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Design and synthesis of novel Gefitinib analogues with improved anti-tumor activity

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ABSTRACT

There is an urgent need to design and develop new and more potent EGFR inhibitors with improved anti-tumor activity. Here we describe the design and synthesis of two series of 4-benzothienyl amino quinazolines as new analogues of the EGFR inhibitor Gefitinib. The anti-tumor activity of these novel Gefitinib analogues in 6 human cancer cell lines was examined. Compared with the parental Gefitinib, most of the new compounds show a markedly increased cytotoxicity to cancer cells. Furthermore, several of the series B compounds that side chains at position 7 contain either a methyl or ethyl group are potent pan-RTK inhibitors. Two representative compounds in this class, **15** and **17**, have an enhanced capability to inhibit cancer cell growth and induce apoptosis in vitro and inhibit tumor formation in vivo in human cancer cells with high HER-2, as compared with the parental Gefitinib. Thus they may be promising lead compounds to be developed as an alternative for current Gefitinib therapy or for Gefitinb-resistant patients, potentially via simultaneously blocking multiple RTK signaling pathways.

1. Introduction

The epidermal growth factor receptor (EGFR) belongs to the ErbB family of receptor tyrosine kinases (RTK), which includes four members: ErbB-1/EGFR, ErbB-2/HER-2/neu, ErbB-3/HER-3, and ErbB-4/HER-4.[1,2] Activation of EGFR (ligand-dependent or ligand-independent) leads to the phosphorylation at cytoplasmic tails of the receptor and subsequent recruitment of adapter moleculars, which results in pro-growth/survival signal transduction via several pathways. The major pathways implicated in EGFR signaling include RAS–RAF–MAPK (mitogen-activated protein kinase), PI3K (phosphatidylinositol 3-kinase)–AKT, and JAK–STAT (signal transducers and activators of transcription).[2–4]

EGFR overexpression occurs frequently in human epithelial malignancies and its activation plays a significant role in the development and progression of human cancers, since EGFR signaling pathways are associated with cell proliferation, survival promotion and apoptosis inhibition.[5,6] Therefore, EGFR is a very attractive molecular target for cancer therapy. Over the past 20 years, numerous small molecular inhibitors and monoclonal antibodies targeting EGFR have been successfully developed. The 4-anilino quinazolines derivatives, Iressa (ZD1839, Gefitinib) and Tarceva (OSI-774, Erlotinib) (Fig. 1), are two selective EGFR inhibitors approved by the FDA in 2003 and 2004 for locally advanced or metastatic non-small-cell lung cancer (NSCLC) therapy, and are currently under evaluation in clinical trials for other tumor types.[7,8] Clinical data show that 10–20% of all NSCLC patients partially respond to these two EGFR inhibitors,[9,10] but only Erlotinib prolongs the survival of patients with recurrent NSCLC.[11] Moreover, most of the patients who responded to initial treatment eventually developed resistance to the EGFR inhibitors.[12] Thus there is an urgent need to design and develop new and more potent EGFR inhibitors with improved anti-tumor activity.

Numerous novel derivatives have been synthesized using Gefitinib as a leading compound. Most modifications of the structure of Gefitinib are focused on substitution of the benzene ring, but its replacement with another aromatic ring is rarely reported. In this study, we designed and synthesized two series of 4-benzothienyl amino quinazoline derivatives that show significantly improved anti-tumor activity. New compounds that side chains at position 7 contain either a methyl or ethyl group were identified as potential pan-RTK inhibitors with enhanced apoptosis-inducing capability.

2. Chemistry

Based on the structure–activity relationships (SAR) and quantitative structure–activity relationships (QSAR) of the 4-anilinoqui-

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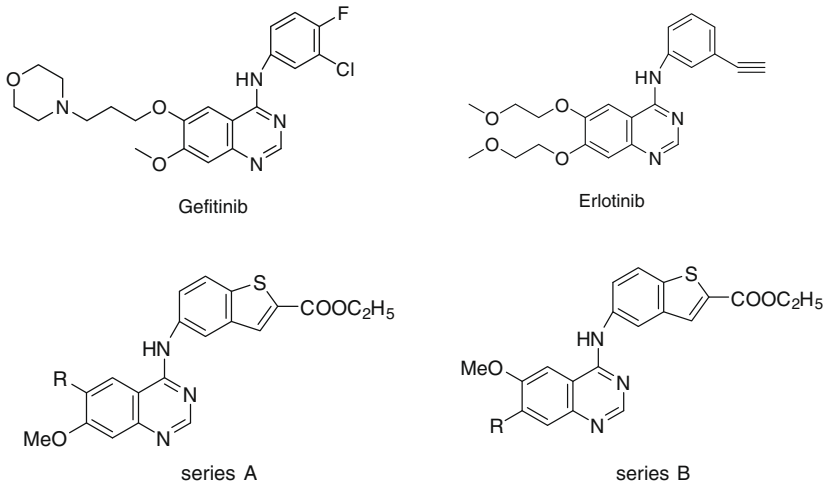
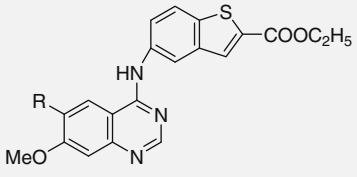


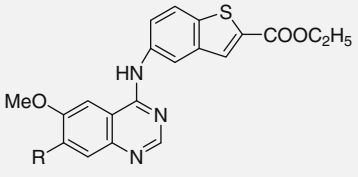
Figure 1. Structure of Gefitinib, Erlotinib and core structure of analogues.

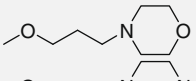
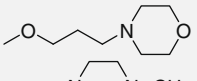
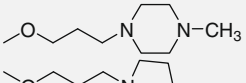
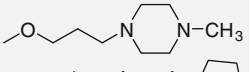
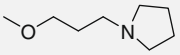
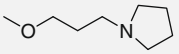
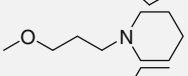
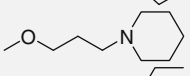
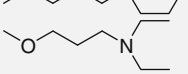
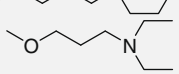
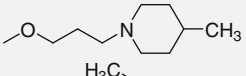
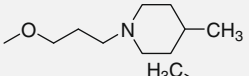
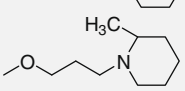
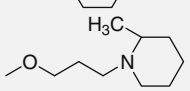
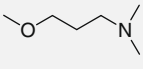
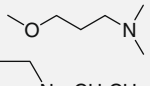
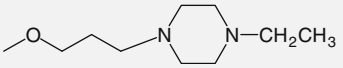
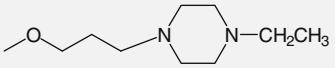
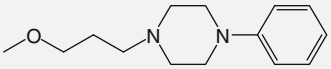
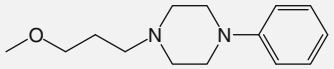
nazolines reported previously,[13–21] and using Gefitinib as a leading compound, we designed two series of novel analogues: series A, replacement of the benzene ring with benzothiophene, secondary amino-substituted propoxy side chain at position 6 and methoxy group at position 7 of the quinazoline nucleus; series B, replacement of the benzene ring with benzothiophene, methoxy group at position 6 and secondary amino-substituted propoxy side chain at position 7 of the quinazoline nucleus. Our objective was to determine whether these compounds favor greater inhibition of cell proliferation and higher induction of cell death.

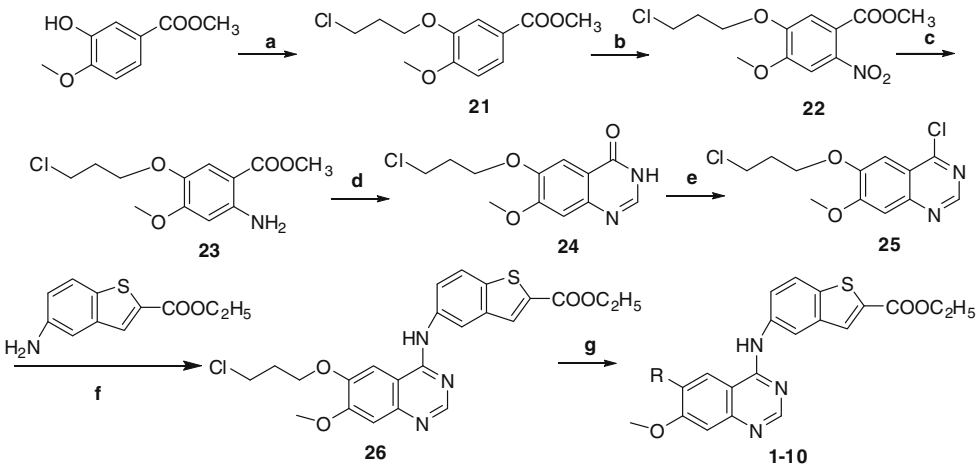
As summarized in Table 1, up to 20 compounds in series A (1–10) and series B (11–20) were synthesized by two main routes, as illustrated in Schemes 1 and 2, respectively. Methyl 3-hydroxy-4-methoxy benzoate as starting material was alkylated with 1-bromo-3-chloropropane to give **21** in 95% yield. Nitration of **21** with nitric acid in acetic acid afforded **22**, which was then reduced by powdered iron in acetic acid to give **23** in satisfactory yield (77%). In contrast, catalytic hydrogenation using Raney/Ni or 5% Pd/C gave incomplete conversions, even after a long reaction time. Cyclization of **23** with formamidine acetate and chlorination with

Table 1
Structures and yields of compounds **1–20**

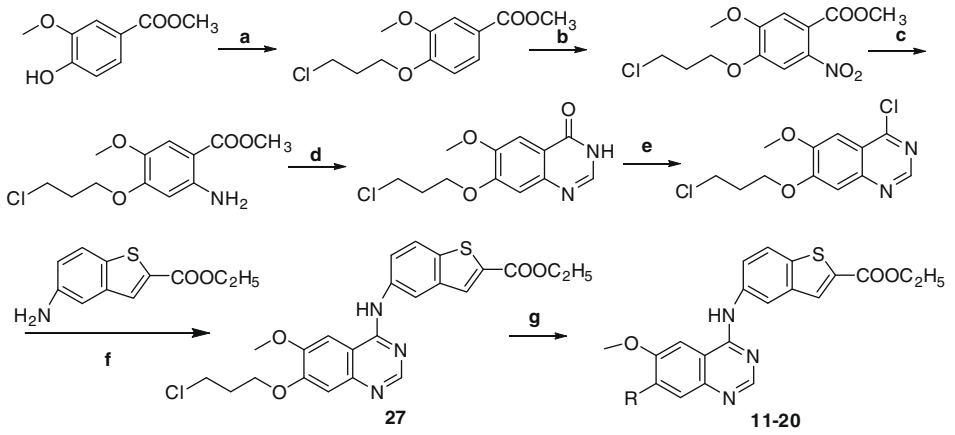




Compd	R	Yield (%)	Compd	R	Yield (%)
1		77	**11**		68
2		68	**12**		62
3		58	**13**		51
4		71	**14**		68
5		69	**15**		72
6		64	**16**		65
7		59	**17**		60
8		61	**18**		52
9		65	**19**		67
10		59	**20**		59



Scheme 1. Reagents and conditions: (a) $ClCH_2CH_2CH_2Br$, K_2CO_3, 70 °C; (b) HNO_3, AcOH, Ac_2O, 0–5 °C; (c) Fe, AcOH, MeOH, N_2, 50 °C; (d) formamidine acetate, EtOH, reflux; (e) $SOCl_2$, DMF, reflux; (f) *i*-PrOH, reflux; (g) aliphatic amine, KI, 70 °C.



Scheme 2. Reagents and conditions: (a) $ClCH_2CH_2CH_2Br$, K_2CO_3, 70 °C; (b) HNO_3, AcOH, Ac_2O, 0–5 °C; (c) Fe, AcOH, MeOH, N_2, 50 °C; (d) formamidine acetate, EtOH, reflux; (e) $SOCl_2$, DMF, reflux; (f) *i*-PrOH, reflux; (g) aliphatic amine, KI, 70 °C.

thionyl chloride gave **25**. Aminolysis of **25** was performed using excess ethyl 5-aminobenzo[*b*]thiophene-2-carboxylate to afford **26** in 93% yield. The final step was nucleophilic displacement of the chlorine atom with different aliphatic amines to yield the corresponding target compounds series A **1–10**.

Starting from methyl 4-hydroxy-3-methoxybenzoate (Scheme 2), the compounds **11–20** could be obtained via the same synthetic methods as described above. This is the first study to replace the benzene ring of Gefitinib with benzothiophene to design completely new compounds. All the target compounds are new chemical entities and obtained in satisfactory yields (Table 1).

3. Results and discussion

3.1. New compounds showed higher cytotoxicity in different cancer cell lines compared with Gefitinib and Erlotinib

We first examined the cytotoxicity of the 20 new compounds, as well as the parental compounds Gefitinib and Erlotinib, in 6 human cancer cell lines by the MTT-based cytotoxicity assay (Table 2). The IC_{50} values of most of the compounds in those 6 cell lines were reduced compared to the two parental drugs, indicating that the anti-tumor activity of these new compounds was significantly improved.

3.2. Series B compounds that side chains at position 7 contain either a methyl or ethyl group are pan-RTK inhibitors

As Gefitinib was a selective EGFR inhibitor, we determined the EGFR inhibitory activity of novel Gefitinib analogues using an EGFR Kinase Assay Kit. Unexpectedly, the inhibitory activity of the new compounds on EGFR tyrosine kinase was partially reduced (Table 2). The compounds were then tested for their ability to inhibit two other EGFR-related receptor tyrosine kinases, HER-2 and MET. As also shown in Table 2, most series B compounds maintained or increased HER-2 inhibitory ability while increasing MET inhibitory ability compared to Gefitinib and Erlotinib, while series A compounds appeared to have less inhibitory potency in both kinases. Our result is consistent with an earlier report[22] that Gefitinib analogues with the secondary amino-substituted propoxy side chain at position 7 were more potent RTK inhibitors than the compounds substituted at position 6. However, we replaced the benzene ring with benzothiophene to generate 4-benzothienyl amino quinazoline derivatives, which are totally new class of analogues.

Based on the above results, we tried to summarize preliminary SAR: replacement of the benzene ring with benzothiophene increased cytotoxicity but decreased EGFR inhibitory activity. Shifting the secondary amino-substituted propoxy side chain at position 6 (series A compounds **1–10**) to position 7 (series B com-

Table 2
Cytotoxicity in different cell lines and enzyme inhibition activity of Gefitinib, Erlotinib and compounds **1–20**

Compd	Cytotoxicity in different cell lines (IC_{50}, μM)[a]						Enzyme inhibition[a,b] (%)		
	Miapaca2	Panc1	DU145	PC3	A549	NCI-H661	EGFR	HER-2	MET
Gefitinib	50.62	40.42	42.17	22.86	37.93	31.06	98.37	81.11	28.18
Erlotinib	82.47	>100	19.40	36.76	33.03	12.71	96.95	77.94	39.76
1	14.11	27.72	19.69	18.17	19.23	17.70	22.43	46.02	24.11
2	5.208	15.66	5.756	6.216	8.026	9.670	45.38	67.61	32.27
3	3.090	18.75	7.780	2.538	5.828	9.353	41.02	62.35	38.65
4	6.813	18.52	27.20	7.375	21.12	7.746	18.07	47.32	21.09
5	3.295	13.17	7.132	2.453	7.695	7.066	22.69	47.82	21.98
6	2.511	10.86	8.64	6.810	6.812	8.841	30.47	57.01	12.80
7	2.272	17.26	20.00	7.703	10.94	7.719	33.73	54.74	25.16
8	4.640	4.220	3.035	3.051	3.929	6.966	40.28	63.20	21.62
9	2.331	5.948	8.529	6.238	9.043	7.013	37.61	77.45	44.64
10	3.561	12.25	29.99	2.278	9.120	10.58	25.40	59.54	35.21
11	31.84	53.28	>100	32.46	62.98	36.79	8.27	71.22	61.51
12	4.096	6.756	6.669	3.872	5.826	15.76	72.91	83.39	51.02
13	7.556	39.70	61.85	33.91	64.79	20.53	13.26	69.33	44.86
14	2.273	6.694	24.84	6.002	8.976	6.745	11.33	74.46	52.25
15	3.694	7.276	6.552	6.685	8.518	13.52	62.74	86.85	57.26
16[c]	87.19	>100	>100	>100	>100	>100	1.52	19.52	14.14
17	2.630	7.502	5.320	2.716	4.147	6.533	70.47	86.08	69.80
18	7.105	18.45	27.30	16.16	21.47	26.28	67.88	74.82	44.96
19	2.892	4.029	8.977	2.342	5.680	5.744	60.14	82.81	51.62
20	3.044	11.11	30.67	5.621	10.26	7.030	19.59	73.01	44.20

Miapaca2, Panc1: human pancreatic cancer cell line; DU145, PC3: human prostate cancer cell line; A549, NCI-H661: human lung cancer cell line.
[a] Values are averages of at least two independent experiments, SD <10%.
[b] Compounds tested at a concentration of 10 μM.
[c] Low solubility in DMSO stock.

pounds **11–20**) increased HER-2 and MET inhibitory ability but decreased EGFR inhibitory potency with an exception: the side chains at position 7 contain either a methyl or ethyl group (compounds **12**, **15**, **17**, **18** and **19**; compound **16** was an exception due to its low solubility). Thus compounds **12**, **15**, **17**, **18** and **19** might be pan-RTK inhibitors.

Since crosstalk between EGFR and HER-2/MET was one of the mechanisms of resistance to Gefitinnib,[23,24] using these compounds as pan-RTK inhibitors might overcome this problem, allowing compounds **12**, **15**, **17**, **18** and **19** to serve as potential alternatives to Gefitinib. Based on IC_{50} and RTK inhibitory activity, the compounds **15** and **17** were chosen for further characterization and mechanism studies.

3.3. Compounds 15 and 17 exhibit inhibitory effect on HER-2 kinase and selectively inhibit the growth of breast cancer cells transfected with HER-2

The compounds **15** and **17** were examined for their effects on the HER-2 kinase activity. As shown in Figure 2A and B, the IC_{50} values are 2.29 μM and 1.32 μM for **15** and **17**, respectively. The parental compounds showed much lower activity. To further validate their inhibitory effect on the proliferation function of HER-2, we compared the cytotoxicity of **15** and **17** in HER-2 negative human breast cancer cell line MCF-7 and that with HER-2-transfected MCF-7 clone that is highly tumorigenic and dependent on HER-2 (MCF-HER2, Fig. 2C). Representative results of two independent experiments are shown in Figure 2D and E. MCF-HER2 cells were more sensitive to **15** and **17** than HER-2-negative MCF-7 cells. **17** appears to be more potent and selective than **15,** in terms of cytotoxicity to MCF-HER2 versus MCF-7 (the IC_{50} of **15** and **17** are 5.9 and 2.7 μM for MCF-HER2 cells, 7.3 and 6.3 μM for MCF-7 cells, respectively). This apparent selectivity is correlated with their HER-2 kinase inhibitory activity shown above. The IC_{50} of the parental compounds were all >30 μM in these cells. **15** and **17** showed limited cytotoxicity to normal breast epithelial cell line MCF-10A (IC_{50} >30 μM). The responses of MCF-7-vector cells to these compounds were similar to that of MCF-7 cells (data not shown). Taken together, these results strongly support that HER-2 is one of the targets of **15** and **17**.

3.4. Compounds 15 and 17 significantly suppressed pancreatic cancer Miapaca2 cell proliferation

To examine the effects of **15** and **17** on cancer cells, we first carried out the MTT-based cytotoxicity (Fig. 3A), the cell growth curves (Fig. 3B) and colony formation assays (Fig. 3C and D) with human pancreatic cancer Miapaca2 cells. After treatment with **15** or **17**, Miapaca2 cell growth was significantly suppressed and colony formation was reduced in a dose-dependent manner, while Gefitinib had very limited effects. These results suggested that **15** and **17** markedly suppress the proliferation of Miapaca2 cells that are HER-2 positive and low cMET.

3.5. Compounds 15 and 17 potently induced apoptosis in Miapaca2 cells

We next examined the mechanism of **15** and **17** in cell growth inhibition. As shown in Figure 4A and B, **15** or **17** potently induced apoptosis in a dose- and time-dependent manner. In contrast, Gefitinib did not induce any significant apoptosis comparing with DMSO control. Cleaved PARP (Fig. 4C) provided further evidence of **15** and **17**-induced apoptosis. Furthermore, as PARP was one of the substrates of caspase-3, our functional caspase-3 activation assay showed a similar result. Caspase-3 activity increased more than eight-fold after treatment with 20 μM **15** or **17**, whereas Gefitinib had only a minor effect (Fig. 4D). The above data demonstrate that **15** and **17** are potent apoptosis inducers. Further studies are ongoing to delineate the exact mechanism(s) of action and determine whether these compounds have other molecular targets.

3.6. Compounds 15 and 17 inhibited Miapaca2 tumor formation in vivo

To further confirm the anti-tumor activity of **15** and **17**, Miapaca2 cells were treated by 2.5 μM Gefitinib, **15** or **17** for 48 h

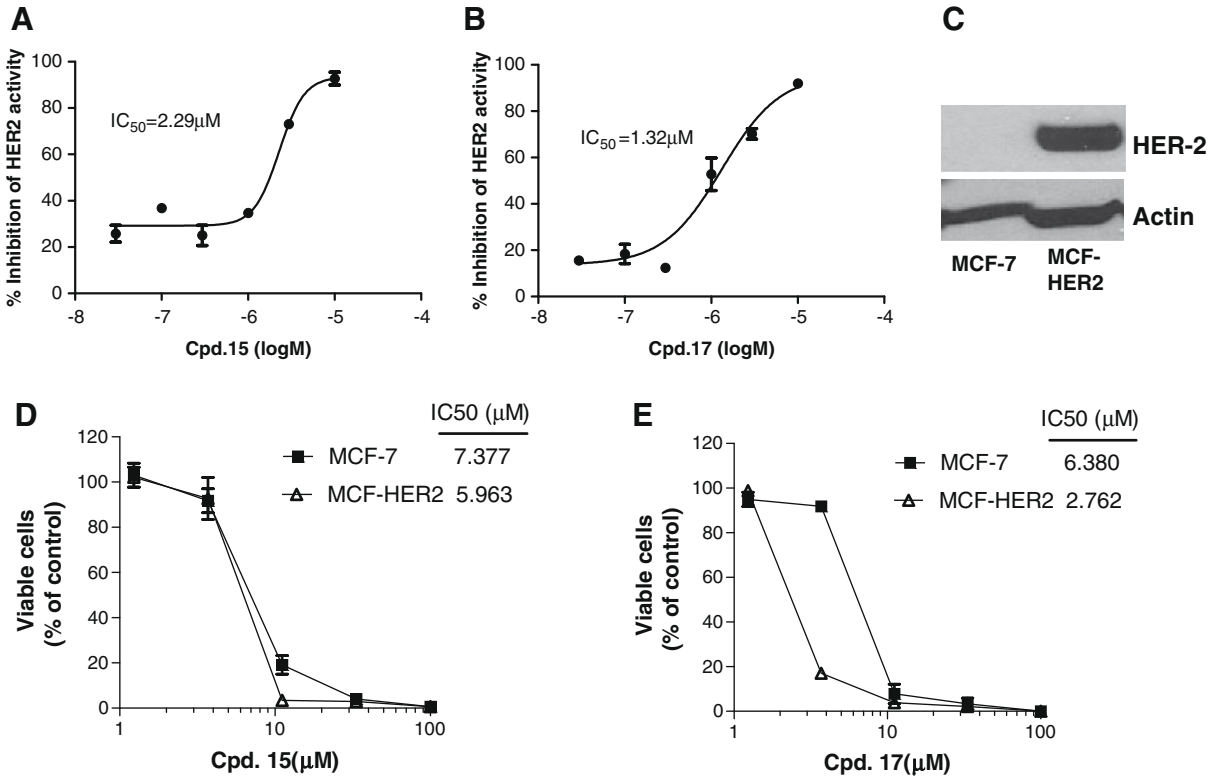


Figure 2. Compounds **15** and **17** exhibit inhibitory effect on HER-2 kinase and selectively inhibits the growth of cancer cells with high HER-2. (A, B) Representative inhibition curve of HER-2 kinase by **15** and **17** by a HER-2 Kinase Assay Kit (repeated three times). (C) Western blot analysis of HER-2 protein expression in MCF-7 and HER-2 transfected stable clone of MCF-7 (MCF-HER2) cells. Actin is shown as a loading control. (D, E) The MTT-based cytotoxicity assay in MCF-7 and MCF-HER-2 cells was carried out as described in Section 5. MCF-HER2 cells were more sensitive to **15** and **17** than HER-2 negative MCF-7 cells.

and then implanted into nude mice subcutaneously. Tumor incidence and tumor sizes were measured. Notably, pre-treatment with **15** and **17** effectively delayed Miapaca2 tumor formation in vivo (Fig. 5A and B) and inhibited tumor growth (Fig. 5C and D). The data demonstrate the improved anti-tumor activity as compared with the parent compound Gefitinb. Compound **17** showed even better activity than **15**, which is correlated with its better inhibitory activity on HER-2.

4. Conclusion

In our study, two series of 20 novel analogues of Gefitinb were designed and synthesized, and certain structure–activity relationships were proposed. Most of the new compounds show improved anti-tumor activity in the 6 human cancer cell lines tested. More significantly, the series B compounds that side chains at position 7 contain either a methyl or ethyl group were identified to be potent pan-RTK inhibitors. Two representative compounds, **15** and **17,** showed more potent and selective apoptosis-inducing capability and improved anti-tumor activity in cancer cells with HER-overexpression, as compared with the parental Gefitinb. The novel pan-RTK inhibitors discovered in our study hold promise as alternatives to current Gefitinib therapy or for treatment of Gefitinib-resistant patients, potentially via simultaneously blocking multiple RTK signaling pathways.

5. Experimental

5.1. Synthesis

All reagents were purchased from commercial sources and used without further purification. Melting points were measured in open capillaries and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 500 spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS), used as an internal standard. Mass spectra (MS) were obtained from Agilent 1100LC/MS. IR spectra were run on FI-IR Spectrometer (Perkin–Elmer). Element analysis was run on Elementa Vario ELIII element analyzer. All compounds were routinely checked by TLC with silica gel GF-254 glass plates and viewed under UV light at 254 nm.

5.1.1. Methyl 3-(3-chloropropoxy)-4-methoxybenzoate (21)

A mixture of methyl 3-hydroxy-4-methoxybenzoate (84.6 g, 0.47 mol), 1-bromo-3-chloropropane (101.6 g, 0.65 mol), and potassium carbonate (138.1 g, 1.0 mol) in DMF (500 mL) was heated at 70 °C for 4 h. The reaction mixture was cooled to room temperature, then poured slowly into ice water (3 L) while stirring constantly. The solid formed was filtered off and washed with cold water. The off-white product was recrystallized from ethyl acetate (200 mL) to give 113.9 g of **21** in 95% yield. Mp: 111–113 °C; ^{1}H NMR δ: 2.02–2.22 (tt, 2H, $-CH_2CH_2CH_2-$), 3.65 (t, 2H, $-CH_2Cl$), 3.79 (s, 3H, $-OCH_3$), 3.88 (s, 3H, $-OCH_3$), 4.10 (t, 2H, $-CH_2O$), 6.84 (d, 1H, HAr), 7.49 (s, 1H, HAr), 7.71 (d, 1H, HAr).

5.1.2. Methyl 5-(3-chloropropoxy)-4-methoxy-2-nitrobenzoate (22)

Nitric acid (84.5 ml, 66%) was added dropwise at 0–5 °C to a solution of methyl 3-(3-chloropropoxy)-4-methoxybenzoate (**21**, 93.0 g, 0.36 mol) in a mixture of acetic acid (300 mL) and acetic anhydride (100 mL). This mixture was stirred at room temperature for 6 h, then slowly poured into ice water (2 L) and extracted with ethyl acetate (4 × 200 mL). The combined organic layer was washed with saturated sodium bicarbonate (2 × 200 mL) and brine

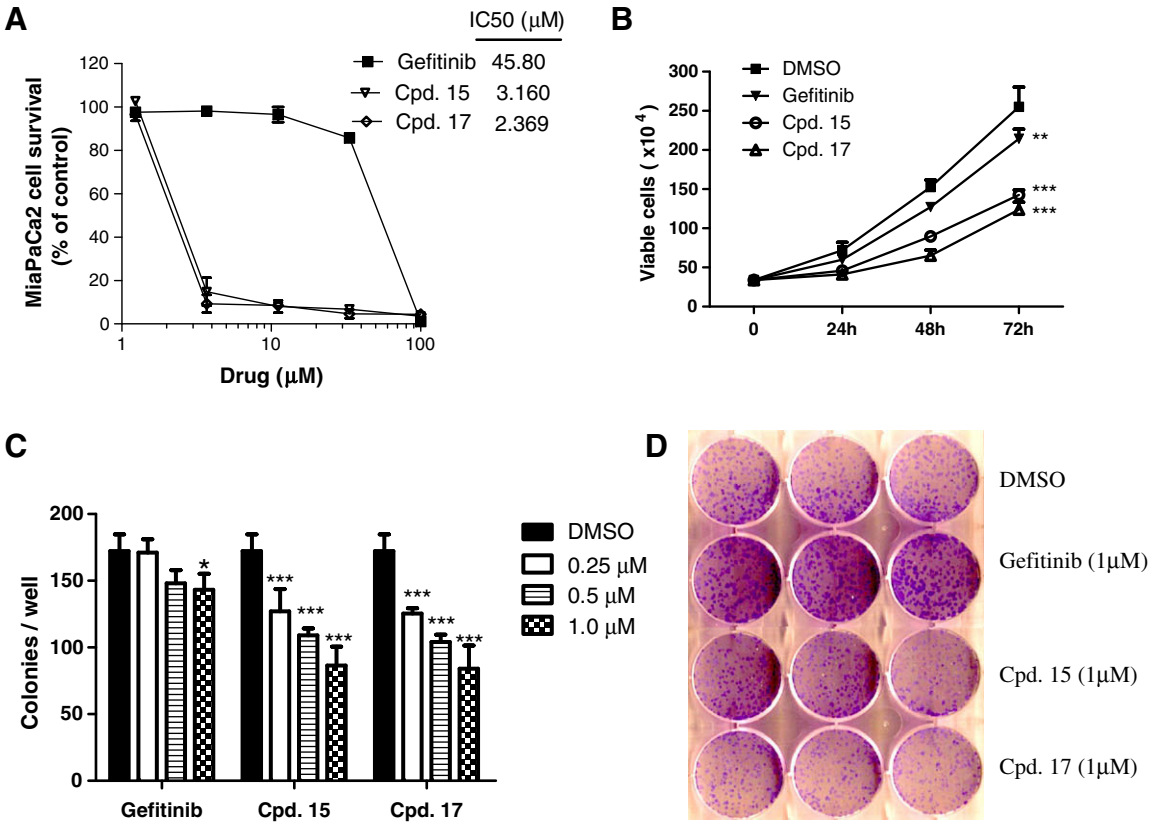


Figure 3. Compounds **15** and **17** significantly suppress HER-2(+) human pancreatic cancer Miapaca2 cell proliferation as compared with Gefitinib. (A) The MTT-based cytotoxicity assay in Miapaca2 cells was carried out as described in Section 5. (B) Growth curve of Miapaca2 cells after treatment with 2.5 μM Gefitinib, **15** or **17**. Miapaca2 cells were seeded in 6-well plates and, after treatment for the indicated time, trypsinized and counted. Mean ± SD ($n = 3$). $^{**}P$ <0.01, $^{***}P$ <0.001, versus DMSO control, two-way ANOVA. (C) Miapaca2 cells were seeded in 6-well plates and treated with Gefitinib, **15** or **17** at indicated doses. After 9–10 days incubation, the plates were gently washed with PBS and stained with 0.1% crystal violet. Colonies with over 50 cells were counted. Mean ± SD ($n = 3$). $^{*}P$ <0.05, $^{***}P$ <0.001, versus DMSO control, two-way ANOVA. (D) Representative pictures of the colonies.

(2 × 100 mL) and dried (Na_2SO_4). The ethyl acetate was evaporated to give a yellow oil that solidified after standing in a refrigerator for 12 h and was then recrystallized from ethyl acetate/petroleum ether to afford the product **22** as light yellow crystals (97.1 g, 89% yield). Mp: 54–56 °C; ^{1}H NMR δ: 2.03–2.24 (tt, 2H, $-CH_2CH_2CH_2-$), 3.66 (t, 2H, $-CH_2Cl$), 3.78 (s, 3H, $-OCH_3$), 3.89 (s, 3H, $-OCH_3$), 4.12 (t, 2H, $-CH_2O$), 7.82 (s, 1H, HAr), 8.01 (d, 1H, HAr).

5.1.3. Methyl 5-(3-chloropropoxy)-2-amino-4-methoxybenzoate (23)

Powdered iron (50 g, 0.89 mol) was added to acetic acid (500 mL). The resulting suspension was stirred for 15 min at 50 °C under an atmosphere of N_2, and a solution of methyl 5-(3-chloropropoxy)-4-methoxy-2-nitrobenzoate (**22**, 90.0 g, 0.30 mol) in methanol (300 mL) was added dropwise. The mixture was stirred for another 30 min at 50–60 °C. The catalyst was filtered, and the filtrate was slowly poured into water (4 L) and extracted with ethyl acetate (4 × 200 mL). The organic phase was washed with a saturated solution of sodium carbonate (2 × 100 mL) and brine (2 × 100 mL) and then dried (Na_2SO_4). The solvent was removed under vacuum and the brown solid residue was recrystallized from ethyl acetate/petroleum ether to give the product **23** as light brown crystals (63.1 g, 77% yield). Mp: 96–98 °C; ^{1}H NMR δ: 1.98–2.20 (tt, 2H, $-CH_2CH_2CH_2-$), 3.62 (t, 2H, $-CH_2Cl$), 3.76 (s, 3H, $-OCH_3$), 3.85 (s, 3H, $-OCH_3$), 4.07 (t, 2H, $-CH_2O$), 5.10–5.35 (br, 2H, $-NH_2$), 6.09 (d, 1H, HAr), 7.21 (s, 1H, HAr).

5.1.4. 6-(3-Chloropropoxy)-7-methoxyquinazolin-4(3*H*)-one (24)

A solution of methyl 5-(3-chloropropoxy)-2-amino-4-methoxybenzoate (**23**, 98.2 g, 0.36 mol) and formamidine acetate (52.6 g, 0.51 mol) in ethanol (800 mL) was heated at reflux for 6 h with overhead stirring. The mixture was allowed to stand in the refrigerator overnight. The precipitate was then collected by filtration, washed with ethanol and air dried to give **24** as a white power (88.7 g, 92% yield). Mp: 218–219 °C; ^{1}H NMR δ: 2.10–2.31 (tt, 2H, $-CH_2CH_2CH_2-$), 3.72 (t, 2H, $-CH_2Cl$), 3.83 (s, 3H, $-OCH_3$), 4.02 (t, 2H, $-CH_2O$), 6.98 (d, 1H, HAr), 7.89 (s, 1H, HAr), 8.02 (d, 1H, HAr), 9.03–9.42 (br, 1H, –NH–).

5.1.5. 4-Chloro-6-(3-Chloropropoxy)-7-methoxyquinazoline (25)

6-(3-Chloropropoxy)-7-methoxyquinazolin-4(3*H*)-one (**24**, 102 g, 0.38 mol) was added to thionyl chloride (500 mL) with magnetic stirring. DMF (20 mL) was then slowly added dropwise and the mixture was heated to reflux for 4 h. Most of the excess of thionyl chloride was then removed under reduced pressure and the yellow residue was dissolved in chloroform (500 mL), washed with a saturated solution of sodium carbonate (2 × 100 mL) and water (2 × 100 mL), and dried (Na_2SO_4). The chloroform was then removed under reduced pressure to give an off-white power, which was recrystalized from ethyl acetate to give the product **25** (93.5 g, 86% yield). Mp: 150–152 °C; ^{1}H NMR δ: 2.43 (tt, 2H, $-CH_2CH_2CH_2-$), 3.85 (t, 2H, $-CH_2Cl$), 4.09 (s, 3H, $-OCH_3$), 4.39 (t, 2H, $-OCH_2$), 7.43 (s, 1H, HAr), 7.47 (s, 1H, HAr), 8.91 (s, 1H, HAr).

5.1.6. Ethyl 5-(6-(3-chloropropoxy)-7-methoxyquinazolin-4-ylamino)benzo[*b*]thiophene-2-carboxylate (26)

Ethyl 5-aminobenzo[*b*]thiophene-2-carboxylate (16.1 g, 73 mmol) was added to a solution of 4-chloro-6-(3-chloropropoxy)-7-methoxyquinazoline (**25**, 14.9 g, 52 mmol) in isopropanol (300 mL). The mixture was heated to reflux for 3 h, and then left standing

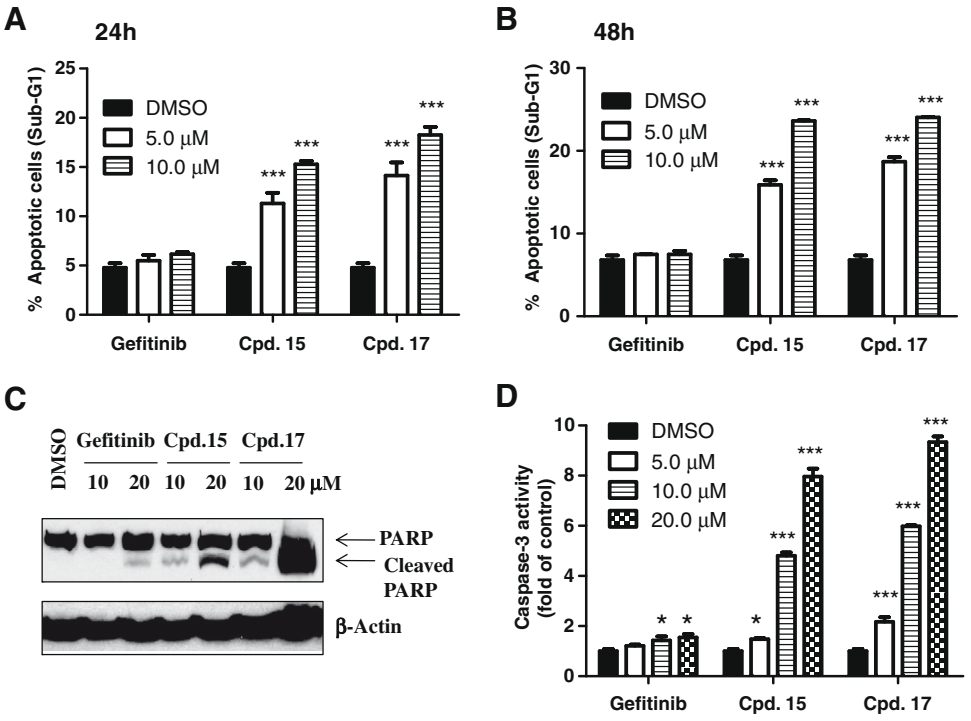


Figure 4. Compounds **15** and **17** potently induced cell apoptosis. Miapaca2 cells were seeded in 6-well plates and incubated with Gefitinib, **15** or **17** at doses of 5, 10, or 20 μM, respectively. 24 h or 48 h after incubation, cells were harvested and processed for further detection. (A, B) Apoptotic cell population after 24 h and 48 h treatment. Miapaca2 cells were stained with propidium iodide after ethanol fixation and analyzed by flow cytometry. Mean ± SD (n = 2). ***P <0.001, two-way ANOVA versus DMSO control. (C) Western blot analysis of apoptosis-related protein PARP after 24 h treatment. Actin is shown as a loading control. (D) Caspase-3 activation after 24 h treatment. Whole-cell lysates (20 μg) were reacted with fluorogenic substrate DEVD-AFC. After incubation at 37 °C for 2 h, released AFC was monitored by a microplate reader (BMG LABTECH). Fold increase of fluorescence signal was expressed by normalizing activity to DMSO control. Mean ± SD (n = 2). *P <0.05, ***P <0.001, two-way ANOVA versus DMSO control.

in the refrigerator overnight; the precipitate was collected by filtration, washed with chilled isopropanol (2 × 150 mL), and recrystalized from ethyl acetate to give an off-white power (22.8 g, 93% yield). Mp: >270 °C.

5.1.7. Ethyl 5-(7-methoxy-6-(3-morpholinopropoxy)quinazolin-4-ylamino)benzo[*b*]thiophene-2-carboxylate (1)

Ethyl 5-(6-(3-chloropropoxy)-7-methoxyquinazolin-4-ylamino)-benzo[*b*]thiophene-2-carboxylate **(26,** 0.80 g, 2 mmol**)** and potassium iodide (0.2 g) were added to the solution of morpholine (5 mL) in DMF (15 mL).The solution was stirred at 70 °C for 30 min. The excess morpholine was then removed under reduced pressure and the residue dissolved in chloroform (60 mL), washed with water (2 × 20 mL), and then dried (Na_2SO_4). The solvent was removed under vacuum. The crude product was purified by column chromatography on silica gel, eluting with ethyl acetate/triethylamine (20:1) to afford white powder (**1**, 0.81 g, 77% yield). Mp: 192–194 °C; ^{1}H NMR ($CDCl_3$) δ: 1.43 (t, 3H), 2.12 (dd, 2H), 2.49 (t, 4H), 2.58 (t, 2H), 3.72 (t, 4H), 4.00 (s, 3H), 4.19 (t, 2H), 4.42 (q, 2H), 7.13 (s, 1H), 7.37 (s, 1H), 7.70 (d, 1H), 7.85 (d, 1H), 8.04 (s, 1H, HAr), 8.30 (d, 1H), 8.67 (s, 1H); ^{13}C NMR ($CDCl_3$) δ: 14.21, 26.23, 35.79, 55.40, 56.22, 60.39, 66.96, 67.76, 100.79, 108.19, 109.04, 118.07, 122.71, 123.14, 130.22, 135.19, 136.07, 138.23, 139.48, 147.67, 149.15, 153.63, 155.29, 156.47, 162.74; IR (cm^{-1}): 3445, 3367, 3027, 2976, 2870, 2109, 1710, 1621, 1592, 1526, 1503, 1424, 1391, 1240, 1141, 1110, 996, 957, 857, 754; *M/z*: 523.2 ([M+H]$^+$, 100%). Elem. Anal. Calcd: C, 62.05; H, 5.79; N, 10.72. Found: C, 61.57; H, 6.04; N, 10.09.

5.1.8. Ethyl 5-(7-methoxy-6-(3-(4-methylpiperazin-1-yl)propoxy)-quinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (2)

As in the procedure described for **1,** compound **2** was prepared as white powder (0.72 g, 68% yield). Mp: 169–171 °C; ^{1}H NMR ($CDCl_3$) δ: 1.42 (t, 3H), 2.10 (q, 2H), 2.27 (s, 3H), 2.45–2.54 (br, 8H), 2.55 (t, 2H), 3.99 (s, 3H), 4.22 (t, 2H), 4.42 (q, 2H), 7.23 (s, 1H), 7.34 (s, 1H), 7.73 (dd, 1H), 7.82 (d, 1H), 7.95 (br, 1H), 8.02 (s, 1H, HAr), 8.31 (d, 1H), 8.65 (s, 1H); ^{13}C NMR ($CDCl_3$) δ: 14.29, 26.43, 45.98, 53.20, 54.87, 55.05, 56.16, 61.62, 67.84, 100.90, 108.04, 118.07, 122.76, 123.06, 130.22, 136.08, 149.10, 153.59; IR (cm^{-1}): 3406, 3235, 3209, 2941, 2806, 2149, 1708, 1624, 1593, 1522, 1510, 1428, 1237, 1147, 1070, 1008, 962, 866, 756; *M/z*: 536.2 ([M+H]$^+$, 100%). Elem. Anal. Calcd: C, 62.78; H, 6.21; N, 13.07. Found: C, 62.09; H, 6.25; N, 13.25.

5.1.9. Ethyl 5-(7-methoxy-6-(3-(pyrrolidin-1-yl)propoxy)quinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (3))

As in the procedure described for **1,** compound **3** was prepared as light yellow powder (0.52 g, 58% yield). Mp: 162–164 °C; ^{1}H NMR ($CDCl_3$) δ: 1.41 (t, 3H), 1.81 (m, 4H), 2.12 (p, 2H), 2.54 (br, 4H), 2.65 (t, 2H), 3.98 (s, 3H), 4.17 (t, 2H), 4.40 (q, 2H), 7.18 (s, 1H), 7.53 (s, 1H), 7.71 (dd, 1H), 7.84 (d, 1H), 8.02 (s, 1H), 8.30 (d, 1H), 8.67 (s, 1H); ^{13}C NMR ($CDCl_3$) δ: 14.30, 23.45, 28.28, 52.90, 54.25, 56.16, 61.60, 67.91, 101.12, 107.91, 109.16, 118.04, 122.77, 122.96, 130.27, 134.92, 136.23, 138.04, 139.39, 147.43, 148.97, 153.54, 155.09, 156.59, 162.76; IR (cm^{-1}): 3452, 3313, 3280, 2871, 2186, 1705, 1624, 1587, 1513, 1438, 1413, 1288, 1225, 1140, 921, 855; *M/z*: 507.2 ([M+H]$^+$, 100%). Elem. Anal. Calcd: C, 64.01; H, 5.97; N, 11.06. Found: C, 63.59; H, 6.05; N, 10.85.

5.1.10. Ethyl 5-(7-methoxy-6-(3-(piperidin-1-yl)propoxy)quinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (4)

As in the procedure described for **1,** compound **4** was prepared as white powder (0.67 g, 71% yield). Mp: 153–155 °C; ^{1}H NMR ($CDCl_3$) δ: 1.40–1.46 (m, 5H), 1.57–1.65 (m, 4H), 2.12 (t, 2H), 2.44 (br, 4H), 2.53 (t, 2H), 4.00 (s, 3H), 4.19 (t, 2H), 4.43 (q, 2H), 7.20 (s, 1H), 7.51 (s, 1H), 7.73 (dd, 1H), 7.85 (d, 1H), 8.04 (s, 1H),

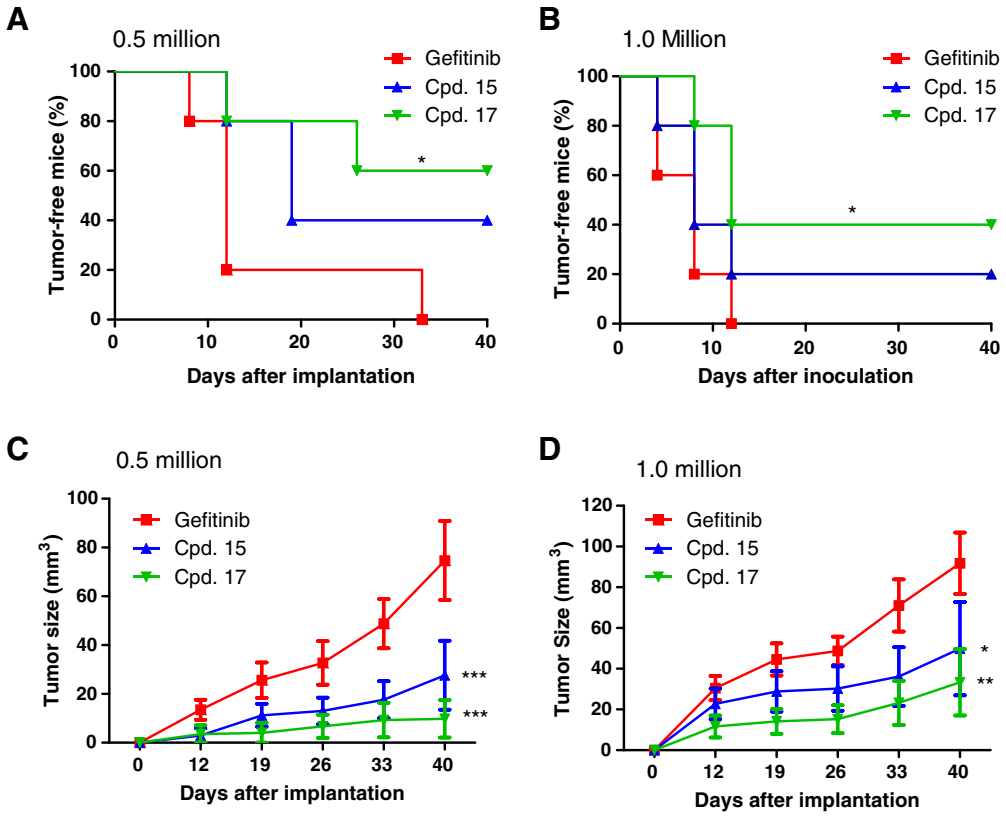


Figure 5. Compounds **15** and **17** inhibited tumor formation and tumor growth. Miapaca2 cells were treated with 2.5 μM Gefitinib, compound **15** or **17** for 48 h. Cells were collected, counted for live cells and inoculated into nude mice subcutaneously (sc) on both flanks, 0.5×10^6 cells or 1×10^6 cells/0.2 ml, respectively. (A, B) Kaplan–Meier curves show effects of Gefitinib, compound **15** or **17** pre-treatment on tumor formation. $^*P$ <0.05 versus Gefitinib, Mantel-Cox test. (C, D) The tumor sizes were measured using a caliper. Tumor volume was calculated using the formula: (length × width^2)/2. Mean ± SEM (n = 5). $^*P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001 versus Gefitinib, two-way ANOVA.

8.32 (d, 1H), 8.67 (s, 1H); ^{13}C NMR ($CDCl_3$): 14.35, 24.35, 25.90, 26.52, 54.68, 55.67, 56.21, 61.64, 68.13, 100.98, 108.13, 109.10, 118.08, 122.78, 123.09, 130.28, 135.09, 136.14, 138.18, 139.47, 147.62, 149.15, 153.60, 155.29, 156.49, 162.76; IR (cm^{-1}): 3466, 2930, 2850, 2071, 1706, 1622, 1594, 1525, 1507, 1427, 1390, 1238, 1194, 1147, 1065, 1010, 951, 862, 756; *M/z*: 521.3 ([M+H]$^+$, 100%). Elem. Anal. Calcd: C, 64.59; H, 6.20; N, 10.76. Found: C, 64.24; H, 6.17; N, 10.91.

5.1.11. Ethyl 5-(6-(3-(diethylamino)propoxy)-7-methoxyquinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (5)

As in the procedure described for **1,** compound **5** was prepared as white powder (0.56 g, 69% yield). Mp: 144–146 °C; ^{1}H NMR ($CDCl_3$) δ: 1.01 (t, 6H), 1.41 (t, 3H), 2.02 (p, 2H), 2.57 (hex, 6H), 3.97 (s, 3H), 4.14 (t, 2H), 4.40 (q, 2H), 7.24 (s, 1H), 7.67 (s, 1H), 7.70 (dd, 1H), 7.82 (d, 1H), 8.00 (s, 1H), 8.26 (d, 1H), 8.67 (s, 1H); ^{13}C NMR ($CDCl_3$) δ: 11.62, 14.28, 26.82, 46.99, 49.07, 56.12, 61.60, 67.79, 100.81, 107.90, 109.09, 118.06, 122.74, 122.99, 130.20, 134.97, 136.09, 138.09, 139.37, 147.44, 149.14, 153.49, 155.20, 156.51, 162.73; IR (cm^{-1}): 2950, 2795, 1711, 1623, 1574, 1521, 1445, 1425, 1389, 1232, 1192, 1142, 1065, 1009, 954, 867, 759; *M/z*: 509.2 ([M+H]$^+$, 100%). Elem. Anal. Calcd: C, 63.76; H, 6.34; N, 11.02. Found: C, 63.89; H, 6.34; N, 11.61.

5.1.12. Ethyl 5-(7-methoxy-6-(3-(4-methylpiperidin-1-yl)propoxy)-quinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (6)

As in the procedure described for **1,** compound **6** was prepared as white powder (0.61 g, 64% yield). Mp: 170–172 °C; ^{1}H NMR ($CDCl_3$) δ: 0.90 (d, 3H), 1.20–1.23 (m, 2H), 1.34 (m, 1H), 1.41 (t, 3H), 1.60 (d, 2H), 1.90 (t, 2H), 2.06 (p, 2H), 2.46 (t, 2H), 2.86 (d, 2H), 3.96 (s, 3H), 4.12 (t, 2H), 4.40 (q, 2H), 7.22 (s, 1H), 7.23 (s, 1H), 7.70 (dd, 1H), 7.74 (s, 1H), 7.80 (d, 1H), 7.99 (d, 1H), 8.27 (d, 1H); ^{13}C NMR ($CDCl_3$) δ: 14.29, 21.79, 26.63, 30.75, 34.30, 54.04, 55.27, 61.60, 68.00, 100.92, 107.88, 109.10, 118.12, 122.79, 122.97, 130.20, 13.95, 136.09, 138.09, 139.36, 147.44, 149.07, 153.30, 155.18, 156.54, 162.73; IR (cm^{-1}): 3282, 3094, 2944, 2913, 1713, 1624, 1577, 1527, 1516, 1451, 1428, 1393, 1295, 1241, 1202, 1146, 1066, 1003, 920, 886, 857, 752; *M/z*: 535.2 ([M+H]$^+$, 100%). Elem. Anal. Calcd: C, 65.14; H, 6.41; N, 10.48. Found: C, 65.02; H, 6.39; N, 10.79.

5.1.13. Ethyl 5-(7-methoxy-6-(3-(2-methylpiperidin-1-yl)propoxy)-quinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (7)

As in the procedure described for **1,** compound **7** was prepared as white powder (0.56 g, 59% yield). Mp: 177–178 °C; ^{1}H NMR ($CDCl_3$) δ: 1.05 (d, 3H), 1.25–1.30 (m, 2H), 1.41 (t, 3H), 1.60–1.66 (m, 2H), 2.02 (t, 2H), 2.13 (t, 1H), 2.42 (br, 1H), 2.44 (p, 1H), 2.54 (s, 2H), 2.85 (p, 2H), 3.95 (s, 3H), 4.06–4.14 (m, 2H), 4.40 (q, 2H), 7.22 (s, 1H), 7.32 (s, 1H), 7.73 (dd, 1H), 7.99 (s, 1H), 8.06 (d, 1H), 8.28 (d, 1H), 8.65 (d, 1H); ^{13}C NMR ($CDCl_3$) δ: 14.28, 23.76, 25.35, 25.96, 34.38, 50.20, 52.11, 56.11, 61.59, 68.00, 101.30, 107.76, 109.21, 118.09, 122.82, 122.91, 130.24, 136.24, 138.01, 139.34, 147.37, 148.92, 153.51, 156.67, 162.76; IR (cm^{-1}): 3331, 2977, 2931, 2849, 2156, 1704, 1626, 1595, 1577, 1524, 1453, 1427, 1392, 1290, 1235, 1221, 1191, 1142, 1077, 1014, 942, 887, 862, 754; *M/z*: 535.2 ([M+H]$^+$, 100%). Elem. Anal. Calcd: C, 65.14; H, 6.41; N, 10.48. Found: C, 65.17; H, 6.60; N, 10.34.

5.1.14. Ethyl 5-(6-(3-(dimethylamino)propoxy)-7-methoxyquinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (8)

As in the procedure described for **1,** compound **8** was prepared as white powder (0.47 g, 61% yield). Mp: 201–203 °C; ^{1}H NMR

($CDCl_3$) δ: 1.14 (t, 3H), 2.07 (t, 2H), 2.25 (s, 6H), 2.45 (t, 3H), 3.98 (s, 3H), 4.17 (t, 2H), 4.40 (q, 2H), 7.24 (s, 1H), 7.67 (s, 1H), 7.70 (dd, 1H), 7.82 (d, 1H), 8.01 (s, 1H), 8.29 (d, 1H), 8.67 (s, 1H); ^{13}C NMR ($CDCl_3$) δ: 14.21, 27.22, 45.56, 56.16, 56,21, 61.65, 67.74, 100.85, 108.05, 109.15, 118.04, 122.71, 123.06, 130.28, 136.14, 138.12, 139.45, 147.49, 149.17, 153.58, 155.16, 156.50, 162.78; IR (cm^{-1}): 3452, 3281, 3089, 2962, 2753, 2099, 1702, 1625, 1579, 1517, 1473, 1430, 1395, 1292, 1233, 1148, 1067, 993, 888, 862, 754; *M/z*: 509.2 ($[M+H]^+$, 100%). Elem. Anal. Calcd: C, 62.48; H, 5.87; N, 11.66. Found: C, 62.42; H, 5.93; N, 10.92.

5.1.15. Ethyl 5-(6-(3-(4-ethylpiperazin-1-yl)propoxy)-7-methoxyquinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (9)

As in the procedure described for **1,** compound **9** was prepared as white powder (0.54 g, 65% yield). Mp: 174–175 °C; ^1H NMR ($CDCl_3$) δ: 1.06 (t, 3H), 1.41 (t, 3H), 2.08 (t, 3H), 2.35–2.53 (m, 12H), 3.97 (s, 3H), 4.15 (t, 2H), 4.41 (q, 2H), 7.19 (s, 1H), 7.24 (s, 1H), 7.61 (br, 1H), 7.70 (d, 1H), 7.83 (d, 1H), 8.01 (s, 1H), 8.28 (s, 1H), 8.67 (s, 1H); ^{13}C NMR ($CDCl_3$) δ: 11.94, 14.30, 26.56, 52.29, 52.83, 53.32, 54.95, 56.15, 61.61, 67.96, 100.99, 108.13, 109.08, 118.06, 122.73, 123.04, 130.18, 136.14, 138.17, 139.45, 147.63, 149.19, 153.58, 155.35, 156.50, 162.71; IR (cm^{-1}): 3296, 3181, 3117, 2965, 2934, 2810, 1710, 1625, 1578, 1522, 1506, 1472, 1450, 1428, 1393, 1332, 1233, 1199, 1144, 1066, 994, 923, 845, 752, 639; *M/z*: 550.3 ($[M+H]^+$, 100%). Elem. Anal. Calcd: C, 63.37; H, 6.42; N, 12.74. Found: C, 62.95; H, 6.32; N, 12.84.

5.1.16. Ethyl 5-(7-methoxy-6-(3-(4-phenylpiperazin-1-yl)propoxy)-quinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (10)

As in the procedure described for **1,** compound **10** was prepared as white powder (0.61 g, 59% yield). Mp: 204–206 °C; ^1H NMR ($CDCl_3$) δ: 1.41 (t, 3H), 1.97 (br, 2H), 2.13 (p, 2H), 2.59–2.65 (m, 6H), 3.20 (t, 4H), 3.99 (s, 3H), 4.20 (t, 2H), 4.41 (q, 2H), 6.82–6.89 (m, 3H), 7.20–7.23 (m, 3H), 7.58 (s, 1H), 7.68 (dd, 1H), 7.80 (d, 1H), 8.00 (s, 1H), 8.27 (d, 1H), 8.67 (s, 1H); ^{13}C NMR ($CDCl_3$) δ: 14.30, 26.51, 49.13, 53.33, 54.90, 56.17, 61.60, 67.90, 101.22, 108.20, 109.13, 116.04, 118.06, 119.80, 122.72, 123.05, 129.10, 130.20, 136.16, 139.46, 147.69, 149.16, 151.22, 153.62, 155.38, 162.70; IR (cm^{-1}): 3454, 3347, 2968, 2815, 1697, 1678, 1623, 1597, 1574, 1528, 1508, 1473, 1434, 1392, 1240, 1219, 1142, 1076, 1008, 916, 866, 758, 692; *M/z*: 598.2 ($[M+H]^+$, 100%). Elem. Anal. Calcd: C, 66.31; H, 5.90; N, 11.72. Found: C, 65.32; H, 5.79; N, 11.81.

5.1.17. Ethyl 5-(7-(3-chloropropoxy)-6-methoxyquinazolin-4-ylamino)benzo[*b*]thiophene-2-carboxylate (27)

Ethyl 5-aminobenzo[*b*]thiophene-2-carboxylate (15.0 g, 68 mmol) was added to a solution of 4-chloro-7-(3-chloropropoxy)-6-methoxyquinazoline (15.2 g, 52 mmol) in isopropanol (300 mL). The mixture was heated to reflux for 3 h, and then left standing in the refrigerator overnight. The precipitate was then collected by filtration, washed with chilled isopropanol (2 × 150 mL), and recrystalized from ethanol to give an off-white power (21.5 g, 86% yield). Mp: >270 °C.

5.1.18. Ethyl 5-(6-methoxy-6-(7-morpholinopropoxy)quinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (11)

Ethyl 5-(7-(3-chloropropoxy)-6-methoxyquinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate **(27,** 0.78 g, 2 mmol**).** and potassium iodide (0.2 g) were added to the solution of morpholine (5 mL) in DMF (15 mL). The solution was stirred at 70 °C for 30 min, the excess morpholine was removed under reduced pressure, and the residue was dissolved in chloroform (60 mL), washed with water (2 × 20 mL), and then dried (Na_2SO_4). The solvent was removed under vacuum. The crude product was purified by column chromatography on silica gel, eluting with ethyl acetate/triethylamine (20:1) to afford white powder (**11**, 0.71 g, 68%). Mp: 155–157 °C; ^1H NMR ($CDCl_3$) δ: 1.41 (t, 3H), 2.09 (t, 2H), 2.48 (t, 4H), 2.54 (t, 2H), 3.72 (t, 4H), 3.97 (s, 3H), 4.21 (t, 2H), 4.40 (q, 2H), 7.13 (s, 1H), 7.55 (s, 1H), 7.69 (d, 1H), 7.83 (d, 1H), 8.01 (s, 1H), 8.26 (d, 1H), 8.6 (s, 1H); ^{13}C NMR ($CDCl_3$) δ: 14.27, 25.92, 53.67, 55.24, 56.32, 61.62, 66.93, 67.38, 99.60, 108.67, 108.88, 118.07, 122.73, 123.03, 130.16, 135.04, 136.01, 138.15, 139.37, 147.47, 149.84, 153.48, 154.34, 156.46, 162.71; IR (cm^{-1}): 3780, 3456, 2953, 2853, 2064, 1706, 1679, 1629, 1521, 1453, 1416, 1228, 1114, 1069, 1010, 858; *M/z*: 523.2 ($[M+H]^+$, 100%). Elem. Anal. Calcd: C, 62.05; H, 5.79; N, 10.72. Found: C, 61.77; H, 6.05; N, 9.99.

5.1.19. Ethyl 5-(6-methoxy-7-(3-(4-methylpiperazin-1-yl)propoxy)-quinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (12)

As in the procedure described for **11,** compound **12** was prepared as white powder (0.60 g, 62% yield). Mp: 173–174 °C; ^1H NMR ($CDCl_3$) δ: 1.42 (t, 3H), 2.10 (p, 2H), 2.31 (s, 3H), 2.25 (br, 8H), 2.57 (t, 2H), 4.01 (s, 3H), 4.24 (t, 2H), 4.42 (q, 2H), 7.07 (s, 1H), 7.33 (s, 1H), 7.70 (dd, 1H), 7.86 (d, 1H), 8.05 (d, 1H), 8.31 (d, 1H), 8.67 (s, 1H); ^{13}C NMR ($CDCl_3$) δ: 14.35, 26.35, 46.00, 53.13, 54.85, 55.16, 56.45, 61.67, 67.61, 99.45, 108.89, 108.95, 118.02, 122.69, 123.14, 130.23, 136.08, 138.22, 139.49, 147.71, 149.98, 153.59, 154.49, 156.40, 162.75; IR (cm^{-1}): 3302, 3181, 3103, 2940, 2838, 2241, 1712, 1624, 1587, 1521, 1454, 1417, 1245, 1229, 1147, 1057, 1010, 922, 854, 755; *M/z*: 536.2 ($[M+H]^+$, 100%). Elem. Anal. Calcd: C, 62.78; H, 6.21; N, 13.07. Found: C, 62.72; H, 6.15; N, 12.85.

5.1.20. Ethyl 5-(6-methoxy-7-(3-(pyrrolidin-1-yl)propoxy)quinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (13)

As in the procedure described for **11,** compound **13** was prepared as yellow powder (0.45 g, 51% yield). Mp: 205–207 °C; ^1H NMR ($CDCl_3$) δ: 1.48 (t, 3H), 1.80 (m, 4H), 2.14 (s, 2H), 2.56 (br, 4H), 2.68 (q, 2H), 3.73 (t, 2H), 4.02 (s, 3H), 4.23 (t, 2H), 7.24 (s, 1H), 7.40 (s, 1H), 7.51 (dd, 1H), 7.68 (d, 1H), 8.01 (d, 1H), 8.16 (d, 1H), 8.60 (s, 1H); ^{13}C NMR ($CDCl_3$) δ: 14.29, 23.54, 24.17, 26.62, 47.44, 49.28, 52.93, 54.25, 56.57, 67.62, 100.73, 108.59, 109.20, 118.56, 122.43, 122.66, 125.79, 136.07, 136.47, 139.56, 136.89, 139.56, 139.89, 147.50, 149.80, 153.65, 154.29, 157.05, 162.74; IR (cm^{-1}): 3422, 3332, 3139, 2869, 2178, 1706, 1625, 1588, 1511, 1450, 1417, 1296, 1224, 1140, 913, 852; *M/z*: 507.2 ($[M+H]^+$, 100%). Elem. Anal. Calcd: C, 64.01; H, 5.97; N, 11.06. Found: C, 63.59; H, 6.34; N, 10.58.

5.1.21. Ethyl 5-(6-methoxy-7-(3-(piperidin-1-yl)propoxy)quinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (14)

As in the procedure described for **11,** compound **14** was prepared as off-white powder (0.64 g, 68% yield). Mp: 166–167 °C; ^1H NMR ($CDCl_3$) δ: 1.39–1.46 (m, 5H), 1.57–1.65 (m, 4H), 2.12 (t, 2H), 2.44 (br, 4H), 2.54 (t, 2H), 4.00 (s, 3H), 4.21 (t, 2H), 4.41 (q, 2H), 7.10 (s, 1H), 7.41 (s, 1H), 7.70 (dd, 1H), 7.85 (d, 1H), 8.04 (s, 1H), 8.30 (d, 1H), 8.66 (s, 1H); ^{13}C NMR ($CDCl_3$) δ: 14.33, 24.33, 25.84, 26.23, 54.58, 55.65, 56.43, 61.66, 67.73, 99.49, 108.84, 108.88, 118.04, 122.75, 122.80, 123.10, 130.24, 136.07, 138.19, 139.44, 147.62, 149.90, 153.54, 154.40, 156.41, 162.76; IR (cm^{-1}): 3466, 3352, 3110, 2930, 2856, 2107, 1708, 1681, 1628, 1578, 1526, 1451, 1419, 1234, 1219, 1140, 1067, 1012, 944, 856, 756; *M/z*: 521.3 ($[M+H]^+$, 100%). Elem. Anal. Calcd: C, 64.59; H, 6.20; N, 10.76. Found: C, 64.67; H, 6.34; N, 10.18.

5.1.22. Ethyl 5-(7-(3-(diethylamino)propoxy)-6-methoxyquinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (15)

As in the procedure described for **11,** compound **15** was prepared as white powder (0.71 g, 72% yield). Mp: 181–183 °C; ^1H NMR ($CDCl_3$) δ: 1.03 (t, 6H), 1.42 (t, 3H), 2.05 (m, 2H), 2.55 (q,

4H), 2.65 (t, 3H), 3.99 (s, 3H), 4.21 (t, 2H), 4.42 (q, 2H), 7.12 (s, 1H), 7.50 (s, 1H), 7.70 (dd, 1H), 7.84 (d, 1H), 8.02 (d, 1H), 8.28 (d, 1H), 8.67 (s, 1H); *M/z*: 509.2, ([M+H]$^+$, 100%); ^{13}C NMR ($CDCl_3$) δ: 11.86, 14.22, 14.34, 26.61, 47.09, 47.15, 49.17, 56.43, 61.67, 67.68, 99.57, 108.86, 108.90, 118.06, 122.76, 123.10, 130.24, 135.11, 136.13, 138.18, 139.46, 147.70, 149.98, 153.57, 154.54, 156.47, 162.77; IR (cm^{-1}): 3453, 3281, 3198, 3093, 2957, 2165, 1705, 1625, 1577, 1522, 1450, 1417, 1234, 1194, 1147, 1065, 1018, 955, 869, 753; *M/z*: 509.2 ([M+H]$^+$, 100%). Elem. Anal. Calcd: C, 63.76; H, 6.34; N, 11.02. Found: C, 63.64; H, 6.34; N, 10.60.

5.1.23. Ethyl 5-(6-methoxy-7-(3-(4-methylpiperidin-1-yl)propoxy)-quinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (16)

As in the procedure described for **11,** compound **16** was prepared as white powder (0.62 g, 65% yield). Mp: 166–168 °C; ^{1}H NMR ($CDCl_3$) δ: 0.91 (d, 3H), 1.23–1.26 (m, 2H), 1.35 (m, 1H), 1.41 (t, 3H), 1.61 (d, 2H), 1.95 (t, 2H), 2.09 (p, 2H), 2.51 (t, 2H), 2.89 (d, 2H), 3.97 (s, 3H), 4.19 (t, 2H), 4.41 (q, 2H), 7.11 (s, 1H), 7.50 (s, 1H), 7.69 (dd, 1H), 7.83 (s, 1H), 8.01 (s, 1H), 8.27 (d, 1H), 8.75 (s 1H); ^{13}C NMR ($CDCl_3$) δ: 14.30, 21.83, 26.48, 30.80, 34.32, 54.02, 55.29, 56.37, 61.63, 67.80, 99.59, 108.76, 108.87, 118.06, 122.74, 123.05, 130.21, 136.08, 139.41, 153.51, 156.45; IR (cm^{-1}): 3431, 3359, 2947, 2918, 2861, 2171, 1709, 1680, 1627, 1579, 1525, 1453, 1419, 1236, 1143, 1067, 1015, 980, 849, 756; *M/z*: 535.2 ([M+H]$^+$, 100%). Elem. Anal. Calcd: C, 65.14; H, 6.41; N, 10.48. Found: C, 65.04; H, 6.67; N, 10.00.

5.1.24. Ethyl 5-(6-methoxy-7-(3-(2-methylpiperidin-1-yl)propoxy)-quinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (17)

As in the procedure described for **11,** compound **17** was prepared as off-white powder (0.51 g, 60% yield). Mp: 160–161 °C; ^{1}H NMR ($CDCl_3$) δ: 1.06 (d, 3H), 1.26–1.32 (m, 2H), 1.42 (t, 3H), 1.51–1.67 (m, 4H), 2.01–2.21 (m, 4H), 2.54 (p, 1H), 2.90 (p, 2H), 3.97 (s, 3H), 4.10–4.20 (m, 2H), 4.41 (q, 2H), 7.12 (s, 1H), 7.55 (s, 1H), 7.69 (dd, 1H), 7.84 (d, 1H), 8.02 (s, 1H), 8.27 (s, 1H), 8.67 (s, 1H); ^{13}C NMR ($CDCl_3$) δ: 14.34, 19.02, 23.98, 25.31, 26.20, 34.66, 50.23, 52.25, 54.66, 55.96, 56.42, 61.67, 67.84, 99.66, 108.82, 108.92, 118.09, 122.77, 123.09, 130.24, 135.10, 136.13, 138.19, 139.45, 147.67, 149.98, 153.57, 154.53, 156.60, 162.78; IR (cm^{-1}): 3450, 3253, 3076, 2928, 2166, 1725, 1702, 1621, 1576, 1525, 1506, 1452, 1418, 1242, 1222, 1146, 1067, 1008, 958, 852, 752; *M/z*: 535.2 ([M+H]$^+$, 100%). Elem. Anal. Calcd: C, 65.14; H, 6.41; N, 10.48. Found: C, 64.48; H, 6.40; N, 10.17.

5.1.25. Ethyl 5-(7-(3-(dimethylamino)propoxy)-6-methoxyquinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (18)

As in the procedure described for **11,** compound **18** was prepared as white powder (0.47 g, 52% yield). Mp: 176–178 °C; ^{1}H NMR ($CDCl_3$) δ: 1.43 (t, 3H), 2.37 (t, 2H), 2.76 (s, 6H), 3.15 (d, 3H), 4.05 (s, 3H), 4.26 (t, 2H), 4.41 (q, 2H), 7.18 (s, 1H), 7.60 (s, 1H), 7.84–7.91 (m, 2H), 8.05 (s, 1H), 8.43 (s, 1H), 8.54 (s, 1H); ^{13}C NMR ($CDCl_3$) δ: 13.70, 24.31, 38.96, 39.51, 35.79, 42.97, 54.90, 56.04, 60.88, 65.57, 101.83, 107.50, 109.19, 118.04, 121.92, 123.05, 129.63, 133.94, 136.27, 136.97, 138.55, 146.06, 148.74, 152.84, 156.47, 162.00; IR (cm^{-1}): 3487, 2825, 2662, 1709, 1622, 1587, 1513, 1453, 1418, 1230, 1146, 1065, 1015, 958, 859, 756; *M/z*: 509.2 ([M+H]$^+$, 100%). Elem. Anal. Calcd: C, 62.48; H, 5.87; N, 11.66. Found: C, 62.02; H, 5.51; N, 11.92.

5.1.26. Ethyl 5-(7-(3-(4-ethylpiperazin-1-yl)propoxy)-6-methoxy-quinazolin-4-ylamino)benzo[*b*] thiophene-2-carboxylate (19)

As in the procedure described for **11,** compound **19** was prepared as white powder (0.56 g, 67% yield). Mp: 155–156 °C; ^{1}H NMR ($CDCl_3$) δ: 1.07 (t, 2H), 1.38 (t, 2H), 2.06 (p, 2H), 2.20 (t, 2H), 2.37–2.55 (m, 12H), 3.93 (s, 3H), 4.17 (t, 2H), 4.39 (q, 2H), 7.17 (s, 1H), 7.24 (s, 1H), 7.67 (dd, 1H), 7.71 (s, 1H), 7.80 (d, 1H), 7.97 (s, 1H), 8.23 (d, 1H), 8.64 (s, 1H); ^{13}C NMR ($CDCl_3$) δ: 11.87, 14.26, 26.31, 52.25, 52.77, 53.15, 54.84, 56.34, 61.60, 67.57, 99.86, 108.72, 108.95, 118.09, 122.77, 122.97, 130.17, 135.01, 136.14, 138.12, 139.37, 147.57, 149.89, 153.49, 154.44, 156.54, 162.73; IR (cm^{-1}): 3420, 3335, 2975, 2948, 2808, 1719, 1697, 1626, 1596, 1579, 1527, 1511, 1453, 1419, 1395, 1237, 1220, 1201, 1162, 1145, 1061, 1012, 923, 866, 756, 649; *M/z*: 550.3 ([M+H]$^+$, 100%). Elem. Anal. Calcd: C, 63.37; H, 6.42; N, 12.74. Found: C, 63.33; H, 6.32; N, 12.40.

5.1.27. Ethyl 5-(6-methoxy-7-(3-(4-phenylpiperazin-1-yl)propoxy)-quinazolin-4-ylamino)benzo[*b*]thiophene-2-carboxylate (20)

As in the procedure described for **12,** compound **20** was prepared as white powder (0.48 g, 59% yield). Mp: 170–172 °C; ^{1}H NMR ($CDCl_3$) δ: 1.43 (t, 3H), 1.69 (br, 2H), 2.16 (p, 2H), 2.61–2.67 (m, 6H), 3.22 (t, 4H), 4.02 (s, 3H), 4.27 (t, 2H), 4.42 (q, 2H), 6.86 (t, 31),6.92 (s, 1H), 6.95 (s, 1H), 7.09 (s, 1H), 7.24–7.30 (m, 2H), 7.34 (s, 1H), 7.70 (dd, 1H), 7.85 (d, 1H), 8.04 (s, 1H), 8.30 (d, 1H), 8.67 (s, 1H); ^{13}C NMR ($CDCl_3$) δ: 14.30, 26.37, 49.20, 53.30, 54.94, 56.45, 61.61, 67.58, 70.47, 99.64, 109.04, 116.08, 119.65, 122.64, 123.09, 129. 09, 130,17, 136.12, 138.21, 139.49, 147.73, 150.01, 151.39, 153.58, 154.50, 156.42, 162.70; IR (cm^{-1}): 3428, 3329, 3180, 2939, 2820, 1693, 1624, 1599, 1557, 1494, 1453, 1395, 1384, 1313, 1278, 1238, 1212, 1139, 1042, 1011, 927, 898, 783, 694; *M/z*: 598.3 ([M+H]$^+$, 100%). Elem. Anal. Calcd: C, 66.31; H, 5.90; N, 11.72. Found: C, 65.35; H, 5.99; N, 12.50.

5.2. Biological evaluation

5.2.1. In vitro kinase assays

In vitro kinase inhibitory ability was determined using the HTScan EGFR Kinase Assay Kit, HTScan HER-2 Kinase Assay Kit, and HTScan MET Kinase Assay Kit (Cell Signaling Technology), following the manufacturer's instructions.

5.2.2. Cell culture and reagents

Human pancreatic cancer cell lines, prostate cancer cell lines, lung cancer cell lines and human breast cell line MCF-7 were purchased from American Type Culture Collection and cultured in high-glucose DMEM (HyClone) supplemented with 10% fetal bovine serum (FBS; HyClone) in a 5% CO_2 humidified incubator at 37 °C. All media were also supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin. MCF-7-HER2 cells were obtained by transfecting MCF-7 cells with plasmid containing human HER-2 cDNA under CMV promoter and selected for highly proliferating and tumorigenic clone.

5.2.3. Cell cytotoxicity assay

Cells were seeded in 96-well culture plates (5000 cells/well) and treated with serially diluted testing compounds in triplicates. After 4–5 days culture, cell growth medium was removed, proliferation reagent WST-8 (Sigma) was added to each well and incubated at 37 °C for 1–3 h. Absorbance was measured with a plate reader at 450 nm with correction at 650 nm. The results were expressed as the percentage of absorbance of treated wells versus that of vehicle control. IC_{50}, the drug concentration causing 50% growth inhibition, was calculated via sigmoid curve fitting using GRAPHPAD PRISM 5.0, as we described previously.[25]

5.2.4. Colony formation assay

For colony formation assay, Miapaca2 cells were seeded in 6-well plates (200 cells/well, in triplicate) and treated with Gefitinib, compound **15** or **17** at different doses. 0.5 ml FBS was added per well on Day 5. After 9–10 days incubation, plates were gently

washed with PBS and stained with 0.1% of crystal violet. Colonies with over 50 cells were manually counted. Plating efficiency was calculated by dividing the number of colonies formed in the treated group by that in the control, as we previously described.[26–28]

5.2.5. Apoptosis analysis

For apoptosis analysis by flow cytometry, MiaPaCa2 cells were treated with Gefitinib, compound **15** or **17** at different doses for 24 h or 48 h, then trypsinized and washed with phosphate-buffered saline and fixed in 70% ethanol on ice. After centrifugation, cells were stained with 50 μg/ml propidium iodide and 0.1 μg/ml RNase A, and analyzed by flow cytometry using a FACStar Plus™. Each histogram was constructed with the data from at least 5000 events. Data were analyzed to calculate the percentage of cell population in each phase using the CellQuest software, as we described previously.[25]

5.2.6. Western blot analysis

Cells were washed with PBS and lysed in an ice-cold RIPA lysis buffer (1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS, and Complete Protease Inhibitor cocktail 100 μg/ml, in PBS) for 15 min on ice. The cell lysates were then cleared by centrifugation at 13,000*g* for 10 min at 4 °C. The supernatants were collected. Protein concentrations were determined with the Bradford method (Bio-Rad); 40 μg of protein were electrophoresed by 10% SDS–PAGE. Separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad). After blocking with 5% milk, blots were probed with anti-HER-2, anti-PARP (Cell Signaling Technology) and anti-β-Actin (Sigma), followed by horseradish peroxidase-conjugated secondary antibody (Pierce), and detected with the SuperSignal West Pico chemiluminescence substrate (Pierce) as we described previously.[27]

5.2.7. Caspase-3 activation assay

Caspase activation in MiaPaCa2 cells treated with Gefitinib, compound **15** or **17** at different doses was determined following the instructions of a Caspase-3 activation assay kit (BioVision). Twenty-four hours after treatment, cells were lysed and the whole cell lysates (20 μg) were incubated with 25 μM fluorogenic substrate DEVD-AFC in a reaction buffer (containing 5 mM DTT) at 37 °C for 2 h. Proteolytic release of AFC was monitored at λ_{ex} = 405 nm and λ_{em} = 500 nm using a fluorescence microplate reader. Fold increase of fluorescence signal was calculated by dividing the normalized signal in each treated sample with that in the DMSO control, as we described previously.[27,29]

5.2.8. Animal studies

Five- to six-week old female athymic NCr-nu/nu nude mice were purchased from NCI. MiaPaCa2 cells were treated with 2.5 μM Gefitinib, compound **15** or **17** for 48 h. Cells were collected and inoculated into nude mice subcutaneously (sc) on both flanks after alcohol preparation of the skin, using a sterile 22-gauge needle with 0.2 ml cell suspension of 0.5×10^6 or 1×10^6 cells, with manual restraint. Tumor sizes were measured using a caliper. Tumor volume was calculated using the formula: (length × width^2)/2, as we described previously.[29] All animal experiments were done according to the protocol approved by the University of Michigan Guidelines for Use and Care of Animals.

5.2.9. Statistical analysis

Two-way ANOVA was employed to analyze the cell assay data using PRISM 5.0 software (GRAPHPAD). Kaplan–Meier analysis and Mantel–Cox test were used for animal studies. P <0.05 was defined as statistically significant.

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