general, inhibit the activity of ErbB-2, those compounds that showed higher cytotoxic activity in the SKBR-3 and BT474 tumor cell lines in general had the most potent ErbB-2 inhibitory activity (Fig. 2 and Table 1).

ErbB-2 over-expression is associated with the activation of downstream proliferative pathways involving the activation of ERK1/2 MAP kinases.[16] It was therefore of interest to determine whether coumarin-3-carboxamide mediated inhibition of ErbB-2 affected the activation of these proteins as well. When cell extracts from SKBR-3 and BT474 cells, incubated for 24 h in the presence of coumarin-3-carboxamides **6c**, **6e**, **6g**, **6j**, and **6l**, were subjected to immunoprecipitation followed by kinase assays (Fig. 2), we observed that these compounds inhibited the activation of ERK1 by more than 80%. Taken together, these results suggest that coumarin-3-carboxamides effectively inhibit ErbB-2 mediated signaling in cells over-expressing this protein.

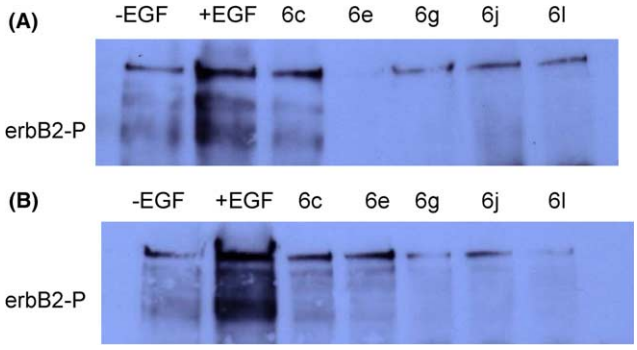

Figure 1. Inhibition of ErbB-2 phosphorylation by coumarin-3-carboxamides in SKBR-3 (A), BT474 (B) cells. Cells were treated with 50 μM carboxamide for 24 h and cell lysates were analyzed by immunoprecipitating with an ErbB-2-specific antibody followed by western blotting with a phosphotyrosine antibody. Data shown are representative of three independent experiments.

4. Discussion

Our current study shows that coumarin-3-carboxamides exert a cytotoxic effect by inhibiting ErbB-2 phosphorylation in tumor cell lines that over-express the receptor, thereby causing growth arrest and cell death. In this study, we investigated the effect of coumarin carbox-

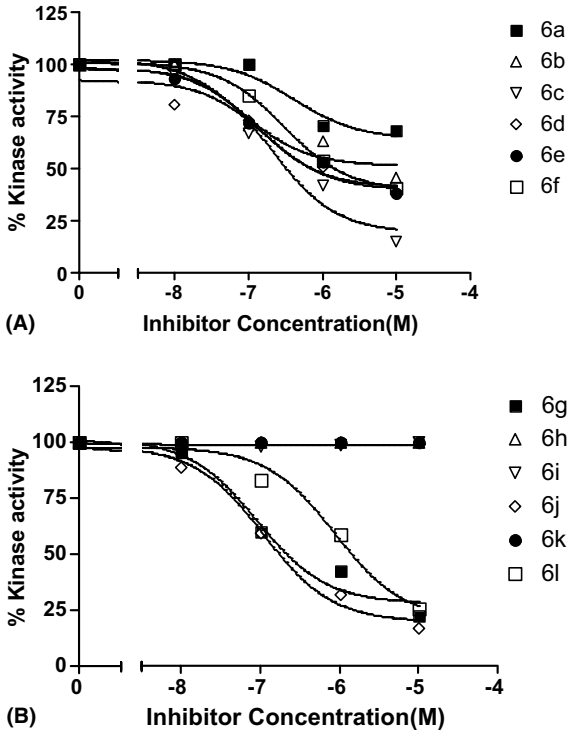

Figure 2. Inhibition of ErbB-2 kinase activity in vitro by coumarin carboxamides. Recombinant ErbB-2 was incubated with various concentrations of carboxamides and kinase reaction was performed using poly(Glu:Tyr,4:1) as substrate. Data shown is a representative of two independent experiments.

amides on downstream survival and proliferation pathways to characterize these compounds and elucidate their mechanism of action.

The ErbB-2 family of growth factors receptors, EGFR and ErbB-2/HER2 in particular, are known to play important roles in breast cancer pathogenesis.[17] In order to develop a targeted drug, it is important to have a clear understanding of the growth factor receptor-dependent pathways that are activated in breast tumor cells. Most mitogenic signals transduced through growth factor receptor activation ultimately converge on common downstream effectors, the ERK1/2 MAP kinases.[18] Activated ERK1/2 control many processes that are central to tumor cell proliferation, survival, and tumor progression.[19] Increased expression of activated ERK1/2 has been demonstrated in a number of human malignancies[20] and over-expression of ErbB-2 in tumor cell lines results in the up-regulation of activated ERK1/2.[21] Our data show that coumarin carboxamides inhibited ERK1 activation in EGFR/ErbB-2-over-expressing tumor cell lines (Fig. 3).

Furthermore, although EGF stimulated the activation of ErbB-2 and ERK1 in SKBR-3 and BT474 breast cancer cells, exposure to coumarin carboxamides completely inhibited these effects (Fig. 2). These results highlight the importance of ligand induced growth effects on the level of receptor activation in tumor cells. They also show that ERK1 may be a useful downstream

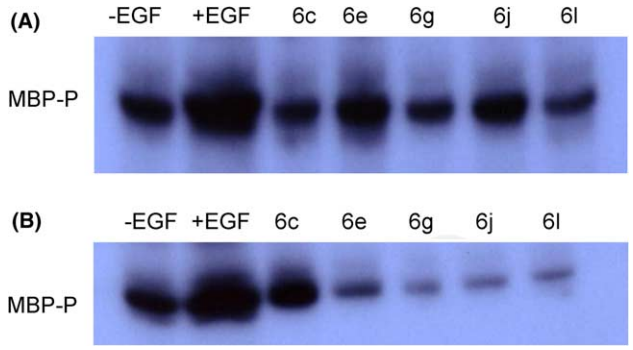

Figure 3. Inhibition of ERK1 phosphorylation by coumarin-3-carboxamides in BT474 (A), SKBR-3 (B) cells. Cells were treated with 50 μM carboxamide for 24 h and cell lysates were analyzed by immunoprecipitating with an ERK1 antibody followed by an in vitro kinase assay using Myelin Basic Protein (MBP) as a substrate. Data shown are representative of three independent experiments.

biomarker that correlates with the ability of coumarin-3-carboxamides to inhibit tumor cell proliferation.

Results presented in this manuscript also demonstrate that the inhibitory activity of coumarin carboxamides can be considerably improved through substitutions on the coumarin ring and on the phenyl ring, which in turn results in improved tumor cell-killing ability of the molecules (Table 1). The demonstration that cell-killing activity of many of these inhibitors is restricted to tumor cells with no demonstrable cytotoxicity against normal human fibroblasts suggests that these compounds are tumor-specific. This in turn appears to be due to the over-expression of ErbB family of receptors on tumor cells, which provides these cells with growth advantage.

5. Conclusions

We have identified a new series of compounds, coumarin-3-(*N*-aryl) carboxamides that are novel classes of potent ErbB-2 and ERK1 MAP kinase inhibitors. The compounds, **6c**, **6e**, **6g**, **6j**, and **6l** in particular, demonstrated a 4–5-fold increase in cytotoxic activity as compared to **6a** and **6b**. These results show that substitutions on the coumarin ring and also on the phenyl group can drastically alter the cytotoxic property of the compounds, and suggest that a coumarin carboxamide with increased potency can be designed and synthesized by analyzing structure–activity relationship. Such a molecule is likely to have much higher ErbB-2 and ERK1 MAPK inhibitory activities, which in turn could result in enhanced anti-tumor activity.

6. Experimental

6.1. Chemistry

6.1.1. General information. Unless otherwise noted, chemicals were commercially available and used as received without further purification. Thin-layer chromatography was performed on precoated silica gel F254

plates (Sigma–Aldrich) with a UV indicator. Silica gel column chromatography was performed using silica gel 60A (Aldrich 70–230 mesh). Melting points were determined in open glass capillaries using Mel-Temp apparatus and are uncorrected. ^{1}H NMR spectra were recorded using a Brucker 400 MHz spectrometer in DMSO-d_6 or $CDCl_3$ as a solvent and TMS as an internal standard. Chemical shifts (δ) are given in parts per million relative to TMS. Elemental analyses for these compounds were performed by Quantitative Technologies Inc., Whitehouse, New Jersey, USA, and are within 0.4% of theoretical values.

6.1.2. General procedure for preparation of *N*-aryl 3-oxo propionic acids (4a–c). Methyl-3-chloro-3-oxopropionate (100 mmol) was added dropwise to a solution of dichloromethane (100 mL) containing substituted aniline (100 mmol) and triethylamine (100 mmol) at 0 °C stirred for an additional 5 h. The resulting reaction mixture was washed with cold water (3 × 50 mL), dried (Na_2SO_4), filtered, and the organic layer evaporated under reduced pressure to obtain methyl *N*-aryl 3-oxopropionate (**3**) quantitatively. Methyl *N*-aryl 3-oxopropionate (**3**) upon hydrolysis with a 10% KOH solution followed by neutralization with dil HCl produced *N*-aryl 3-oxo propionic acid in 78–86% yield.

6.1.2.1. 4-Iodoanilino-3-oxopropionic acid (4a). Yield 82%, mp 162–164 °C; ^{1}H NMR (400 MHz, DMSO): 10.64 (br s, 1H), 7.58 (d, J = 9.0 Hz, 2H), 7.35 (d, J = 9.0 Hz, 2H), 3.13 (s, 2H).

6.1.2.2. 4-Bromoanilino-3-oxopropionic acid (4b). Yield 86% mp 130–132 °C; ^{1}H NMR (400 MHz, DMSO): 10.48 (br s, 1H), 7.50 (d, J = 9.0 Hz, 2H), 7.42 (d, J = 9.0 Hz, 2H), 3.14 (s, 2H).

6.1.2.3. 4-Chloro-3-nitroanilino 3-oxopropionic acid (4c). Yield 78%, mp 118–120 °C; ^{1}H NMR (400 MHz, DMSO):11.5 (br s, 1H), 8.36 (s, 1H), 7.70 (d, J = 8.5 Hz, 1H), 7.62 (d, J = 8.5 Hz, 1H), 3.15 (s, 2H).

6.1.3. General procedure for the preparation of coumarin 3-carboxamides (6a–l)

6.1.3.1. Method A. To a solution of *N*-(aryl)-3-oxopropionic acid (7.75 mmol) and salicylaldehyde (7.75 mmol) in 12 mL glacial acetic acid was added catalytic amount of benzyl amine (300 μL) and refluxed for 5–8 h. Most of the times the condensed product comes out of the solution, which was filtered, washed with 2-propanol (3 × 20 mL) to get the pure product. Some cases where solid is not formed, the reaction mixture was diluted with 75 mL diethyl ether, washed with saturated $NaHCO_3$ (2 × 25 mL), 5% HCl (2 × 20 mL), water (2 × 25 mL), and brine (2 × 25 mL), dried (Na_2SO_4), filtered, and evaporated in vacuo to get pure (60–80%) of the title compounds.

6.1.3.2. Method B. To a solution of salicylaldehyde (7.75 mmol) in 30 mL warm ethanol, was added methyl *N*-(aryl)-3-oxopropionate (7.75 mmol) and catalytic amount of piperidine (0.75 mmol) and refluxed the contents for 1 h. The precipitated product that comes out after cooling was filtered, washed with 2-propanol (3 × 20 mL) and diethyl ether (2 × 20 mL) to obtain a pure product (68–88%).

6.1.3.3. Coumarin 3-(*N*-4-bromophenyl)carboxamide (6a). Coumarin 3-(*N*-4-bromophenyl)carboxamide (**6a**) was prepared according to method A starting from *N*-(4-bromophenyl)-3-oxopropionic acid (2.0 g, 7.75 mmol) and salicylaldehyde (0.946 g, 7.75 mmol), afforded 2.03 g (76%) of (**6a**): mp 269–271 °C; ^{1}H NMR (400 MHz, DMSO): δ 10.67 (br s, 1H), 8.88 (s, 1H), 7.98 (dd, J = 8.0, 1.5 Hz, 1H), 7.76 (m, 1H), 7.69 (d, J = 7.0 Hz, 2H), 7.56 (d, J = 7.0 Hz, 2H), 7.53 (m, 1H), and 7.45 (t, J = 7.0 Hz, 1H). Anal. ($C_{16}H_{10}NO_3Br$) C, H, N.

6.1.3.4. Coumarin 3-(*N*-4-iodophenyl)carboxamide (6b). Coumarin 3-(*N*-4-iodophenyl)carboxamide (**6b**) was prepared according to method B starting from methyl *N*-(4-iodophenyl)-3-oxopropionate (2.47 g, 7.75 mmol) and salicylaldehyde (0.946 g, 7.75 mmol), afforded 2.67 g (88%) of (**6b**): mp 281–282 °C; ^{1}H NMR (400 MHz, DMSO): δ 10.89 (br s, 1H), 9.09 (s, 1H), 8.20 (d, J = 7.7 Hz, 1H), 8.08 (t, J = 8.5 Hz, 1H), 7.92 (d, J = 8.6 Hz, 2H), 7.77 (d, J = 8.6 Hz, 2H), 7.75 (m, 1H), and 7.64 (t, J = 8.5 Hz, 1H). Anal. ($C_{16}H_{10}NO_3I$) C, H, N.

6.1.3.5. 8-Ethoxycoumarin 3-(*N*-4-bromophenyl)carboxamide (6c). 8-Ethoxycoumarin 3-(*N*-4-bromophenyl)-carboxamide (**6c**) was prepared according to method A starting from *N*-(4-bromophenyl)-3-oxopropionic acid (2.0 g, 7.75 mmol) and 3-ethoxysalicylaldehyde (1.29 g, 7.75 mmol), afforded 1.80 g (60%) of (**6c**): mp 242–244 °C; ^{1}H NMR (400 MHz, DMSO): δ 10.71 (br s, 1H), 8.83 (s, 1H), 7.68 (d, J = 8.9 Hz, 2H), 7.53 (d, J = 8.9 Hz, 2H), 7.49 (d, J = 7.8 Hz, 1H), 7.41 (d, J = 7.6 Hz, 1H), 7.34 (t, J = 7.8 Hz, 1H), 4.23 (q, J = 7.0 Hz, 2H), and 1.38 (t, J = 6.9 Hz, 3H). Anal. ($C_{18}H_{14}NO_4Br$) C, H, N.

6.1.3.6. 8-Ethoxycoumarin 3-(*N*-4-chloro 3-nitrophenyl)-carboxamide (6d). 8-Ethoxycoumarin 3-(*N*-4-chloro 3-nitrophenyl)carboxamide (**6d**) was prepared according to method A starting from *N*-(4-chloro 3-nitrophenyl)-3-oxopropionic acid (2.0 g, 7.75 mmol) and 3-ethoxysalicylaldehyde (1.29 g, 7.75 mmol), afforded 2.41 g (80%) of (**6d**): mp 260–262 °C; ^{1}H NMR (400 MHz, DMSO): δ 11.08 (br s, 1H), 8.90 (s, 1H), 8.41 (d, J = 2.5 Hz, 1H), 7.71 (dd, J = 8.8, 2.5 Hz, 1H), 7.27–7.15 (m, 3H), 7.43 (d, J = 8.8 Hz, 1H), 4.14 (q, J = 7.0 Hz, 2H), and 1.46 (t, J = 7.0 Hz, 3H). Anal. ($C_{18}H_{14}N_2O_6Cl$) C, H, N.

6.1.3.7. 8-Ethoxycoumarin 3-(*N*-4-chloro 3-aminophenyl)carboxamide (6e). A solution of compound **6d** (1.0 g, 2.57 mmol) in 1:2 water/acetone (30 mL) mixture was heated at 50 °C for 30 min and sodium hydrosulfite (8.95 g, 51.4 mmol) was added and maintained at 50 °C for another 30 min. After cooling to room temperature, the reaction mixture was diluted with 50 mL of

water and extracted with ethyl acetate (100 mL). The aqueous phase was further extracted with ethyl acetate (2 × 50 mL). The combined organic layer was washed successively with water (3 × 50 mL), brine (2 × 25 mL) and dried over anhydrous sodium sulfate. Removal of the solvent on a rotavapor yielded a crude product that was further purified on silica gel column using petroleum ether and ethyl acetate mixture (9:1) afforded 0.276 g (30%) of pure product **6e**: mp 182–186 °C; ^{1}H NMR (400 MHz, DMSO): δ 10.21 (br s, 1H), 8.20 (s, 1H), 8.11 (d, J = 2.5 Hz, 1H), 7.71 (dd, J = 8.8, 2.5 Hz, 1H), 7.27–7.15 (m, 3H), 5.36 (br s, 2H),7.43 (d, J = 8.8 Hz, 1H), 4.14 (q, J = 7.0 Hz, 2H), and 1.46 (t, J = 7.0 Hz, 3H). Anal. ($C_{18}H_{16}N_2O_4Cl$) C, H, N.

6.1.3.8. 8-Ethoxycoumarin 3-(*N*-4-iodophenyl)carboxamide (6f). 8-Ethoxycoumarin 3-(*N*-4-iodophenyl)carboxamide (**6f**) was prepared according to method B starting from methyl *N*-(4-iodophenyl)-3-oxopropionate (2.47 g, 7.75 mmol) and 3-ethoxysalicylaldehyde (1.29 g, 7.75 mmol), afforded 2.29 g (68%) of (**6f**): mp 252–255 °C; ^{1}H NMR (400 MHz, DMSO): δ 10.73 (br s, 1H), 8.89 (s, 1H), 7.75 (d, J = 8.7 Hz, 2H), 7.59 (d, J = 8.7 Hz, 2H), 7.56 (d, J = 7.8 Hz, 1H), 7.48 (d, J = 8.2 Hz, 1H), 7.30 (t, J = 7.6 Hz, 1H), 4.25 (q, J = 7.0 Hz, 2H), and 1.45 (t, J = 7.0 Hz, 3H). Anal. ($C_{18}H_{14}NO_4I$) C, H, N.

6.1.3.9. 6-Bromocoumarin 3-(*N*-4-iodophenyl)carboxamide (6g). 6-Bromocoumarin 3-(*N*-4-iodophenyl)carboxamide (**6g**) was prepared according to method B starting from methyl *N*-(4-iodophenyl)-3-oxopropionate (2.47 g, 7.75 mmol) and 5-bromosalicylaldehyde (1.56 g, 7.75 mmol), afforded 2.73 g (75%) of (**6g**): mp 258–260 °C; ^{1}H NMR (400 MHz, DMSO): δ 10.52 (br s, 1H), 8.70 (s, 1H), 8.14 (d, J = 2.6 Hz, 1H), 7.79 (dd, J = 8.9, 2.2 Hz, 1H), 7.59 (d, J = 8.5 Hz, 2H), 7.43 (d, J = 8.6 Hz, 2H), and 7.39 (d, J = 8.9 Hz, 1H). Anal. ($C_{16}H_9NO_3BrI$) C, H, N.

6.1.3.10. 6-Chlorocoumarin 3-(*N*-4-bromophenyl)carboxamide (6h). 6-Chlorocoumarin 3-(*N*-4-bromophenyl)-carboxamide (**6h**) was prepared according to method A starting from *N*-(4-bromophenyl)-3-oxopropionic acid (2.0 g, 7.75 mmol) and 5-chlorosalicylaldehyde (1.21 g, 7.75 mmol), afforded 2.03 g (69%) of (**6h**): mp 168–172 °C; ^{1}H NMR (400 MHz, DMSO): 12.74 (br s, 1H), 9.01 (s, 1H), 7.84 (d, J = 2.6 Hz, 1H), 7.79 (dd, J = 8.9, 2.2 Hz, 1H), 7.72 (d, J = 8.5 Hz, 2H), 7.43 (d, J = 8.6 Hz, 2H), and 7.09 (d, J = 8.9 Hz, 1H). Anal. ($C_{16}H_9NO_3BrCl$) C, H, N.

6.1.3.11. 6-Chlorocoumarin 3-(*N*-4-iodophenyl)carboxamide (6i). 6-Chlorocoumarin 3-(*N*-4-iodophenyl)carboxamide (**6i**) was prepared according to method B starting from methyl *N*-(4-iodophenyl)-3-oxopropionate (2.47 g, 7.75 mmol) and 5-chlorosalicylaldehyde (1.21 g, 7.75 mmol), afforded 2.37 g (72%) of (**6i**): mp 267–269 °C; ^{1}H NMR (400 MHz, DMSO): δ 10.52 (br s, 1H), 8.71 (s, 1H), 8.01 (d, J = 2.5 Hz, 1H), 7.68 (dd, J = 8.9, 2.5 Hz, 1H), 7.59 (d, J = 8.8 Hz, 2H), 7.46 (d, J = 9.0 Hz, 2H), and 7.43 (d, J = 8.8 Hz, 1H). Anal. ($C_{16}H_9NO_3ClI$) C, H, N.

6.1.3.12. 6-Chlorocoumarin 3-(*N*-4-chloro 3-nitrophenyl)-carboxamide (6j). 6-Chlorocoumarin 3-(*N*-4-chloro 3-nitrophenyl)carboxamide (**6j**) was prepared according to A starting from *N*-(4-chloro, 3-nitrophenyl)-3-oxopropionic acid (2.0 g, 7.75 mmol) and 5-chlorosalicylaldehyde (1.21 g, 7.75 mmol), afforded 2.35 g (80%) of (**6j**): mp 293–295 °C; ^{1}H NMR (400 MHz, DMSO): δ 11.03 (br s, 1H), 8.92 (s, 1H), 8.64 (d, J = 2.5 Hz, 1H), 8.21 (d, J = 2.5 Hz, 1H), 8.05 (dd, J = 8.8, 2.5 Hz, 1H), 7.89 (dd, J = 8.8, 2.5 Hz, 1H), 7.67 (d, J = 8.0 Hz, 1H), and 7.44 (d, J = 8.0 Hz, 1H). Anal. ($C_{16}H_8N_2O_5Cl_2$) C, H, N.

6.1.3.13. 6-Bromocoumarin 3-(*N*-4-bromophenyl)carboxamide (6k). 6-Bromocoumarin 3-(*N*-4-bromophenyl)-carboxamide (**6k**) was prepared according to method A starting from *N*-(4-bromophenyl)-3-oxopropionic acid (2.0 g, 7.75 mmol) and 5-bromosalicylaldehyde (1.56 g, 7.75 mmol), afforded 2.36 g (72%) of **6k**: mp 253–256 °C; ^{1}H NMR (400 MHz, DMSO): δ 10.65 (br s, 1H), 8.81 (s, 1H), 8.24 (d, J = 2.5 Hz, 1H), 7.89 (dd, J = 9.0, 2.5 Hz, 1H), 7.67 (d, J = 9.5 Hz, 2H), 7.52 (d, J = 9.5 Hz, 2H), and 7.50 (d, J = 8.5 Hz, 1H). Anal. ($C_{16}H_9NO_3Br_2$) C, H, N.

6.1.3.14. 6-Chlorocoumarin 3-(*N*-4-chloro 3-aminophenyl)carboxamide (6l). To a solution of the compound **6j** (0.974 g, 2.57 mmol) in 1:2 water/acetone (30 mL), at 50 °C for 30 min, sodium hydrosulfite (8.95 g, 51.4 mmol) was added and continued at 50 °C for another 30 min. After cooling to room temperature, 50 mL of water was added and extracted with ethyl acetate (100 mL). The aqueous layer was further extracted with ethyl acetate (2 × 50 mL). The combined organic phase was washed successively with water (3 × 50 mL), brine (2 × 25 mL), dried over sodium sulfate, and evaporated to obtain crude product, which was further purified on silica gel column using petroleum ether and ethyl acetate mixture (9:1) afforded 0.27 g (30%) pure product **6l**: mp 186–190 °C; ^{1}H NMR (400 MHz, DMSO): δ 10.21 (br s, 1H), 8.60 (s, 1H), 8.41 (d, J = 2.5 Hz, 1H), 7.71 (dd, J = 8.8, 2.5 Hz, 1H), 7.27–7.15 (m, 3H), 5.46 (br s, 2H), and 7.43 (d, J = 8.8 Hz, 1H). Anal. ($C_{16}H_{10}N_2O_3Cl_2$) C, H, N.

6.1.4. Cell lines. HFL human lung fibroblasts, BT474 and SKBR-3 breast carcinoma cells were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 0.01 mg/mL streptomycin at 37 °C under humidified conditions in 5% CO_2.

6.1.5. In vitro cytotoxicity. Cells lines (1×10^5) were seeded into six well dishes and 24 h later, each compound was added at five different concentrations over a 2 log dilution (1–100 mM). The total number of viable cells was determined 96 h post-treatment by trypan blue exclusion. The percentage of viable cells remaining was calculated as follows: # viable cells (compound treated)/# viable cells (DMSO treated) * 100. Dose response curves were generated by plotting the percentage of cells at each concentration versus the concentration tested.

6.1.6. Analysis of ErbB-2 tyrosine phosphorylation by immunoprecipitation followed by western blotting. Immunoprecipitation and western blot analysis was performed according to the procedure as described by VaraPrasad et al.[22] Briefly, SKBR-3/BT474 cells were grown in 100 mm plates to 80% confluence and serum starved for 18 h. The cells were incubated with the appropriate compound (50 μM) for 2 h and stimulated with 10 ng/mL EGF. After 15 min, the cells were washed twice with ice cold PBS and lysed in lysis buffer.[22] Two hundred micrograms of the lysate was immunoprecipitated with an ErbB-2 antibody at 4 °C overnight in the presence of 50 μL protein A beads. The immune complex was washed twice with PBS, resuspended in 50 μL of Laemmli buffer, and boiled for 3 min. The samples were electrophoresed on a 10% polyacrylamide gel and transferred onto a PVDF membrane. The membrane was blocked with 3% milk and the level of tyrosine phosphorylated ErbB-2 was determined using an anti-phosphotyrosine antibody (clone 4G10; Upstate Cell Signaling Solutions, Lake Placid, NY).

6.1.7. In vitro kinase kinase assay for ErbB-2 inhibition. Recombinant ErbB-2 was expressed in baculovirus-infected Sf9 cells and purified according to the supplier's instructions (BD Biosciences). One hundred nanograms of purified ErbB-2 was incubated with various concentrations of coumarin carboxamides for 30 min at room temperature. After incubation, kinase reactions were performed at 30 °C for 20 min in the presence of 100 μM ATP, 40 μCi ^{32}P-γATP, and 0.25 μg/mL poly-(Glu:Tyr,4:1), (PGT; Sigma Chemical Co). The reaction was terminated by addition of 0.25 mM EDTA and spotted onto P81 phosphocellulose filters. The filters were washed thrice for 5 min each with 0.75% phosphoric acid, once with acetone and the level of radioactivity determined using a liquid scintillation counter. Kinase assays were performed in triplicate and represent an average of two independent experiments. Non-specific binding was determined by conducting the assay in the absence of enzyme and the value subtracted from each of the experimental values. The level of kinase activity is expressed as a percent of the maximal kinase activity.

6.1.8. In vitro kinase assay for ERK1 inhibition. SKBR-3 and BT474 cell lysates were prepared as mentioned above and immunoprecipitated using an ERK1/2 antibody for 2 h at 4 °C. The immune complexes were washed and incubated in kinase reaction buffer containing 100 μM ATP, 40 μCi ^{32}P-γATP, and 2.5 μg MBP at 30 °C for 20 min. The reactions were terminated by the addition of Laemmli buffer, boiled for 5 min, and electrophoresed on a 15% sodium dodecyl sulfate–polyacrylamide gel followed by autoradiography.

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