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Enhancement of EGFR tyrosine kinase inhibition by C–C multiple bonds-containing anilinoquinazolines

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ABSTRACT

A series of 4-anilinoquinazolines with C–C multiple bond substitutions at the 6-position were synthesized and investigated for their potential to inhibit epidermal growth factor receptor (EGFR) tyrosine kinase activity. Among the compounds synthesized, alkyne **6d** and allenes **7d** and **7f** significantly inhibited EGFR tyrosine kinase activity. These compounds inhibited EGF-mediated phosphorylation of EGFR in A431 cells, resulting in cell-cycle arrest and apoptosis induction. The C–C multiple bonds substituted at the C-6 position of the anilinoquinazoline framework were essential for the significant inhibitory activity. Compounds with long carbon chains ($n = 3–6$), such as **6c–f**, **7c–f**, **11**, and **12**, displayed prolonged inhibitory activity.

1. Introduction

Epidermal growth factor receptor (EGFR) tyrosine kinase plays a fundamental role in signal transduction pathways,[1,2] and the uncontrolled activation of this EGFR-mediated signaling may be due to the overexpression of the receptors in numerous tumors, including head and neck, lung, breast, bladder, prostate, and kidney cancers; mutations resulting in their constitutive activation in brain tumors; or the overexpression of the ligands, including EGF or TGF-α, in pancreatic, prostate, lung, ovarian, and colon cancers.[3,4] Therefore, EGFR tyrosine kinase is an attractive target for novel anticancer drug development.[5] Gefitinib (Iressa™)[6,7] and erlotinib (Tarceva™)[8] are reversible binding inhibitors of EGFR kinase activity and have been approved for non-small-cell lung cancer (NSCLC) therapy. Lapatinib is a dual reversible ErbB inhibitor with potent activity toward EGFR and Her-2 kinases, and has been approved for breast cancer therapy.[9] Although the therapeutic response to those inhibitors can persist for as long as 2–3 years, most patients who show recurrence ultimately develop acquired resistance to those agents. In particular, a mutation, T790M, within the EGFR kinase domain was found in ~50% of the patients.[10] In this regard, irreversible EGFR inhibitors have attracted attention in recent years because of their potential to inhibit the activity of EGFR tyrosine kinase with the T790M mutation.[11] EKB-569,[12] HKI-272,[13] and CI-1033[14,15] have been investigated as irreversible inhibitors and phase I/II clinical trials are under way (Fig. 1).

We have been trying to uncover the efficiency of carbon–carbon multiple bonds, including alkenes, alkynes, and allenes, in medicinal chemistry.[16,17] Allenes, in particular, have extraordinary properties, such as the potential for axial chirality and a higher reactivity than alkenes.[18] Therefore, there is much expectation that the introduction of an allenic moiety to an existing molecular scaffold would improve the biological and pharmacological properties of the compound.[19] Recently, we developed a simple protocol for the introduction of an allene moiety into various electrophiles under palladium-catalyzed conditions.[20–22] Using this protocol, we found that the introduction of allene into 4-anilinoquinazolines selectively inhibited EGFR tyrosine kinase activity.[17] In this paper, we demonstrated the introduction of various C–C multiple bond substituents into the 6-position of the anilinoquinazoline framework and investigated their potential to inhibit EGFR tyrosine kinase activity.

2. Chemistry

6-Alkoxyanilinoquinazoline derivatives **5** were synthesized from 6,7-dimethoxyquinazoline **2** via selective deprotection of the methyl group from the 6-methoxy group of **2** according to the procedure in the literature, with modification (Scheme 1).[6] Briefly, **2** was treated with D,L-methionine in methanesulfonic acid to give 6-hydroxy-7-methoxyquinazoline, and acetyl protection of the phenolic hydroxyl group followed by chlorination with $POCl_3$ gave **3** in 36% yield in three steps. Treatment of 4-chloroquinazoline **3** with 3-chloroaniline or 3-chloro-4-fluoroaniline in isopropyl alcohol gave corresponding 4-anilinoquinazolines **4a** and **4b** in 69%

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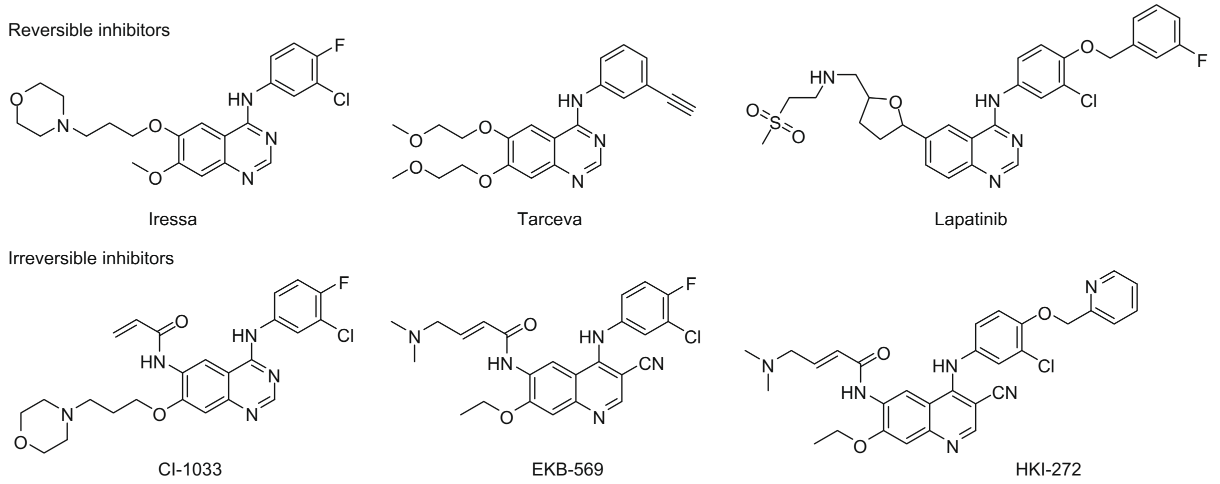
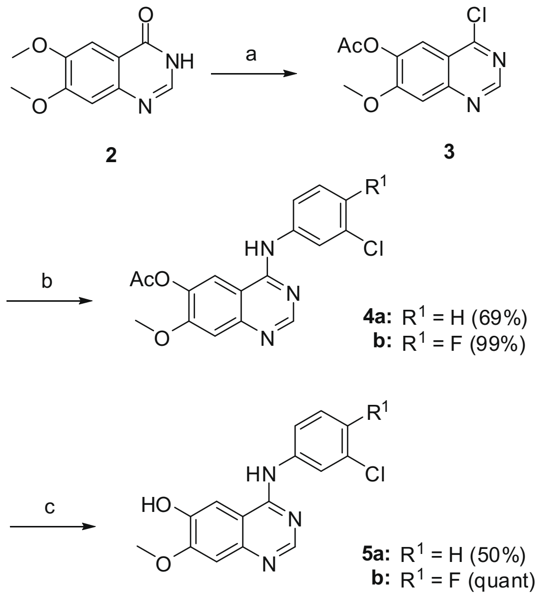
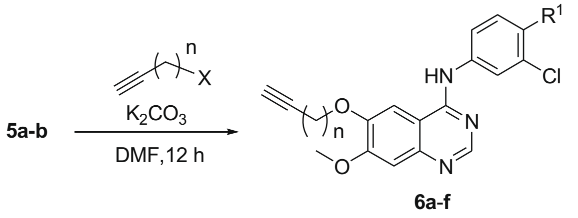


Figure 1. Structures of EGFR tyrosine kinase inhibitors.



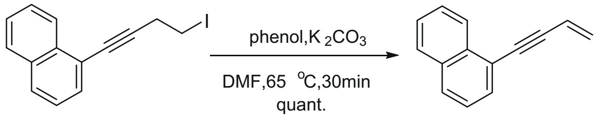
Scheme 1. Reagents and conditions: (a) (i) D,L-methionine, CH_3SO_3H, 120 °C, 12 h; (ii) Ac_2O, DMAP, pyridine, 0 °C to rt, 12 h; (iii) $POCl_3$, DIPEA, toluene, 120 °C, 14 h, three steps, 36%; (b) 3-chloroaniline or 3-chloro-4-fluoroaniline, *i*PrOH, 90 °C, 2 h; (c) 25% NH_3 aq, MeOH, rt, 12 h.

Table 1
Synthesis of alkynes from 6-hydroxyquinazolines **5**



Compound	R^1	*n*	X	Yield[a] (%)
6a	H	1	Br	88
6b	F	1	Br	>99
6c	H	3	I	78
6d	F	3	I	59
6e	F	4	Cl	74
6f	F	5	I	48

[a] Isolated yields based on **5**.



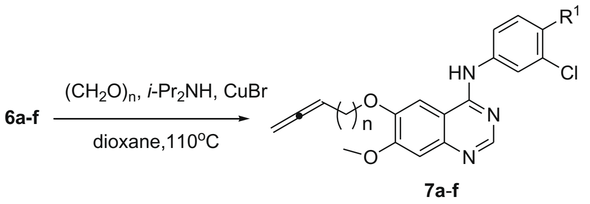
Scheme 2. Reaction of 1-(4-iodo-1-butynyl)naphthalene with phenol in the presence of K_2CO_3 as a base.

and 99% yields, respectively. The removal of the acetyl group with 25% aqueous ammonia in methanol afforded 4-anilino-6-hydroxy-7-methoxyquinazoline derivatives **5a** and **5b**. As shown in Table 1, **5a** and **5b** reacted with various haloalkynes in the presence of K_2CO_3 as a base to give corresponding alkynyl ether derivatives **6a–f** (*n* = 1–5) in good to high yields. Interestingly, ether formation was not observed whereas quantitative recovery of **5a** (or **5b**) was observed in the case of the reaction with 4-iodo-1-butyne (*n* = 2), although 4-iodo-1-butyne was consumed readily. Indeed, the ether formation of alcohols upon reacting with β-halogenated alkynes has not been reported so far. Therefore, we conducted a model reaction as shown in Scheme 2 to examine the reaction in greater detail. We synthesized 1-(4-iodo-1-butynyl)naphthalene and reacted it with phenol in the presence of K_2CO_3 as a base in DMF at 65 °C. 1-(4-Iodo-1-butynyl)naphthalene was consumed in 30 min and the corresponding elimination (E2) product was obtained quantitatively. The results indicate that β-halogenated alkynes undergo not the S_N2 reaction with alkoxy anions but the E2 reaction to afford conjugated enynes.

Resultant terminal alkynes **6** were converted into corresponding allenes **7** by treatment with paraformaldehyde and *N*,*N*-diisopropylamine in the presence of 2 equiv of copper bromide in dioxane at 110 °C in 29–50% yields.[23,24] Recently, we found that the copper–bromide-catalyzed allene transformation was accelerated by microwave irradiation.[25] Therefore, we examined the transformation of **6d** into **7d** under microwave condition. The reaction was carried out in a sealed vial and the temperature of the microwave apparatus was set at 150 °C. The reaction was completed in 5 min and **7d** was obtained in 52% yield (Table 2).

Acetylene-conjugated anilinoquinazoline **10** was also synthesized from 4-anilino-6-hydroxy-7-methoxyquinazoline derivative

Table 2
Synthesis of allenes from alkynes **6**



Compound	R^1	*n*	Time (h)	Yield[a] (%)
7a	H	1	4	50
7b	F	1	4	38
7c	H	3	4	22
7d	F	3	4 (5 min)[b]	40 (52)[b]
7e	F	4	4	39
7f	F	5	5	29

[a] Isolated yields based on **6**.

[b] The reaction was carried out in the microwave reactor and the reaction time and yield were indicated in the parenthesis.

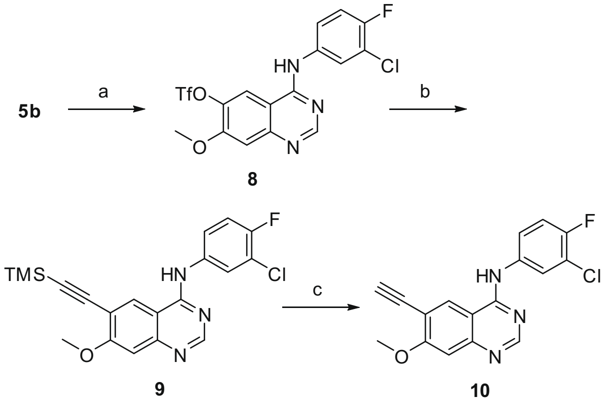
5b, as shown in Scheme 3. Compound **5b** was treated with trifluoromethanesulfonic anhydride to furnish triflate **8**, and **8** underwent the Sonogashira coupling reaction with ethynyltrimethyl silane in the presence of $Pd(PPh_3)_4$ catalyst in THF under reflux condition to give **9** in 68% yield. Removal of the TMS group with K_2CO_3 in MeOH gave **10**, quantitatively.

To compare the inhibitory effects of the C–C multiple bond moieties on EGFR tyrosine kinase activity, hexenyloxy- and hexyloxy-group-conjugated anilinoquinazolines **11** and **12** were synthesized from **5b** (Scheme 4).

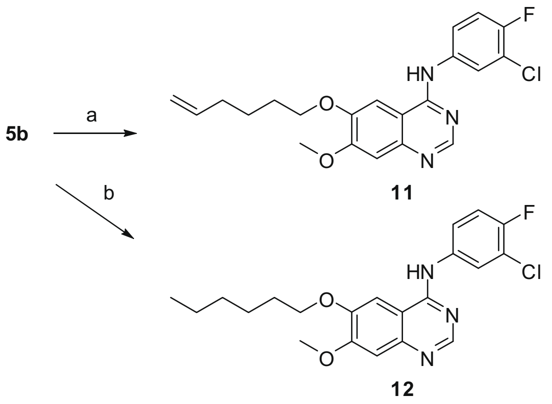
3. Results and discussion

3.1. Assay for in vitro kinase inhibitory activity

The inhibitory effect on EGFR tyrosine kinase activity was determined by measuring the extent of phosphorylation of the tyrosine-kinase-specific peptide poly(Glu/Tyr) in vitro.[26] Table 3 shows the results of assay for the EGFR tyrosine kinase inhibitory activity of the synthesized compounds and four EGFR tyrosine kinase inhibitors, Tarceva, AG1476, PD168393, and Iressa. Compounds **6a–f** and **7a–f** inhibited EGFR tyrosine kinase activity with subnanomolar IC_{50} values. Especially, compounds **6d** (*n* = 3), **7d** (*n* = 3), and **7f** (*n* = 5) showed significant inhibition with IC_{50} values of 2.53, 3.16, and 4.45 nM, respectively. Their inhibitory activities are similar to that of AG1476 and PD168393 (IC_{50} = 6.30 and 2.62 nM, respectively), known EGFR tyrosine kinase inhibitors, and are higher than that of Tarceva and Iressa (IC_{50} = 20.3, 21.0 nM, respectively). Compound **10**, in which an acetylene group is directly conjugated to the C-6 position of the quinazoline ring, did not display EGFR tyrosine kinase inhibitory activity (IC_{50} = >1000 nM). To the contrary, it should be noted that an acetylene group directly conjugated to the aniline ring such as Tarceva is essential for EGFR tyrosine kinase inhibition. We also tested compound **11**, which has a terminal alkene group substitution at the C-6 position, and compound **12**, which has a hexyl group substitution at the C-6 position. Compound **11** displayed higher EGFR tyrosine kinase inhibitory activity than **6e** but lower activity than **7d**, although all these three compounds have six carbon substituents at the C-6 positions of their quinazoline rings. Meanwhile, compound **12** did not display significant kinase inhibitory activity (IC_{50} = 106 nM). The results indicate that alkyne and allene substitutions at the C-6 position are essential to induce the EGFR tyrosine kinase inhibitory activity, and alkene and alkane substituents are less effective although they have the same carbon number.[27]



Scheme 3. Reagents and conditions: (a) Tf_2O, DMAP, pyridine, 0 °C to rt, 12 h, 94%; (b) ethynyltrimethylsilane, TEA, $Pd(PPh_3)_4$, CuI, THF, reflux, 12 h; (c) K_2CO_3, MeOH/CH_2Cl_2, rt, 3 h, two steps, 74%.



Scheme 4. Reagents and conditions: (a) 6-chloro-1-hexene, K_2CO_3, KI, DMF, 60 °C, 12 h, 44%; (b) 1-chlorohexane, K_2CO_3, KI, DMF, 60 °C, 12 h, 52%.

Table 3
In vitro inhibition of EGFR tyrosine kinase activity[a]

Compd	EGFR inhibition (IC_{50}/nM)
6a	25.9 ± 1.49
7a	16.9 ± 0.82
6b	14.8 ± 5.15
7b	23.8 ± 1.61
6c	17.7 ± 4.85
7c	28.5 ± 0.86
6d	2.53 ± 0.58
7d	3.16 ± 1.33
6e	87.1 ± 1.30
7e	65.5 ± 2.81
6f	32.7 ± 1.55
7f	4.45 ± 2.15
10	>1000
11	32.6 ± 1.68
12	106 ± 11.3
Tarceva	20.3 ± 1.02
AG1476	6.30 ± 0.10
PD168393	2.62 ± 0.36
Iressa	21.0 ± 0.01

[a] The drug concentration required to inhibit the phosphorylation of the poly(Glu/Tyr) substrate by 50% (IC_{50}) was determined from semi-logarithmic dose–response plots. The results represent mean ± SD of three independent experiments performed in duplicate.

3.2. Inhibition of EGF-induced EGFR phosphorylation

To clarify the EGFR tyrosine kinase inhibitory activity of the compounds at the cellular level, we examined the effects of compounds **6a–f** and **7a–f** on EGF-induced EGFR autophosphorylation in EGFR-overexpressing A431 cells. The immunoblot analysis in Figure 2a revealed that all the synthesized compounds suppressed the EGF-induced EGFR phosphorylation (Tyr1173) in a concentration-dependent manner and almost complete inhibition was observed at 100 nM without affecting EGFR levels. AG1476, Tarceva, PD168393, and Iressa also down-regulated EGFR phosphorylation in a manner similar to the synthesized compounds. Interestingly, the prolonged inhibitory activity was observed in an experiment where A431 cells were treated with the compounds and washed prior to the assay.[28] After continuous incubation of A431 cells with the synthesized compounds, AG1476, Tarceva, PD168393, and Iressa at 10 and 100 nM for 1 h, followed by EGF stimulation, each compound inhibited the autophosphorylation of EGFR in A431 cells, as shown by the immunoblot analysis (Fig. 2b). When the cells were washed to remove unbound compounds from the cell medium and incubated for an additional 5 h prior to EGF stimulation, EGFR autophosphorylation activity was restored in the case of compounds **6a–b**, **7a–b**, AG1476, and Tarceva. Meanwhile, for cells treated with compounds **6c–f**, **7c–f**, and Iressa, the autophosphorylation activity was still inhibited in a manner similar to PD168393, an irreversible EGFR tyrosine kinase inhibitor.[29] These results suggest that compounds **6c–f**, **7c–f**, and Iressa, showed prolonged inhibition of EGFR tyrosine kinase activity, whereas other compounds, such as compounds **6a–b**, **7a–b**, AG1476, and Tarceva, reversibly interacted with EGFR tyrosine kinase. We speculated that the prolonged inhibitory activity may be caused by the length of the side chains substituted at the C-6 position of the quinazoline rings. Therefore, we compared the prolonged inhibitory activities of compounds **6e**, **7d**, **11**, and **12**, which have the same carbon number at the C-6 position of the molecules, in a wash-out experiment. As shown in Figure 3, EGFR autophosphorylation activity was restored by compounds **6e**, **7d**, **11**, and **12**. As compounds **6a–b**, **7a–b**, AG1476, and Tarceva, which have short alkyl or hydrophilic chains substituted at the C-6 position, did not show prolonged inhibition (Fig. 2b), the results indicate that the prolonged inhibitory activities of compounds **6c–f**, **7c–f**, **11**, and **12** are induced by the hydrophobic interaction between the long alkyl chains of the compounds and the target protein.

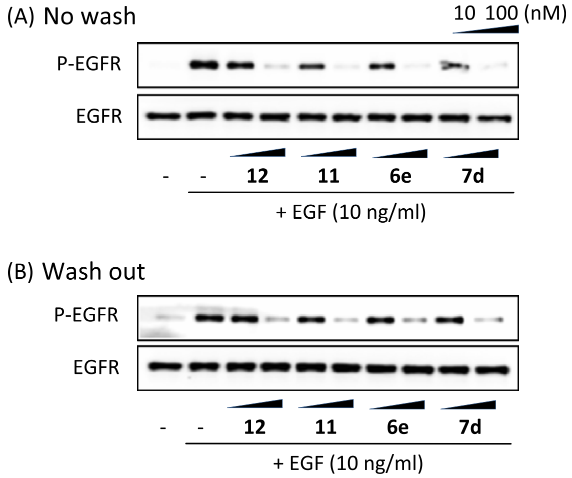


Figure 3. Prolonged inhibition of EGF-induced EGFR phosphorylation by compounds **6e**, **7d**, **11**, and **12**. A431 cells were pre-incubated for 10 min with the indicated concentrations of compounds **6e**, **7d**, **11**, or **12**, and then stimulated with EGF (10 ng/ml) (a) or washed with fresh medium for 5 h prior to stimulation with EGF (b). Levels of phosphorylated (P)-EGFR and EGFR were detected by immunoblot analysis with specific antibody. Similar results were observed in three independent experiments.

3.3. Suppression of cell growth

As EGFR expression is associated with increased tumor cell growth, the inhibition of EGFR expression is expected to lead to a reduction in cell growth. Therefore, we next examined the cell growth inhibitory effect of compounds **6d**, **7d**, and **7f** by the MTT assay. As shown in Table 4, treatment of A431 cells with compounds **6d**, **7d**, and **7f** resulted in decreased growth rate, and their GI_{50} values were 0.221, 0.268, and 0.586 μM, respectively. EGFR tyrosine kinase inhibitors Tarceva, AG1476, and PD168393 showed cell growth inhibitory activity with GI_{50} values of 0.915, 0.302, and 0.021 μM, respectively. These results support the findings of EGFR tyrosine kinase activity (Table 3) and EGF-induced EGFR phosphorylation (Figure 2). It was reported that the lack of autophosphorylation sites resulted in the reduction of internalization and degradation rates of EGFR.[30] Therefore, the prolonged inhibition of EGFR autophosphorylation by **6d**, **7d**, and PD168393 induces

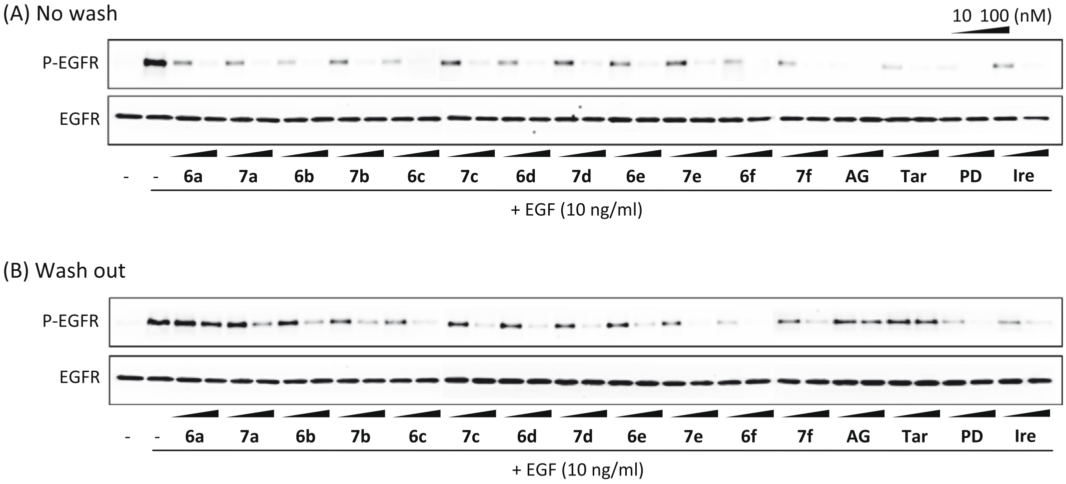


Figure 2. Inhibition of EGF-induced EGFR phosphorylation by synthesized compounds. A431 cells were pre-incubated for 10 min with the indicated concentrations of the synthesized compounds, AG1476 (AG), Tarceva (Tar), or PD168393 (PD), and then stimulated with EGF (10 ng/ml) (a) or washed with fresh medium for 5 h prior to stimulation with EGF (b). Levels of phosphorylated (P)-EGFR and EGFR were detected by immunoblot analysis with specific antibody. Similar results were observed in three independent experiments.

Table 4
Growth inhibition of A431 cells by **6d**, **7d**, and **7f**[a]

Compound	Growth inhibition ($GI_{50}/\mu M$)
6d	0.221 ± 0.023
7d	0.268 ± 0.053
7f	0.586 ± 0.083
Tarceva	0.915 ± 0.171
AG1476	0.302 ± 0.083
PD168393	0.021 ± 0.001
Iressa	0.514 ± 0.045

[a] A431 cells were incubated for 72 h with various concentrations (1 nM to 10 μM) of compounds, and then the rates of viable cells were determined by MTT assay. The drug concentration required to inhibit cell growth by 50% (GI_{50}) was determined from semi-logarithmic dose-response plots. The results represent mean ± SD of triplicate samples.

the extended reduction of internalization and degradation rates of EGFR, which may lead to the prolonged reduction of cell growth.

3.4. Induction of cell-cycle arrest and apoptosis

It has been established that the inhibition of EGFR by tyrosine kinase inhibitors induced G1 cell-cycle arrest and apoptosis in human carcinoma cells.[31] To determine whether the cell growth inhibitory activity of compounds **6d** and **7d** is mediated by the induction of cell-cycle arrest and apoptosis or not, biparametric cytofluorimetric analysis was performed using FITC-labeled annexin V and propidium iodide (PI), which, respectively, stain phosphatidylserine residues and DNA.[32] As shown in Figure 4, compounds **6d** and **7d** induced cell-cycle arrest in the G1 phase in a concentration-dependent manner. Furthermore, the population of early apoptotic cells (annexin V positive and PI negative, B4 area) increased to 13.5% and 13.9% on treatment with compounds **6d** and **7d**, respectively (Fig. 5). PD168393 also showed similar activity for G1 cell-cycle arrest and apoptosis induction. These results indicate that the inhibition of cell growth by compounds **6d** and **7d** is due to the induction of cell-cycle arrest and apoptosis in A431 cells.

4. Conclusion

We have succeeded in the synthesis of various anilinoquinazolines with C–C multiple bond substitutions at the 6-position and investigated their potential to inhibit EGFR tyrosine kinase activity. Among the compounds synthesized, alkyne **6d** and allenes **7d** and **7f** displayed significant inhibitory effects on EGFR tyrosine kinase activity. These compounds inhibited EGF-mediated phosphorylation of EGFR in A431 cells, resulting in cell-cycle arrest and apoptosis induction. The C–C multiple bonds substituted at the C-6 position of the anilinoquinazoline framework were essential for the significant kinase inhibitory activity. Long carbon chains ($n = 3–6$), such as compounds **6c–f**, **7c–f**, **11**, **12**, and Iressa, played an important role to display their prolonged inhibitory activity.

Although the irreversible inhibitor PD168393, which is equipped with a Michael acceptor functional group, is more potent than **6d**, **7d**, and Tarceva for the growth inhibition of A431 cells, the findings presented herein show the potential of introducing C–C multiple bonds, including alkynes and allenes, into pharmacologically active molecular scaffolds for drug development.

5. Experimental

5.1. Chemistry

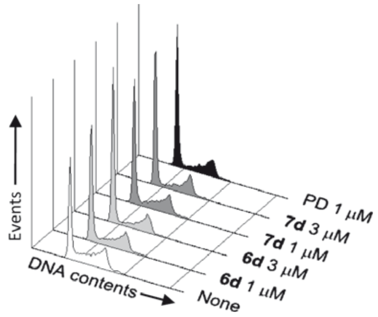
^1H NMR and ^{13}C NMR spectra were measured on JEOL JNM-AL 300 (300 MHz) and Varian Unity-Inova 400 (400 MHz) spectrometers. ^1H NMR and ^{13}C NMR chemical shifts are expressed in δ (ppm), and coupling constant are expressed in Hz. IR spectra were measured on a Shimadzu FTIR-8200A spectrometer. Analytical TLC was performed on glass plates (Merck Kieselgel 60 F_{254} or RP-18 F_{254}, layer thickness: 0.2 mm). Samples were visualized by UV light (254 nm), I_2, or $KMnO_4$. Column chromatography was performed on silica gel (Merck Kieselgel 70–230 mesh). All reactions were carried out under argon atmosphere using standard Schlenk techniques. All the chemicals were of analytical grade and used without further purification.

5.1.1. 6-Acetoxy-4-(3′-chloroanilino)-7-methoxyquinazoline 4a

To a solution of **3** (239 mg, 0.95 mmol) in isopropanol (10 ml) was added 3-chloroaniline (0.11 ml, 1.95 mmol) and the mixture was refluxed for 2 h with stirring. After cooling to room temperature, the precipitated solids were filtered and washed with isopropanol to give **4a** as a white solid (291 mg, 0.85 mmol, 89% yield). ^1H NMR (400 MHz, CD_3OD) δ 8.85 (s, 3H), 8.39 (s, 1H), 7.91 (s, 1H), 7.66–7.69 (d, J = 8.0 Hz, 1H), 7.49–7.53 (t, J = 8.0 Hz, 1H), 7.39–7.42 (m, 1H), 7.38 (s, 1H), 7.13 (s, 3H), 2.42 ppm (s, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ 170.1, 160.9, 160.3, 151.8, 143.2, 140.6, 139.1, 135.5, 131.3, 128.0, 125.7, 123.8, 119.0, 108.5, 101.8, 57.7, 20.2. IR (KBr) 3359, 3024, 2943, 2715, 1774, 1635, 1574, 1535, 1512, 1443, 1365, 1300, 1234, 1157, 1064, 999, 868 cm^{-1}; MS (ESI) m/z 344 ($[M+H]^+$). Anal. Calcd for $C_{17}H_{14}ClN_3O_3$: C, 59.49; H, 4.10; N, 12.22. Found: C, 59.23; H, 3.95; N, 12.05.

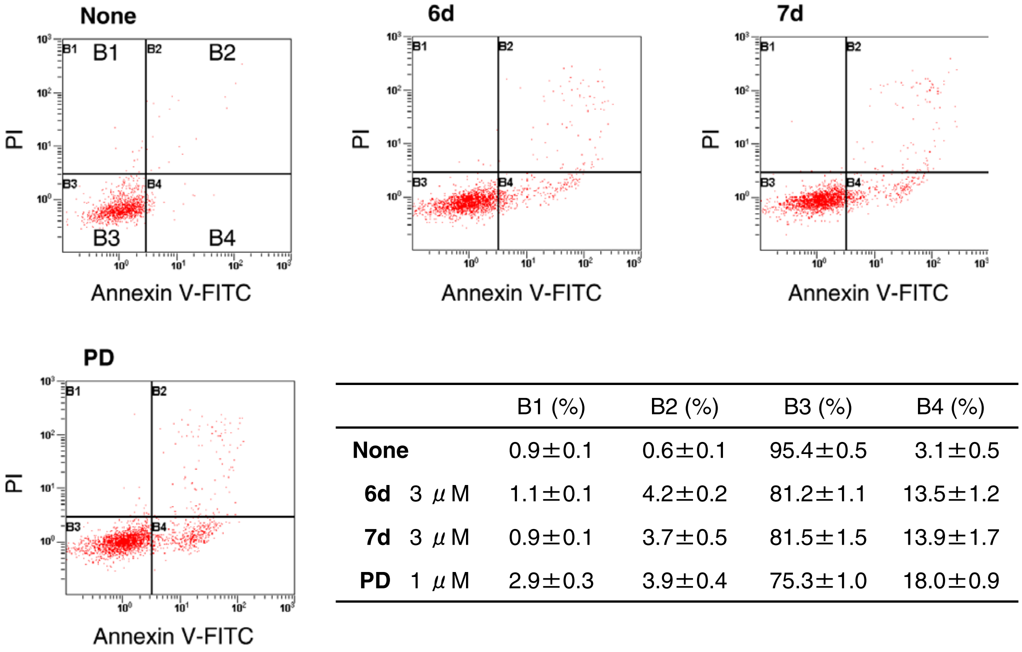
5.1.2. 6-Acetoxy-4-(3′-chloro-4′-fluoroanilino)-7-methoxyquinazoline 4b

Compound **4b** was prepared from **3** (251 mg, 1.0 mmol) and 3-chloro-4-fluoroaniline (175 mg, 1.2 mmol) in isopropanol (12 ml) by the procedure described for **4a** to give **4b** as a white solid (374 mg, 1.03 mmol, >99%). ^1H NMR (400 MHz, CD_3OD) δ 8.83 (s, 1H), 8.34 (s, 1H), 7.98–8.00 (m, 1H), 7.67–7.70 (m, 1H), 7.39–7.43



		G1 (%)	S (%)	G2/M (%)
None		39.8±0.2	48.5±4.2	11.1±3.1
6d	1 μM	53.5±0.5	34.7±2.7	11.7±3.1
	3 μM	65.3±1.9	22.1±2.8	12.5±4.5
7d	1 μM	54.3±0.7	33.6±2.6	12.2±2.8
	3 μM	63.6±1.4	23.0±2.4	13.4±3.6
PD	1 μM	60.0±1.0	27.7±2.0	12.3±2.2

Figure 4. Induction of G1 cell-cycle arrest by compounds **6d** and **7d**. A431 cells were incubated for 24 h with the indicated concentrations of **6d**, **7d**, or PD168393 (PD). The cells were harvested and stained with propidium iodide, and the cell-cycle phases were determined by a flow cytometer. The results represent the mean ± SD of triplicate samples.



	B1 (%)	B2 (%)	B3 (%)	B4 (%)
None	0.9±0.1	0.6±0.1	95.4±0.5	3.1±0.5
6d 3 μM	1.1±0.1	4.2±0.2	81.2±1.1	13.5±1.2
7d 3 μM	0.9±0.1	3.7±0.5	81.5±1.5	13.9±1.7
PD 1 μM	2.9±0.3	3.9±0.4	75.3±1.0	18.0±0.9

Figure 5. Apoptosis induction by **6d** and **7d**. A431 cells were incubated for 12 h with the indicated concentrations of **6d**, **7d**, or PD168393 (PD). The cells were harvested and stained with annexin V-FITC and propidium iodide (PI), and the percentage of stained cells was determined by a flow cytometer. The results represent the mean ± SD of triplicate samples.

(t, *J* = 8.8 Hz, 1H), 7.37 (s, 1H), 4.12 (s, 3H), 2.42 (s, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ 169.9, 161.0, 160.4, 159.5, 156.2, 151.8, 143.2, 140.3, 134.7, 134.6, 128.2, 126.2, 126.1, 122.0, 121.8, 119.0, 118.0, 117.7, 108.4, 101.6, 57.7, 20.2. IR (KBr) 3020, 2623, 1952, 1778, 1639, 1612, 1574, 1501, 1435, 1362, 1319, 1258, 1223, 1177, 1150, 1069, 995, 879 cm^{-1}; MS (ESI) *m*/*z* 362 ([M+H]$^+$). Anal. Calcd for $C_{17}H_{13}ClFN_3O_3$: C, 56.44; H, 3.62, N 11.62. Found: C, 56.13; H, 3.56; N, 11.50.

5.1.3. 4-(3′-Chloroanilino)-6-hydroxy-7-methoxyquinazoline (5a)

To a solution of **4a** (291 mg, 0.85 mmol) in methanol (7 ml) was added aqueous ammonia (25% in H_2O, 0.85 ml) and the mixture was stirred for 13 h at room temperature. Solvents were removed under reduced pressure and the resulting solid was washed with dichloromethane to give **5a** as a white solid (229 mg, 0.76 mmol, 89% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.48 (s, 1H), 7.99 (s, 1H), 7.73 (s, 1H), 7.69–7.71 (d, *J* = 8.0 Hz, 1H), 7.37–7.41 (t, *J* = 8.0 Hz, 1H), 7.23 (s, 1H), 7.16–7.19 (d, *J* = 10.0 Hz, 1H), 4.09 (s, 3H). ^{13}C NMR (75 MHz, DMSO) δ 155.9, 154.0, 151.9, 146.8, 146.2, 141.5, 132.7, 130.0, 122.3, 120.7, 119.7, 109.7, 107.1, 105.4, 55.9. IR (KBr) 3375, 3005, 2496, 1770, 1620, 1582, 1524, 1404, 1277, 1246, 1207, 1150, 1006, 933, 898, 849 cm^{-1}; MS (ESI) *m*/*z* 302 ([M+H]$^+$). Anal. Calcd for $C_{15}H_{12}ClN_3O_2$: C, 59.71; H, 4.01; N, 13.93. Found: C, 59.66; H, 4.21; N, 13.87.

5.1.4. 4-(3′-Chloro-4′-fluoroanilino)- 6-hydroxy-7-methoxyquinazoline (5b)

Compound **5b** was prepared from **4b** (371 mg, 1.03 mmol) and aqueous ammonia (25%, 1 ml) in methanol (8 ml) by the procedure described for **5a** to give **5b** as a white solid (341 mg, 1.07 mmol, >99%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.45 (s, 1H), 8.03–8.06 (m, 1H), 7.70 (s, 2H), 7.26–7.31 (t, *J* = 10.0, 1H), 7.23 (s, 1H), 4.09 (s, 3H). ^{13}C NMR (75 MHz, DMSO) δ 155.9, 154.5, 153.9, 151.9, 151.3, 146.8, 146.2, 137.2, 137.1, 122.8, 121.7, 121.6, 118.8, 118.5, 116.6, 116.3, 109.5, 107.2, 105.3, 55.9. IR (KBr) δ 3386, 2936, 2835, 2484, 1848, 1612, 1585, 1516, 1470, 1339, 1246, 1215, 1153, 1053, 1003, 934, 853 cm^{-1}. MS (ESI) *m*/*z* 320 ([M+H]$^+$). Anal. Calcd for $C_{15}H_{11}ClFN_3O_2$: C, 56.35; H, 3.47; N, 13.14. Found: C, 56.40; H, 3.21; N, 13.06.

5.1.5. 4-(3′-Chloroanilino)-7-methoxy-6-(prop-2-ynyloxy)quinazoline (6a)

To a solution of **5a** (76.5 mg, 0.25 mmol) in DMF (3 ml) were added propargylbromide (28 µl, 0.38 mmol) and K_2CO3 (41.5 mg, 0.30 mmol) and the mixture was stirred for 12 h at room temperature. The reaction was quenched with water and the mixture was extracted with ethyl acetate, dried over anhydrous $MgSO_4$, and concentrated. Purification by column chromatography on silica gel with dichloromethane/methanol (20/1) gave **6a** as a yellow solid (74.4 mg, 0.22 mmol, 88% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.71 (s, 1H), 7.89 (s, 1H), 7.57–7.59 (d, *J* = 8.0 Hz, 1H), 7.32–7.36 (t, *J* = 8.4 Hz, 1H), 7.31 (s, 1H), 7.13–7.16 (d, *J* = 9.2, 1H), 7.10 (s, 1H), 4.94–4.95 (d, *J* = 2.4 Hz, 2H), 4.04 (s, 3H), 2.63 (s, 1H). ^{13}C NMR (75 MHz, CD_3OD) δ 156.1, 155.4, 153.9, 148.2, 147.2, 139.8, 134.7, 130.0, 124.2, 121.6, 119.5, 108.8, 108.5, 102.7, 77.6, 76.6, 57.3, 56.3. IR (KBr) 3449, 1219, 2931, 2124, 1904, 1622, 1597, 1574, 1504, 1483, 1431, 1385, 1230, 1205, 1140, 1099, 1076, 1030, 997, 929, 887, 856, 775, 707, 677, 592, 480 cm^{-1}. MS (ESI) *m*/*z* 340 ([M+H]$^+$). Anal. Calcd for $C_{18}H_{14}ClN_3O_2$: C, 63.63; H, 4.15; N, 12.37. Found: C, 63.83; H, 4.06; N, 12.10.

5.1.6. 4-(3′-Chloro-4′-fluoroanilino)-6-(prop-2-ynyloxy)-7-methoxyquinazoline (6b)

Compound **6d** was prepared from **5b** (120 mg, 0.38 mmol), propargylbromide (43 µl, 0.57 mmol), and K_2CO_3 (79 mg, 0.57 mmol) in DMF (6 ml) by the procedure described for **6a** to give **6b** as a white solid (136 mg, 0.38 mmol, >99%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.67 (s, 1H), 7.86–7.88 (dd, *J* = 2.8, 6.4 Hz, 1H), 7.50–7.54 (m, 1H), 7.27 (s, 2H), 7.14–7.19 (t, *J* = 8.8 Hz, 1H), 4.90–4.91 (d, *J* = 2.4 Hz, 2H), 4.01 (s, 3H), 2.60–2.62 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (75 MHz, CD_3OD) δ 156.2, 155.4, 153.8, 148.1, 147.2, 135.1, 124.2, 121.8, 121.7, 116.8, 116.5, 108.6, 108.5, 102.5, 76.6, 74.6, 57.2, 56.3. IR (KBr) 3271, 2131, 1674, 1628, 1583, 1531, 1502, 1475, 1431, 1342, 1234, 1145, 997, 856, 661, 561 cm^{-1}. MS (ESI) *m*/*z* 358 ([M+H]$^+$). Anal. Calcd for $C_{18}H_{13}ClFN_3O_2$: C, 60.43; H, 3.66; N, 11.75. Found: C, 60.20; H, 3.72; N, 11.90.

5.1.7. 4-(3′-Chloroanilino)-7-methoxy-6-(pent-4-ynyloxy)quinazoline (6c)

To a solution of **5a** (179 mg, 0.59 mmol) in DMF (2 ml) were added 5-iodo-1-pentyne (230 mg, 1.2 mmol) and K_2CO_3 (163 mg, 1.2 mmol) and the mixture was stirred for 12 h at 60 °C. The reaction was quenched with water and the mixture was extracted with ethyl acetate, dried over anhydrous $MgSO_4$, and concentrated. Purification by column chromatography on silica gel eluted with hexane/ethyl acetate (1/1) gave the crude product. Further purification was carried out by recrystallization from dichloromethane and hexane to afford **6c** as a white solid (161 mg, 0.46 mmol, 78% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.66 (s, 1H), 7.87–7.89 (m, 1H), 7.57–7.59 (m, 1H), 7.31–7.35 (t, *J* = 8.0 Hz, 1H), 7.12–7.14 (dd, *J* = 6.0 Hz, 2H), 7.08 (s, 1H), 4.28–4.32 (t, *J* = 6.4 Hz, 2H), 4.02 (s, 3H), 2.49–2.53 (m, 2H), 2.13–2.19 (quint, *J* = 6.4 Hz, 2H), 2.04–2.05 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (75 MHz, CD_3OD) δ 155.9, 155.3, 153.5, 149.1, 147.7, 139.9, 134.7, 130.0, 124.1, 121.5, 119.4, 109.0, 108.2, 100.5, 83.5, 69.3, 67.7, 56.2, 27.8, 15.2. IR (KBr) 3292, 3076, 2928, 2120, 1923, 1626, 1599, 1574, 1506, 1473, 1429, 1393, 1285, 1240, 1209, 1148, 1097, 1069, 1043, 1005, 935, 862, 777, 682, 640, 590 cm^{-1}. MS (ESI) *m*/*z* 368 ([M+H]$^+$). Anal. Calcd for $C_{20}H_{18}ClN_3O_2$: C, 65.31; H, 4.97; N, 11.42. Found: C, 65.27; H, 4.83; N, 11.38.

5.1.8. 4-(3′-Chloro-4′-fluoroanilino)-7-methoxy-6-(pent-4-ynyloxy)quinazoline (6d)

Compound **6d** was prepared from **5b** (197 mg, 0.62 mmol), 5-iodo-1-pentyne (170 mg, 1.23 mmol), and K_2CO_3 (170 mg, 1.23 mmol) in DMF (3 ml) by the procedure described for **6c** to give **6d** as a yellow solid (138 mg, 0.36 mmol, 59%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.66 (s, 1H), 7.88–7.90 (m, 1H), 7.51–7.55 (m, 1H), 7.16–7.20 (t, *J* = 8.8 Hz, 1H), 7.07 (s, 1H), 7.04 (s, 1H), 4.28–4.31 (t, *J* = 6.4 Hz, 2H), 4.02 (s, 3H), 2.49–2.53 (m, 2H), 2.15–2.18 (m, 2H), 2.03–2.05 (t, *J* = 2.8 Hz, 1H). ^{13}C NMR (75 MHz, CD_3OD) δ 156.0, 155.3, 153.4, 149.1, 147.6, 135.2, 124.1, 121.7, 121.6, 116.8, 116.5, 108.8, 108.1, 100.4, 83.5, 69.3, 67.6, 56.2, 27.7, 15.2. IR (KBr) 3854, 3649, 3425, 2934, 2345, 1626, 1583, 1500, 1499, 1474, 1429, 1339, 1217, 1146, 1053, 1016, 954, 935, 853 cm^{-1}. MS (ESI) *m*/*z* 386 ([M+H]$^+$). Anal. Calcd for $C_{20}H_{17}ClFN_3O_2$: C, 62.26; H, 4.44; N, 10.89. Found: C, 62.26; H, 4.34; N, 10.80.

5.1.9. 4-(3′-Chloro-4′-fluoroanilino)-6-(hex-5-ynyloxy)-7-methoxyquinazoline (6e)

Compound **6e** was prepared from **5b** (197 mg, 0.62 mmol), 6-chloro-1-hexyne (0.11 ml, 0.93 mmol), KI (10 mg, 0.06 mmol), and K_2CO_3 (171 mg, 1.24 mmol) in DMF (3 ml) by the procedure described for **6c** to give **6e** as a pale yellow solid (183 mg, 0.46 mmol, 74%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.66 (s, 1H), 7.88–7.91 (m, 1H), 7.52–7.56 (m, 1H), 7.16–7.20 (t, *J* = 8.8 Hz, 1H), 7.00 (s, 2H), 4.17–4.20 (t, *J* = 6.4 Hz, 2H), 7.02 (s, 3H), 2.33–2.37 (m, 2H), 2.05–2.12 (quint, *J* = 6.4 Hz, 2H), 1.99–2.00 (t, *J* = 2.4 hz, 1H), 1.76–1.84 (quint, *J* = 7.6 Hz, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 167.4, 155.4, 155.0, 153.4, 149.2, 147.6, 135.4, 124.0, 121.5, 116.8, 116.5, 108.2, 100.1, 97.5, 84.0, 69.0, 68.9, 56.3, 27.9, 24.9, 18.1. IR (KBr) 3414, 3283, 2955, 2340, 1620, 1578, 1501, 1427, 1394, 1227, 1142, 1005, 929, 845, 816, 640, 552, 515 cm^{-1}. MS (ESI) *m*/*z* 400 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{19}ClFN_3O_2$: C, 63.08; H, 4.79; N, 10.51. Found: C, 62.94; H, 4.53; N, 10.26.

5.1.10. 4-(3′-Chloro-4′-fluoroanilino)-6-(hept-6-ynyloxy)-7-methoxyquinazoline (6f)

Compound **6f** was prepared from **5b** (140 mg, 0.44 mmol), 7-iodoheptyne (137 mg, 0.66 mmol), and K_2CO_3 (122 mg, 0.88 mmol) in DMF (5 ml) by the procedure described for **6c** to give **6f** as a pale yellow solid (88 mg, 0.21 mmol, 48%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.66 (s, 1H), 7.87–7.89 (dd, *J* = 2.8, **6.4** Hz, 1H), 7.52–7.56 (m, 1H), 7.16–7.20 (t, *J* = 8.8 Hz, 1H), 7.06 (s, 1H), 7.00 (s, 1H), 4.12–4.16 (t, *J* = 6.4 Hz, 2H), 4.02 (s, 3H), 2.26–2.27 (q, *J* = 2.8 Hz, 2H), 1.96–1.98 (quint, *J* = 2.8 Hz, 3H), 1.64–1.67 (quint, *J* = 3.6 Hz, 4H). ^{13}C NMR (75 MHz, CD_3OD) δ 156.4, 156.1, 155.3, 153.1, 149.2, 147.5, 135.3, 135.2, 124.1, 121.7, 121.6, 121.2, 121.0, 116.8, 116.5, 108.8, 107.9, 100.3, 84.3, 69.3, 68.5, 56.2, 28.5, 28.0, 25.1, 18.3. IR (KBr) 3649, 3287, 3255, 2943, 2341, 1626, 1578, 1501, 1473, 1425, 1398, 1354, 1246, 1217, 1146, 1070, 997, 927, 862, 813, 690, 658, 546 cm^{-1}. MS (ESI) *m*/*z* 414 ([M+H]$^+$). Anal. Calcd for $C_{22}H_{21}ClFN_3O_2$: C, 63.84; H, 5.11; N, 10.15. Found: C, 63.68; H, 5.19; N, 10.24.

5.1.11. 6-(Buta-2,3-dienyloxy)-4-(3′-chlorophenylamino)-7-methoxyquinazoline (7a)

To a mixture of **6a** (74.4 mg, 0.22 mmol), CuBr (16 mg, 0.11 mmol), and paraformaldehyde (17 mg, 0.55 mmol) in dioxane (2 ml) was added diisopropylamine (62 μl, 0.44 mmol) under argon atmosphere and the mixture was stirred under refluxed condition for 4 h. The reaction mixture was filtered by a short silica gel column chromatography to remove the copper catalysts. Purification by column chromatography on silica gel with hexane/ethyl acetate (2/1) gave **7a** as a yellow solid (31 mg, 0.11 mmol, 50% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.70 (s, 1H), 7.88–7.89 (t, *J* = 2.4 Hz, 1H), 7.55–7.57 (d, *J* = 8.0 Hz, 1H), 7.30–7.34 (t, *J* = 8.0 Hz, 1H), 7.20 (s, 1H), 7.13 (s, 1H), 7.12 (s, 1H), 5.47–5.51 (quint, *J* = 6.4, 1H), 4.92–4.95 (m, 2H), 4.78–4.81 (m, 2H), 4.02 (s, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ 210.2, 156.1, 155.2, 153.5, 148.1, 147.5, 139.9, 134.6, 129.9, 124.0, 121.4, 119.3, 109.0, 107.9, 101.6, 86.3, 76.6, 67.2, 56.2. IR (KBr) 3557, 2926, 1954, 1626, 1578, 1597, 1514, 1429, 1396, 1354, 1292, 1234, 1144, 1067, 929, 856, 773, 681, 660, 556 cm^{-1}. MS (ESI) *m*/*z* 354 ([M+H]$^+$). Anal. Calcd for $C_{19}H_{16}ClN_3O_2$: C, 64.50; H, 4.56; N, 11.88. Found: C, 64.63; H, 4.62; N, 11.62.

5.1.12. 6-(Buta-2,3-dienyloxy)-4-(3′-chloro-4′-fluoroanilino)-7-methoxyquinazoline (7b)

Compound **7b** was prepared from **6b** (132 mg, 0.37 mmol), CuBr (27 mg, 0.19 mmol), paraformaldehyde (28 mg, 0.93 mmol), and diisopropylamine (0.11 ml, 0.74 mmol) in dioxane (4 ml) by the procedure described for **7a** to give **7b** as a pale yellow solid (50 mg, 0.14 mmol, 38% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.67 (s, 1H), 7.88–7.91 (dd, *J* = 2.4, 6.4 Hz, 1H), 7.50–7.52 (m, 1H), 7.14–7.19 (t, *J* = 8.8 Hz, 1H), 7.13 (s, 1H), 5.47–5.50 (quint, *J* = 6.8 Hz, 1H), 4.90–4.93 (m, 2H), 4.75–4.78 (m, 2H), 4.01 (s, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ 210.0, 156.3, 155.2, 153.4, 153.1, 148.2, 135.3, 124.0, 121.6, 121.5, 121.1, 116.7, 116.4, 107.8, 101.7, 86.2, 67.1, 56.1, 50.6. IR (KBr) 3402, 3080, 2932, 2216, 1955, 1625, 1579, 1504, 1472, 1425, 1377, 1336, 1215, 1142, 1167, 987, 927, 858, 816, 816, 792, 783, 579, 441 cm^{-1}. MS (ESI) *m*/*z* 372 ([M+H]$^+$). Anal. Calcd for $C_{19}H_{15}ClFN_3O_2$: C, 61.38; H, 4.07; N, 11.30. Found: C, 61.30; H, 3.90; N, 11.21.

5.1.13. 4-(3′-Chlorophenylamino)-6-(hexa-4,5-dienyloxy)-7-methoxyquinazoline (7c)

Compound **7c** was prepared from **6a** (161 mg, 0.44 mmol), CuBr (32 mg, 0.22 mmol), paraformaldehyde (33 mg, 1.1 mmol), and diisopropylamine (0.12 ml, 0.88 mmol) in dioxane (4 ml) by the procedure described for **7a** to give **7c** as a yellow solid (37 mg, 0.097 mmol, 22% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.70 (br, 1H), 7.86 (s, 1H), 7.57–7.59 (d, *J* = 8.4 Hz, 1H), 7.30–7.34 (t, *J* = 8.0 Hz, 1H), 7.19 (s, 1H), 7.12–7.14 (d, *J* = 8.0 Hz, 1H), 7.04 (s, 1H), 5.17–5.24, (quint, *J* = 6.4 Hz, 1H), 4.71–4.74 (dt, *J* = 3.6, 6.8 Hz, 2H), 4.15–4.19 (t, *J* = 6.8 Hz, 2H), 4.01 (s, 3H), 2.23–2.27 (m, 2H), 2.05–2.08 (quint, *J* = 6.8 Hz, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 208.5, 155.9, 155.3, 149.2, 139.9, 134.6, 130.0, 124.0, 121.6, 119.5, 107.9, 100.4, 89.1, 74.5, 68.6, 56.2, 28.1, 24.4. IR (KBr) 2931, 1954, 1624, 1599, 1578, 1504, 1471, 1429, 1393, 1335, 1240, 1209, 1146, 1069, 928, 849, 773, 679, 584, 556 cm^{-1}. MS (ESI) *m*/*z* 382 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{20}ClN_3O_2$: C, 66.05; H, 5.28; N, 11.00. Found: C, 66.06; H, 5.05; N, 11.00.

5.1.14. 4-(3′-Chloro-4′-fluorophenylamino)-6-(hexa-4,5-dieny loxy)-7-methoxyquinazoline (7d)

Compound **7d** was prepared from **6b** (129 mg, 0.33 mmol), CuBr (24 mg, 0.17 mmol), paraformaldehyde (15 mg, 0.83 mmol), and diisopropylamine (0.093 ml, 0.66 mmol) in dioxane (2 ml) by the procedure described for **7a** to give **7c** as a white solid (53 mg, 0.13 mmol, 40% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.67 (s, 1H), 7.87–7.89 (m, 1H), 7.51–7.55 (m, 1H), 7.16–7.20 (t, *J* = 8.8 Hz, 1H), 7.05 (s, 1H), 7.01 (s, 1H), 5.19–5.23 (quint, *J* = 6.8 Hz, 1H), 4.72–4.74 (quint, *J* = 3.6 Hz, 2H), 4.16–4.19 (t, *J* = 6.8 Hz, 2H), 4.01 (s, 3H), 2.23–2.29 (m, 2H), 2.04–2.11 (quint, *J* = 6.8 Hz, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 208.6, 156.1, 155.4, 153.4, 149.3, 147.5, 135.3, 124.2, 121.7, 116.3, 108.9, 108.0, 100.4, 89.1, 75.6, 68.6, 56.2, 28.2, 24.4. IR (KBr) 3425, 2945, 2345, 1949, 1603, 1578, 1500, 1394, 1329, 1285, 1194, 1136, 1057, 1013, 997, 844, 815 cm^{-1}. MS (ESI) *m*/*z* 400 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{19}ClFN_3O_2$: C, 63.08; H, 4.79; N, 10.51. Found: C, 63.13; H, 4.51; N, 10.40.

5.1.15. 4-(3′-chloro-4′-fluorophenylamino)-6-(hepta-5,6-dieny loxy)-7-methoxyquinazoline (7e)

Compound **7e** was prepared from **6b** (174 mg, 0.43 mmol), CuBr (32 mg, 0.22 mmol), paraformaldehyde (32 mg, 1.1 mmol), and diisopropylamine (0.12 ml, 0.86 mmol) in dioxane (3 ml) by the procedure described for **7b** to give **7e** as a pale yellow solid (70 mg, 0.17 mmol, 39% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.64 (s, 1H), 7.87–7.90 (dd, *J* = 2.8, 6.8 Hz, 1H), 7.52–7.57 (m, 1H), 7.16–7.20 (t, *J* = 8.8 Hz, 2H), 7.00 (s, 1H), 5.13–5.17 (quint, *J* = 6.8 Hz, 1H), 4.68–4.71 (quint, *J* = 2.8 Hz, 2H), 4.14–4.17 (t, *J* = 6.4 Hz, 2H), 4.01 (s, 3H), 2.12–2.14 (m, 2H), 1.98–2.02 (quint, *J* = 7.2 Hz, 2H), 1.65–1.69 (quint, *J* = 7.6 Hz, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 208.6, 156.0, 155.4, 153.4, 149.4, 135.3, 124.1, 121.7, 121.6, 116.8, 116.5, 108.1, 100.1, 89.6, 75.1, 69.3, 56.3, 28.4, 27.9, 25.4. IR (KBr) 3649, 2945, 1953, 1624, 1580, 1502, 1429, 1398, 1145, 1057, 1028, 999, 935, 845, 816, 773, 691, 670, 554 cm^{-1}. MS (ESI) *m*/*z* 414 ([M+H]$^+$). Anal. Calcd for $C_{22}H_{21}ClFN_3O_2$: C, 63.84; H, 5.11; N, 10.15. Found: C, 63.58; H, 4.99; N, 10.34.

5.1.16. 4-(3-Chloro-4-fluorophenylamino)-6-(octa-6,7-dienyl oxy)-7-methoxyquinazoline (7f)

Compound **7f** was prepared from **6b** (88 mg, 0.21 mmol), CuBr (15 mg, 0.11 mmol), paraformaldehyde (16 mg, 0.53 mmol), and diisopropylamine (0.06 ml, 0.42 mmol) in dioxane (3 ml) by the procedure described for **7b** to give **7f** as a white solid (25 mg, 0.1706 mmol, 29% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.66 (s, 1H), 7.86–7.88 (dd, *J* = 2.4, 6.4 Hz, 1H), 7.51–7.54 (m, 1H), 7.15–7.19 (t, *J* = 8.8 Hz, 2H), 7.01 (s, 1H), 5.10–5.13 (quint, *J* = 6.4 Hz, 1H), 4.65–4.68 (quint, *J* = 3.2 Hz, 2H), 4.09–4.13 (t, *J* = 6.4 Hz, 2H), 4.00 (s, 3H), 2.05–2.07 (m, 2H), 1.93–1.96 (quint, *J* = 6.8 Hz, 2H), 1.53–1.55 ppm (m, 4H). ^{13}C NMR (75 MHz, CD_3OD) δ 208.5, 156.5, 155.3, 153.2, 149.4, 135.3, 124.2, 121.8, 121.0, 116.8, 100.3, 89.7, 74.8, 69.4, 56.2, 29.0, 28.8, 28.7, 28.1, 25.4. IR (KBr) 3649, 3281, 3080, 2936, 2339, 1954, 1626, 1578, 1499, 1473, 1429, 1396, 1339, 1244, 1217, 1146, 1070, 995, 849, 815, 691, 548 cm^{-1}. MS (ESI) *m*/*z* 428 ([M+H]$^+$). Anal. Calcd for $C_{23}H_{23}ClFN_3O_2$: C, 64.56; H, 5.42; N, 9.28. Found: C, 64.38; H, 5.51; N, 9.57.

5.1.17. 4-(3′-Chloro-4′-fluorophenylamino)-7-methoxy-6-trifluoromethanesulfonoylquinazoline (8)

To a solution of **5b** (127 mg, 0.40 mmol) in pyridine (4 ml) was added Tf_2O (0.14 ml, 0.84 mmol) at 0 °C dropwise under argon atmosphere and then the mixture was stirred at room temperature for 5 h. After pyridine was removed under reduced pressure, the residue was purified by column chromatography on silica gel eluted with dichloromethane/methanol (30/1) to give **8** as a white solid (170 mg, 0.38 mmol, 94%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.74 (s, 1H), 7.88–7.90 (dd, *J* = 2.8 Hz, 6.4 Hz, 1H), 7.73 (s, 1H), 7.49–7.53 (m, 1H), 7.42 (s, 1H), 7.21 (t, *J* = 8.8 Hz, 1H), 4.07 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.4, 157.0, 156.0, 155.3, 153.7, 150.8, 138.2, 134.2, 127.9, 124.9, 122.4, 122.3, 121.4, 121.2, 120.8, 116.9, 116.6, 115.0, 109.6, 108.3, 56.7. IR (KBr) 3452, 3411, 3126, 3259, 1630, 1504, 1421, 1219, 1132, 895, 629, 498 cm^{-1}. MS (ESI) *m*/*z* 452 ([M+H]$^+$). Anal. Calcd for $C_{16}H_{10}ClF_4N_3O_4S$: C, 42.54; H, 2.23; N, 9.30; S, 7.10. Found: C, 42.71; H, 2.12; N, 9.43; S, 6.96.

5.1.18. 4-(3′-Chloro-4′-fluorophenylamino)-6-ethynyl-7-meth oxyquinazoline (10)

To a mixture of **8** (90 mg, 0.42 mmol), $Pd(PPh_3)_4$ (49 mg, 0.04 mmol), and CuI (16 mg, 0.08 mmol) in THF (5 ml) were added ethynyltrimethylsilane (0.09 ml, 0.63 mmol) and triethylamine (0.23 ml, 1.7 mmol) under argon atmosphere and the mixture was stirred under reflux condition for 12 h. After the solvent was removed under reduced pressure, the residue was filtered with short column chromatography on silica gel eluted with dichloromethane/methanol (10/1) to remove the catalysts. The mixture was again concentrated and dissolved in dichloromethane/methanol (v/v 2/1, 3 ml). K_2CO_3 (59 mg, 0.43 mmol) was added and the mixture was stirred for 3 h at room temperature. The reaction mixture was filtered with Celite and concentrated. Purification by column chromatography on silica gel with dichloromethane/methanol (30/1) gave **10** as a white solid (85 mg, 0.31 mmol, 74% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.70 (s, 1H), 8.00 (s, 1H), 7.93–7.96 (dd, *J* = 2.8, 6.4 Hz, 1H), 7.49–7.53 (m, 1H), 7.16–7.21 (t, *J* = 8.4 Hz, 2H), 4.04 (s, 3H). ^{13}C NMR (75 MHz, CD_3OD): δ 163.0, 153.5, 156.1, 152.2, 134.6, 126.7, 124.3, 121.7, 121.6, 116.9, 116.6, 113.3, 108.9, 107.3, 82.3, 78.8, 56.4. IR (KBr) 3587, 3449, 3288, 3179, 2100, 1624, 1562, 1535, 1500, 1472, 1420, 1383, 1337, 1231, 1132, 1001, 931, 850, 816, 789, 545 cm^{-1}. MS (ESI) *m*/*z* 328 ([M+H]$^+$). Anal. Calcd for $C_{17}H_{11}ClFN_3O$: C, 62.30; H, 3.38; N, 12.82. Found: C, 62.53; H, 3.30; N, 12.68.

5.1.19. 4-(3′-Chloro-4′-fluorophenylamino)-6-(hex-5-enyloxy)-7-methoxyquinazoline (11)

To a mixture of **5b** (80 mg, 0.25 mmol), K_2CO_3 (53 mg, 0.38 mmol), and KI (4.2 mg, 0.03 mmol) in DMF (2 ml) was added 6-chloro-1-hexene (0.05 ml, 0.38 mmol) and the mixture was stirred at 60 °C for 12 h. The reaction was quenched with water and the reaction mixture was extracted with ethyl acetate, dried over $MgSO_4$, and concentrated. Purification by column chromatography on silica gel with dichloromethane/methanol (30/1) gave the crude **11**. Further purification was carried out by recrystallization from methanol to give **11** as a white solid (44 mg, 0.11 mmol, 44% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.64 (s, 1H), 7.87–7.90 (dd, *J* = 2.8, 6.4 Hz, 1H), 7.52–7.57 (m, 1H), 7.16–7.20 (t, *J* = 8.4 Hz, 2H), 7.00 (s, 1H), 5.80–5.89 (q, *J* = 7.2 Hz, 1H), 5.00–5.09 (m, 2H), 4.14–4.17 (t, *J* = 6.8 Hz, 2H), 4.01 (s, 3H), 2.17–2.19 (q, *J* = 7.6 Hz, 2H), 1.95–1.99 (quint, *J* = 7.2 Hz, 2H), 1.65–1.67 (quint, *J* = 8.0 Hz, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 156.2, 155.3, 153.1, 153.0, 149.2, 146.9, 138.2, 135.2, 121.9, 121.8, 121.1, 120.8, 116.7, 116.4, 114.9, 108.9, 107.3, 100.6, 69.2, 56.1, 33.3, 28.3, 25.1. IR (KBr) 3649, 3283, 3078, 2941, 2361, 1626, 1578, 1502, 1474, 1427, 1398, 1354, 1219, 1146, 1070, 997, 927, 907, 849, 794, 691, 546 cm^{-1}. MS (ESI) m/z 402 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{21}ClFN_3O_2$: C, 62.76; H, 5.27; N, 10.46. Found: C, 62.53; H, 5.44; N, 10.20.

5.1.20. 4-(3-Chloro-4-fluorophenylamino)-6-hexyloxy-7-methoxyquinazoline (12)

Compound **12** was synthesized from **5b** (79 mg, 0.24 mmol) with K_2CO_3 (51 mg, 0.37 mmol), KI (4.0 mg, 0.03 mmol), and 1-chlorohexane (0.051 ml, 0.37 mmol) by the procedure, described for **11** to give **12** as a white solid (51 mg, 0.13 mmol, 52% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.61 (s, 1H), 7.88–7.91 (dd, J = 2.8, 6.8 Hz, 1H), 7.56–7.58 (m, 1H), 7.25 (s, 1H), 7.15–7.20 (t, J = 8.8 Hz, 1H), 7.07 (s, 1H), 4.13–4.16 (t, J = 6.8 Hz, 2H), 4.00 (s, 3H), 1.90–1.97 (quint, J = 7.2 Hz, 2H), 1.36–1.52 (m, 6H), 0.91–0.94 (t, J = 6.8 Hz, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ 156.2, 155.5, 152.6, 149.4, 146.3, 135.1, 124.3, 121.9, 116.7, 116.4, 108.7, 107.0, 101.3, 100.5, 69.6, 56.2, 31.5, 28.9, 25.6, 22.5, 14.0. IR (KBr) 3275, 3084, 2934, 2332, 1636, 1578, 1501, 1474, 1427, 1339, 1246, 1219, 1146, 1070, 999, 862, 816, 690, 554 cm^{-1}. MS (ESI) m/z 404 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{23}ClFN_3O_2$: C, 52.45; H, 5.74; N, 10.40. Found: C, 62.45; H, 5.74; N, 10.33.

5.1.20.1. Kinase assay. The kinase activity of various tyrosine kinases was determined by ELISA.[26] EIA/RIA strip-well plates (Corning) were coated with poly(Glu/Tyr, 4:1) peptide (Sigma, 50 μg/ml, 100 μl per well) by incubation overnight at 4 °C in phosphate-buffered saline (PBS, Ca/Mg-free). Kinase reaction was performed in the plates by addition of 50 μl kinase buffer (50 mM HEPES, 125 mM NaCl, 10 mM $MgCl_2$, pH 7.4) containing ATP, 10 ng recombinant EGFR (Invitrogen, catalytic domain), and inhibitors. After 20 min, the plates were washed three times with wash buffer (0.1% Tween 20 in PBS) and incubated for 20 min with HRP conjugated anti-phosphotyrosine antibody (0.2 μg/ml, 50 μl per well, Santa Cruz). After two washes, the plates were developed by the addition of tetramethylbenzidine (50 μl per well, Sigma) and stopped by addition of H_2SO_4 (2 N, 50 μl per well). The absorbance at 450 nm was measured by a 96-well plate reader (Tecan).

5.1.20.2. Cell growth assays. The human epidermoid carcinoma cell line A431 was used for the cell growth assays. The cells (5×10^3 cells per well of a 96-well plate) were incubated at 37 °C for 72 h in 100 μl RPMI-1640 medium containing various concentrations of drugs. After incubation, MTT (10 μl, 5 mg/ml in PBS; MTT [3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma] was added to each well, and the cells were further incubated at 37 °C for 4 h. After removal of the medium, DMSO (100 μl) was added, and the absorbance at 570 nm was determined by a 96-well plate reader.

5.1.20.3. Immunoblot analysis. After drug treatment for a specified period, the cells were washed three times with PBS, dipped in 100 μl ice-cold lysis buffer (20 mM HEPES, pH 7.4, 1% Triton X-100, 10% glycerol, 1 mM sodium vanadate, 5 μg/ml leupeptin, and 1 mM EDTA) for 15 min, and disrupted with a Handy Sonic Disrupter, and the lysate was boiled for 5 min in a sample buffer (50 mM Tris, pH 7.4, 4% sodium dodecyl sulfate (SDS), 10% glycerol, 4% 2-thioethanol, and 0.05 mg/ml bromophenol blue) at a ratio of 4:1. The proteins were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (GE Healthcare). Antibodies used for immunoblotting were phospho-EGFR (Tyr1173) antibody and EGFR antibody (Santa Cruz). After incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz), the blot was treated with ECL kit (GE Healthcare), and protein expression was visualized with a ChemiDoc XRS image analyzer (Bio-Rad).

5.1.20.4. Cell-cycle analysis. After incubation of A431 cells with the drugs, the cells were washed with PBS and fixed with 70% ethanol for 2 h at 4 °C. The cells were incubated for 30 min at 37 °C in 1 ml of RNase solution (0.25 mg/ml in PBS), and further incubated for 30 min at 4 °C in propidium iodide (PI) staining solution (0.05 mg/ml in PBS). The suspension was then passed through a nylon mesh filter (40 μm) and analyzed by a Cytomics FC500 flow cytometer (Beckman Coulter).

5.1.20.5. Apoptosis detection. Phosphatidylserine externalization was measured using an annexin V-FITC kit (Beckman Coulter) according to the manufacturer's instructions. After incubation of A431 cells with the drugs, the cells were suspended in 100 μl 1× binding buffer, and 5 μl annexin V-FITC (5 μg/ml) and 2.5 μl PI (250 μg/ml) were added. The cell suspensions were incubated for 10 min at 4 °C and analyzed by a flow cytometer.

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