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Design, synthesis and biological evaluation of novel 6-alkenylamides substituted of 4-anilinothieno[2,3-*d*]pyrimidines as irreversible epidermal growth factor receptor inhibitors

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ABSTRACT

A novel series of 6-alkenylamides of 4-anilinothieno[2,3-*d*]pyrimidine derivatives was designed, synthesized and evaluated as irreversible inhibitors of the epidermal growth factor receptor (EGFR). Most of the compounds exhibited good potency against EGFR wild type (EGFR wt) and EGFR T790M/L858R. Among these, the half-maximal inhibitory concentration (IC_{50}) values of 17 compounds against EGFR wt were less than 0.020 μM, and those of 12 compounds were less than 0.010 μM. The IC_{50} values of 10 compounds against EGFR T790M/L858R were less than 0.005 μM. Compounds **8l**, **9n**, **9o**, **9q** and **9v** almost completely blocked the phosphorylation of EGFR in the A431 cell line at 1 μM. Compounds **8l**, **9n**, **9o**, **9q** and **9v** blocked the autophosphorylation of EGFR in NCI-H1975 cells at high concentration (1 μM), and compound **8l** was confirmed to be an irreversible inhibitor through the dilution method.

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1. Introduction

The ErbB family is one of the most extensively studied signal-transduction networks[1,2] and plays a critical role in many of the signal transduction pathways that regulate numerous cellular functions, such as proliferation, differentiation, migration, angiogenesis and apoptosis.[3–5] The family contains four membrane-bound receptors: the epidermal growth factor receptor (EGFR, erbB1, Her1), Her2/erbB2/neu, Her3/erbB3, and Her4/erbB4.[6,7] Activation of ErbB signaling is dependent upon ligand-induced stabilization of homo- or hetero-dimerization followed by autophosphorylation of each tyrosine residue within the intracellular kinase domain. These actions can activate downstream signaling, such as the Ras/Raf/MAPK, and PI3K/AKt/mTOR pathways.[2,6] The ErbB family receptors are targets for novel anticancer agents against non-small-cell lung cancer (NSCLC), HER2-positive breast cancer, head and neck squamous cell carcinoma, and colorectal cancer.[8]

Blocking or inhibiting signaling pathways with EGFR inhibitors has resulted in development of several novel anticancer agents[9] (Fig. 1). The first generation of EGFR inhibitors include EGFR-specific inhibitors, **1** (gefitinib),[10] **2** (erlotinib),[11] and **4** (icotinib),[12] for the treatment of non-small-cell lung cancer (NSCLC), and EGFR/HER2 dual inhibitors **3** (lapatinib)[13] for the treatment of HER2 positive breast cancer. Although **1** and **2** are effective in the treatment of NSCLC, especially in patients with tumors possessing EGFR-sensitive mutants (e.g. EGFR L858R), resistance to EGFR-specific inhibitors has been clinically observed and associated with the T790M mutation of an EGFR-specific tyrosine kinase.[14] The second generation of EGFR inhibitors contain a Michael acceptor group at the 6-position, such as compounds **5** (afatinib/BIBW2992),[15] **6** (canertinib),[16] and **7** (neratinib/HKI-272),[17] are irreversible inhibitors, and have been observed to overcome drug resistance. Moreover, afatinib was approved by FDA in July 2013 for first-line treatment of subjects with EGFR exon 19 deletions or exon 21 (L858R) substitution mutations as detected by an FDA-approved test.[18]

We had presented a new sub-family of 4-anilinoquinazolines with C-6 urea-linked side chains as potent irreversible EGFR inhibitors.[19] Although 4-anilinoquinazolines with C-6 urea-linked side chains derivatives showed good to moderate inhibitory activities

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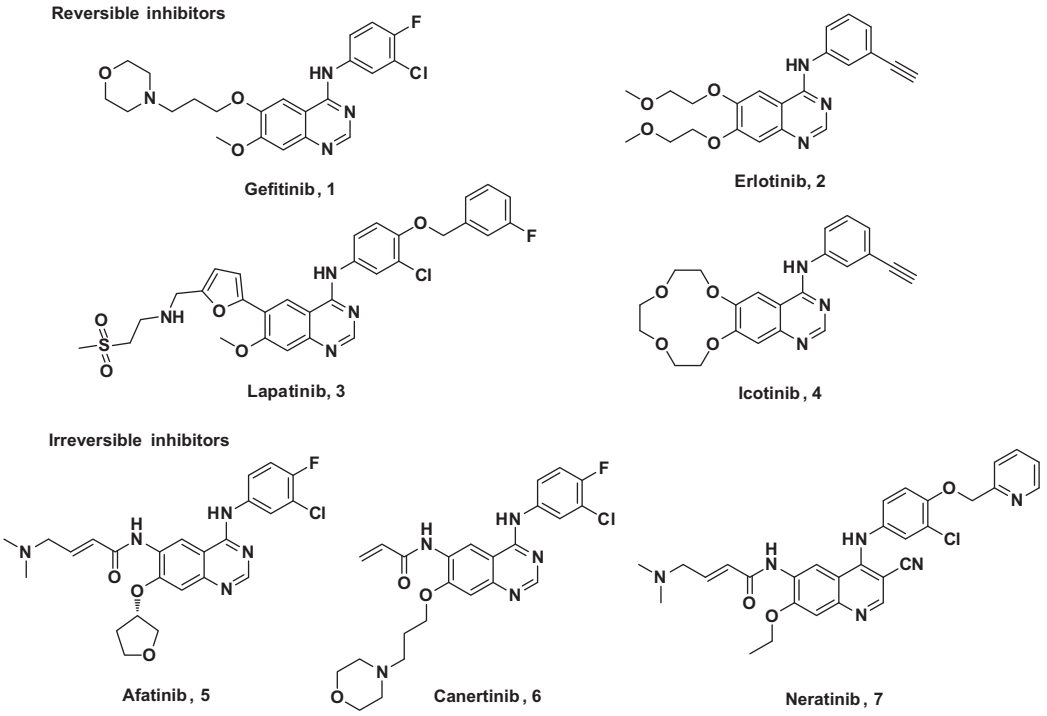


Figure 1. Representative reversible and irreversible EGFR inhibitors.

against EGFR wt and EGFR T790M/L858R, the efficacy on EGF-induced EGFR phosphorylation needs to be improved. Herein, we present 6-alkenylamides substituted of 4-anilinothieno[2,3-*d*]pyrimidines as novel irreversible EGFR inhibitors.

2. Design

Thieno[2,3-*d*]pyrimidine core as an important fragment had been studied widely in antibacterial agents,[20] antioxidant agents[21] and antitumor agents, such as Bcr-Abl inhibitors,[22] c-Met inhibitors[23] and EGFR inhibitors.[24] The 3-chloro-4-fluoro aniline substituent extended into the hydrophobic pocket in the back of the ATP-binding cleft, and the chloro group was surrounded by the side chains of residues Lys745, Leu788, and Thr790, the fluorine extended toward the side chains of Leu788, Met766, and Glu762,[25] which was found in many launched drugs as an important group and studied widely in different areas about the fluorine.[26] The inhibitors contained an alkenylamide functionality that covalently targeted Cys797 in the solvent channel of the kinase, and were thought to overcome the increased ATP affinity of the double mutant, resulting in the potent activity of these compounds in cellular models.[27] On the basis of these three key features, we designed 6-alkenylamides substituted of 4-anilinothieno[2,3-*d*]pyrimidines derivatives in the initial design (Fig. 2). We observed one compound to have good inhibitory activity against EGFR. However, in comparison of the control compound afatinib, the inhibitory activity needed further enhancement. Besides, the 6-alkenylamides of 4-anilinothieno[2,3-*d*]pyrimidines derivatives had solubility problem. Therefore, to improve inhibitory activities and solubility, some polar fragments were introduced into the subsequent modification (Fig. 2).

3. Chemistry

Compounds **8a–o** were synthesized according to Scheme 1. The thieno[2,3-*d*]pyrimidine core **12** was obtained from commercially available compound **10** via cyclization in condition of microwave heating. The key intermediate **16** was generated from compound **12** via nitrification, chlorination, amination and reduction. Compounds **8a–f** and **8l–n** were obtained from compound **16** via condensation with different groups substituted acetyl chlorides. Compound **8o** was obtained from compound **16** via condensation with chloroacetyl chloride. Compounds **8g–k** were obtained from compound **8o**, which underwent a nucleophilic reaction with different amines.

The synthesis of compounds **9a–z** was similar with the synthesis of compounds **8a–o** (Scheme 2). The difference was that compound **20** was obtained from compound **16** via condensation with compound **19**. Then compounds **9a–z** were afforded from compound **20** underwent a nucleophilic reaction with different

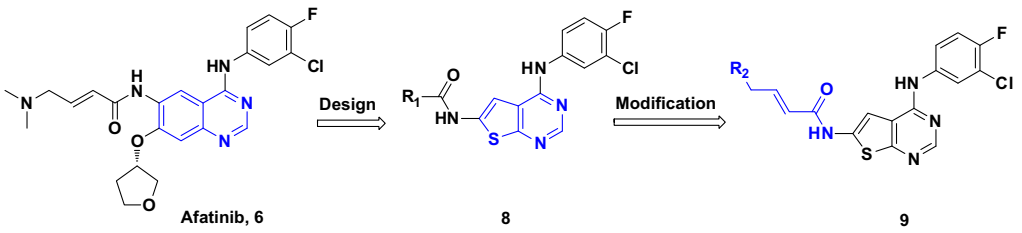
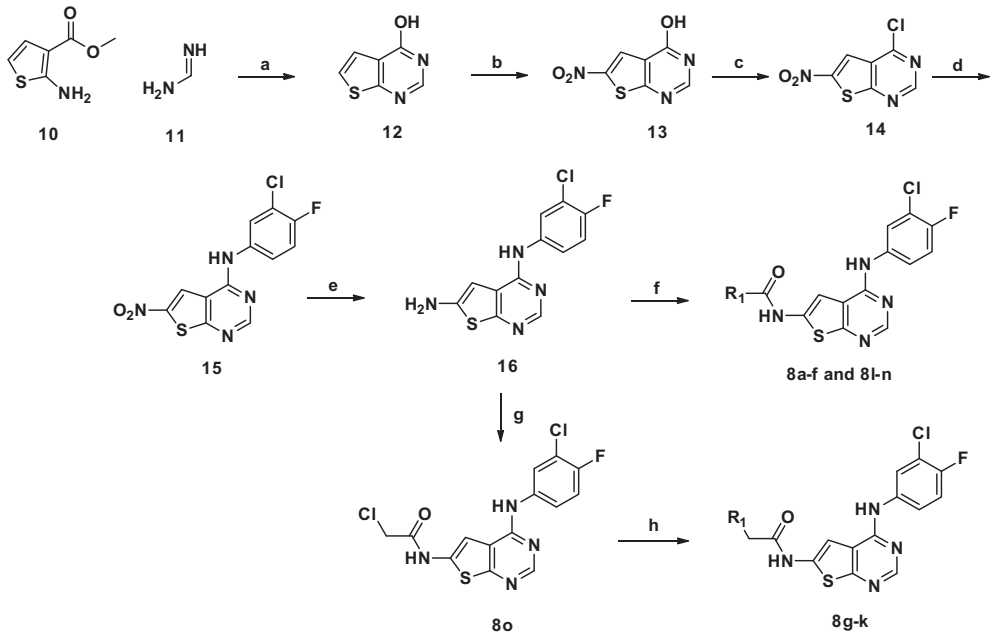
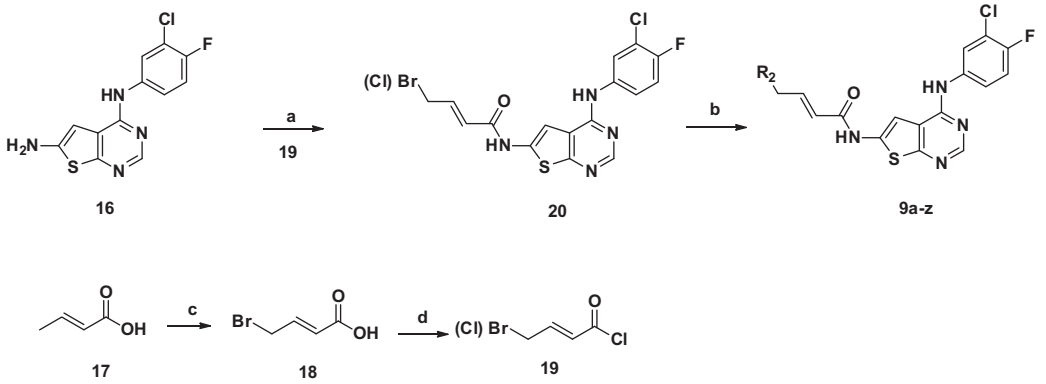


Figure 2. Design strategy and modification of novel irreversible EGFR inhibitors.



Scheme 1. Reagents and conditions: (a) macrowave, 4–5 min; (b) H_2SO_4/HNO_3, 90 °C; (c) $POCl_3$, 110 °C or $POCl_3$, TEA, CH_3CN, 70 °C; (d) 3-chloro-4-fluoroaniline, *i*-PrOH, 80 °C; (e) Fe, NH_4Cl, EtOH, 45 °C; (f) different groups substituted acetyl chloride, TEA, THF; (g) chloroacetyl chloride, pyridine, THF, 60 °C; (h) different amine, THF, 60 °C.



Scheme 2. Reagents and conditions: (a) (*E*)-4-bromobut-2-enoyl chloride, pyridine, THF, 60 °C; (b) different amine, NaI, DMF, overnight; (c) NBS, $(PhCO)_2O$, CCl_4, reflux; (d) $SOCl_2$, DCM, rt.

amines. The intermediate **19** was synthesized from crotonic acid which was similar with synthesis of neratinib.[17]

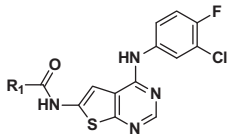
4. Results and discussion

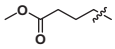
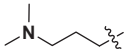
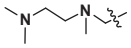
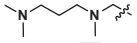
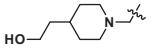
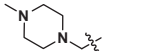
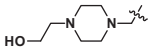
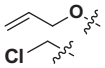
4.1. Kinase inhibition of EGFR wt and EGFR T790M/L858R by compounds

All the synthesized compounds (**8a**–**o** and **9a**–**z**) were evaluated in terms of *in vitro* inhibitory activities on EGFR wt and EGFR T790M/L858R mutant, as well as the anti-proliferative effects on human epithelial carcinoma A431 cells and gefitinib-resistant NCI-H1975 lung adenocarcinoma cells, which expressed high-levels of EGFR wt or EGFR T790M/L858R, respectively (Tables 1 and 2). As shown in Table 1, compounds **8a**–**o** showed good and moderate inhibitory activities against EGFR wt (IC_{50} = 0.016–10.000 μM) and EGFR T790M/L858R (IC_{50} = 0.024–10.000 μM). When R_1 was aliphatic alkane groups (compounds **8a**–**c**), the inhibitory activities against EGFR wt and EGFR T790M/L858R were very weak (IC_{50} >10.000 μM). Introduction of O and N atoms in aliphatic alkane groups (compounds **8d**–**k**), led to improvement in inhibitory activities against EGFR: compound **8f** (EGFR wt, IC_{50} = 0.799 μM), compound **8i** (EGFR wt, IC_{50} = 0.542 μM) and **8k** (EGFR wt, IC_{50} = 0.770 μM). The probable reason was that the O or N atoms could form a hydrogen bond with amino acid residues. When R_1 was an alkene group (compound **8l**, IC_{50} = 0.016 μM), the inhibitory activity showed a considerable improvement. Moreover, the inhibitory activity against EGFR T790M/L858R of compound **8l** was 0.024 μM. However, when a chain of an alkene group was introduced as a substitute group (compound **8m**, IC_{50} = 6.414 μM) or the length of the chain of the alkene group was lengthened (compound **8n**, IC_{50} = 1.792 μM), inhibitory activities were sharply reduced. When R_1 was a chloromethyl group, compound **8o** (EGFR wt, IC_{50} = 0.040 μM) showed good inhibitory activity against EGFR wt. This finding could the chloride group was a good leaving group, and the thiol group of Cys797 could form a covalent bond with the chloromethyl group.

Based on the structure–activity relationships (SARs) of compounds **8a**–**o**, compound **8l** showed good inhibitory activities against EGFR wt (IC_{50} = 0.016 μM) and EGFR T790M/L858R (IC_{50} = 0.024 μM). Moreover, compound **8l** demonstrated good inhibition of A431 cells (IC_{50} = 0.73 μM) and proliferation of

Table 1
Enzymatic and cellular inhibitory activities of compounds **8a–o** against EGFR wt, EGFR T790M/L858R, and A431 and NCI-H1975[a]



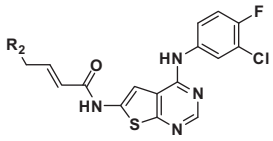
Entries	R_1	EGFR wt (μM)	EGFR T790M/L858R (μM)	A431 (μM)	NCI-H1975 (μM)
8a		>10.000	>10.000	14.21 ± 5.02	>100.00
8b		>10.000	>10.000	22.82 ± 7.45	>100.00
8c		>10.000	>10.000	14.94 ± 3.42	39.30 ± 8.53
8d		4.997 ± 1.595	>10.000	21.33 ± 4.20	>100.00
8e		>10.000	>10.000	27.84 ± 1.22	>100.00
8f		0.799 ± 0.231	1.803 ± 0.337	3.48 ± 1.059	29.61 ± 3.16
8g		4.286 ± 3.774	>10.000	1.59 ± 0.42	17.57 ± 2.78
8h		5.222 ± 1.322	1.699 ± 0.11	2.38 ± 1.27	19.11 ± 2.10
8i		0.542 ± 0.339	>10.000	9.48 ± 6.17	83.43 ± 10.50
8j		3.399 ± 0.812	>10.000	1.70 ± 0.63	21.52 ± 3.55
8k		0.770 ± 0.089	>10.000	10.23 ± 3.65	>100.00
8l		0.016 ± 0.004	0.024 ± 0.001	0.73 ± 0.25	3.00 ± 0.71
8m		6.414 ± 0.238	>10.000	24.18 ± 10.99	>100.00
8n		1.792 ± 0.182	9.859 ± 0.771	26.58 ± 8.46	90.21 ± 8.97
8o		0.040 ± 0.023	0.710 ± 0.205	3.58 ± 1.14	3.01 ± 0.69
Afatinib		0.002 ± 0.001	0.011 ± 0.006	0.02 ± 0.00	0.15 ± 0.04
Erlotinib		0.003 ± 0.002	1.024 ± 0.090	0.56 ± 0.17	7.06 ± 4.00

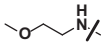
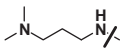
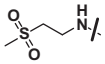
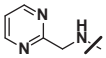
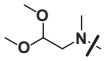
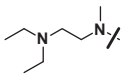
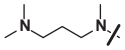
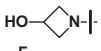
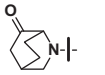
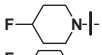
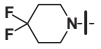
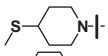
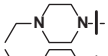
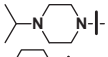
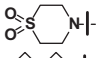
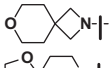
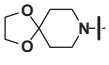
[a] IC_{50} values were obtained by Logit method based on the data obtained from three separate experiments and expressed as means ± SD.

NCI-H1975 cells (IC_{50} = 3.00 μM). However, compared with the control compound afatinib (EGFR wt, IC_{50} = 0.002 μM; EGFR/T790M/L858R, IC_{50} = 0.011 μM), the inhibitory activity should be enhanced further. Also, the solubility of compound **8l** was not good. Therefore, to improve the inhibitory activities and solubility of compound **8l**, some polar groups were introduced to the terminal allyl group in the subsequent modification.

In the subsequent modification, we introduced polar fragments in three ways, i.e. introduction of: (i) the secondary amines in the terminal allyl group; (ii) tertiary amines; (iii) cyclic tertiary amines. Compounds **9a–z** were designed, synthesized and evaluated (Table 2). When R_2 was a 2-methoxyethanamino group (compound **9a**, IC_{50} = 1.040 μM) or 3-(dimethylamino)propyl)amino group (compound **9b**, IC_{50} = 1.196 μM), the inhibitory activities against EGFR wt decreased sharply compared with those of compound **8l** (IC_{50} = 0.016 μM). Interestingly, the inhibitory activities of compounds **9a** (IC_{50} = 0.005 μM) and **9b** (IC_{50} = 0.0007 μM) showed 4.8-, and 34.3-fold improvement against EGFR T790M/L858R compared with compound **8l** (IC_{50} = 0.024 μM). When R_2 was a 2-(methylsulfonyl)ethanamino group (compound **9c**, EGFR wt, IC_{50} = 0.003 μM; EGFR T790M/L858R, IC_{50} = 0.068 μM), the inhibitory activity against EGFR wt showed 5.3-fold improvement, whereas the inhibitory activity against EGFR T790M/L858R showed a 2.8-fold decrease, compared with compound **8l**. Upon introduction of heterocyclic fragments, compounds **9d** (IC_{50} = 0.010 μM), **9e** (IC_{50} = 0.020 μM) and **9f** (IC_{50} = 0.008 μM) showed good inhibitory activities against EGFR wt. However, the inhibitory activity against EGFR T790M/L858R was decreased. Therefore, for the secondary amines in the terminal allyl group, with regard to inhibitory activities against EGFR T790M/L858R, the chain compound was better than cyclic compounds. When R_2 was a dimethylamino group (compound **9g**) or diethylamino group (compound **9h**), the inhibitory activities against EGFR wt were 0.024 and 0.008 μM, respectively. Compound **9i** (IC_{50} = 0.002 μM) showed excellent inhibitory activity against EGFR wt. However, compared with compound **8l**, the inhibitory activities against EGFR T790M/L858R of compounds **9g** (IC_{50} = 0.324 μM), **9h** (IC_{50} = 0.035 μM) and **9i** (IC_{50} = 0.061 μM) were all decreased. For compounds **9j** and **9k**, the inhibitory activities against EGFR wt and EGFR T790M/L858R were both decreased. When R_2 was 3-hydroxyazetidin-1-yl group (compound **9l**) or 3-difluoropyrrolidin-1-yl group (compound **9m**), the inhibitory activities against EGFR wt were 0.038 and 0.028 μM, and these two compounds showed poor inhibitory activities against EGFR T790M/L858R. Compounds **9n** (EGFR wt, IC_{50} = 0.009 μM; EGFR T790M/L858R, IC_{50} = 0.002 μM), **9o** (EGFR wt, IC_{50} = 0.0009 μM; EGFR T790M/L858R, IC_{50} = 0.004 μM), **9p** (EGFR wt, IC_{50} = 0.004 μM; EGFR T790M/L858R, IC_{50} = 0.005 μM) and **9q** (EGFR wt, IC_{50} = 0.007 μM; EGFR T790M/L858R, IC_{50} = 0.004 μM) showed excellent inhibitory activities against EGFR wt and EGFR T790M/L858R. Compared with compound **8l**, the inhibitory activities of compounds **9n**, **9o**, **9p** and **9q** against EGFR T790M/L858R showed 12.0-, 6.0-, 4.8- and 6.0-fold improvement, respectively. When R_2 was 4-(methylthio)piperidin-1-yl group (compound **9r**),

Table 2
Enzymatic and cellular inhibitory activities of compounds **9a–z** against EGFR wt, EGFR T790M/L858R, and A431 and NCI-H1975[a]



Entries	R_2	EGFR wt (μM)	EGFR T790M/L858R (μM)	A431 (μM)	NCI-H1975 (μM)
9a		1.040 ± 0.081	0.005 ± 0.001	4.85 ± 1.05	41.67 ± 10.60
9b		1.196 ± 0.145	0.0007 ± 0.0001	25.40 ± 5.14	>100.00
9c		0.003 ± 0.002	0.068 ± 0.020	19.19 ± 2.18	44.88 ± 7.95
9d		0.010 ± 0.007	0.022 ± 0.006	3.93 ± 0.84	62.20 ± 22.55
9e		0.020 ± 0.002	0.032 ± 0.021	2.21 ± 0.22	7.05 ± 0.38
9f		0.008 ± 0.000	0.055 ± 0.002	3.24 ± 1.71	52.98 ± 4.14
9g		0.024 ± 0.002	0.324 ± 0.059	0.25 ± 0.07	10.11 ± 1.20
9h		0.008 ± 0.002	0.035 ± 0.004	0.64 ± 0.11	11.53 ± 0.18
9i		0.002 ± 0.000	0.061 ± 0.029	0.43 ± 0.15	4.89 ± 0.37
9j		0.139 ± 0.001	0.354 ± 0.052	4.22 ± 0.44	>100.00
9k		0.093 ± 0.025	1.042 ± 0.187	3.41 ± 2.16	>100.00
9l		0.038 ± 0.000	0.305 ± 0.127	1.65 ± 0.45	6.83 ± 0.13
9m		0.028 ± 0.006	1.755 ± 1.032	3.69 ± 1.76	15.83 ± 1.46
9n		0.009 ± 0.003	0.002 ± 0.000	0.13 ± 0.03	6.44 ± 0.44
9o		0.0009 ± 0.0003	0.004 ± 0.004	1.54 ± 0.62	12.05 ± 5.76
9p		0.004 ± 0.001	0.005 ± 0.002	2.40 ± 0.08	10.78 ± 4.12
9q		0.007 ± 0.001	0.004 ± 0.002	1.20 ± 1.13	5.16 ± 1.01
9r		0.018 ± 0.001	0.026 ± 0.008	1.13 ± 0.42	5.22 ± 1.53
9s		0.124 ± 0.021	0.003 ± 0.002	4.47 ± 1.16	34.81 ± 1.31
9t		0.035 ± 0.016	0.004 ± 0.003	4.37 ± 3.21	20.58 ± 0.49
9u		0.034 ± 0.005	0.002 ± 0.001	4.78 ± 2.95	20.74 ± 2.83
9v		0.003 ± 0.002	0.003 ± 0.002	2.17 ± 0.96	11.69 ± 1.38
9w		0.029 ± 0.003	0.007 ± 0.001	8.72 ± 1.43	75.37 ± 1.07
9x		0.007 ± 0.003	0.043 ± 0.013	0.86 ± 0.03	5.54 ± 1.15
9y		2.159 ± 0.018	2.252 ± 0.256	13.37 ± 0.99	39.18 ± 3.18
9z		0.002 ± 0.001	0.020 ± 0.011	0.55 ± 0.14	6.55 ± 0.52
Afatinib		0.001 ± 0.000	0.010 ± 0.006	0.02 ± 0.01	0.15 ± 0.06

[a] IC_{50} values were obtained by Logit method based on the data obtained from three separate experiments and expressed as means ± SD.

4-methylpiperazin-1-yl group (compound **9s**), 4-ethylpiperazin-1-yl group (compound **9t**) or 4-isopropylpiperazin-1-yl group (compound **9u**), the inhibitory activities against EGFR wt were 0.018, 0.124, 0.035 and 0.034 μM, respectively. However, compounds **9s** (IC_{50} = 0.003 μM), **9t** (IC_{50} = 0.004 μM) and **9u** (IC_{50} = 0.002 μM) showed excellent inhibitory activities against EGFR T790M/L858R, showing 8.0-, 6.0-, and 12.0-fold improvement, respectively, compared with compound **8l**. Compound **9v** showed

excellent inhibitory activities against EGFR wt (IC_{50} = 0.003 μM) and EGFR T790M/L858R (IC_{50} = 0.003 μM). When R_2 was 1,1-dioxidothiomorpholino group (compound **9w**), 2-oxa-6-azaspiro[3.3]heptan-6-yl group (compound **9x**), 7-oxa-2-azaspiro[3.5]nonan-2-yl group (compound **9y**), or 1,4-dioxa-8-azaspiro[4.5]decan-8-yl group (compound **9z**), the inhibitory activities against EGFR wt were 0.029, 0.007, 2.159, and 0.002 μM, respectively. The inhibitory activities against EGFR T790M/L858R were 0.007, 0.043, 2.252, and 0.020 μM, respectively. Therefore, for tertiary amines in the terminal allyl group, cyclic tertiary amine compounds were better than chain tertiary amine compounds with regard to inhibitory activities. Among cyclic tertiary amine compounds, hexaheterocyclic compounds were better than five-membered heterocyclic compounds, four-membered heterocyclic compounds and spiro compounds.

4.2. Inhibitory activities of compounds on the EGF-induced activation of EGFR in cancer cells

As the results described above demonstrated, most of the compounds displayed inhibitory effects on EGFR activity *in vitro*. Hence, we further examined their anti-proliferative activities in A431 human epithelial carcinoma cells, which express high-levels of EGFR wt. As shown in Table 1, all of the compounds (**8a–o**) inhibited the proliferation of A431 cells (IC_{50} = 0.73–27.84 μM) moderately. Compounds that contained a nitrogen atom showed better inhibitory activities in A431 cells than compounds which contained an oxygen atom or aliphatic alkane compounds. Also, compound **8l** demonstrated optimal inhibition of proliferation of A431 cell.

In the subsequent modification, compounds **9a–z**, which polar fragments were introduced, demonstrated good inhibition of proliferation of A431 cells (IC_{50} = 0.13–25.40 μM). Compared with compounds **8a–o**, inhibition of proliferation of A431 cells showed improvement. The proposed reason was that introduction of polar fragments improved the solubility of compounds, and the N atom could form a hydrogen bond with amino acid residues. Among these compounds, compounds **9g** (IC_{50} = 0.25 μM), **9h** (IC_{50} = 0.64 μM), **9i** (IC_{50} = 0.43 μM), **9n** (IC_{50} = 0.13 μM), **9x** (IC_{50} = 0.86 μM) and **9z** ((IC_{50} = 0.55 μM)) showed optimal inhibition of proliferation of A431 cells. Also, compounds **9e** (IC_{50} = 2.21 μM), **9l** (IC_{50} = 1.65 μM), **9o** (IC_{50} = 1.54 μM), **9p** (IC_{50} = 2.40 μM), **9q** (IC_{50} = 1.20 μM), **9r** (IC_{50} = 1.13 μM) and **9v** (IC_{50} = 2.17 μM) demonstrated good inhibition of proliferation of A431 cells.

4.3. Inhibition of gefitinib-resistant NCI-H1975 cell

Drug resistance is a critical issue in cancer therapy, and the T790M mutation in EGFR is a key factor in this regard. Therefore, compounds **8a–o** and **9a–z** were evaluated for their ability to inhibit growth of the gefitinib-resistant NCI-H1975 NSCLC cell line harboring the T790M mutation by the Sulforhodamine B (SRB) assay. As shown in Table 1, compound **8l** (IC_{50} = 3.00 μM), which could form a covalent bond with Cys797, showed good inhibition of proliferation of NCI-H1975 cells. Also, compound **8o** (IC_{50} = 3.01 μM), which had the potential to form a covalent bond with Cys797, also showed good inhibition of proliferation of NCI-H1975 cells. Based on the modification of compound **8l**, compounds **9a–z** (IC_{50} = 4.89–100.00 μM), demonstrated moderate to good inhibition of proliferation of NCI-H1975 cells (Table 2). Among these compounds, compounds **9e** (IC_{50} = 7.05 μM), **9g** (IC_{50} = 10.11 μM), **9h** (IC_{50} = 11.53 μM), **9i** (IC_{50} = 4.89 μM), **9l** (IC_{50} = 6.83 μM), **9n** (IC_{50} = 6.44 μM), **9o** (IC_{50} = 12.05 μM), **9p** (IC_{50} = 10.78 μM), **9q** (IC_{50} = 5.16 μM), **9r** (IC_{50} = 5.22 μM), **9v** (IC_{50} = 11.69 μM), **9x** (IC_{50} = 5.54 μM) and **9z** (IC_{50} = 6.55 μM) demonstrated good inhibition of proliferation of NCI-H1975 cells.

In comparison of compounds **8a–o** (Table 1), compound **8l** showed good inhibitory activities against EGFR wt and EGFR T790M/L858R due to having the Michael receptor group at 6-position. However, compared with compound **8l**, most of compounds **9a–z** showed excellent inhibitory activities against EGFR wt and EGFR T790M/L858R (Table 2) because of introduction of some polar groups to the terminal allyl group. The probably reason was the polar groups contained O or N atoms interacted with side chains of residues. Moreover, some of compounds **9a–z** demonstrated better anti-proliferative effects on human epithelial carcinoma A431 cells and gefitinib-resistant NCI-H1975 lung adenocarcinoma cells than that of compounds **8a–o** (Tables 1 and 2). The proposed reason was the improvement of the solubility of some of compounds **9a–z**. Therefore, considering the inhibitory activities against EGFR wt and EGFR T790M/L858R and the anti-proliferative effects on human epithelial carcinoma A431 cells and gefitinib-resistant NCI-H1975 lung adenocarcinoma cells, compounds **8l**, **9n**, **9o**, **9q and 9v** were selected for blocking cellular EGFR phosphorylation and downstream signaling pathways.

4.4. Compounds 8l, 9n, 9o, 9q and 9v block cellular EGFR phosphorylation and downstream signaling pathways

Upon consideration of the potent inhibitory activities against EGFR wt and EGFR T790M/L858R kinases and anti-proliferative effects on cancer cells, compounds **8l**, **9n**, **9o**, **9q** and **9v** were selected for further study to ascertain if they could attenuate EGFR activity and EGFR downstream signaling molecules in A431 and NCI-H1975 cancer cells. When A431 cells were incubated with compounds **8l**, **9n**, **9o**, **9q** and **9v** for 2 h followed by exposure to EGF, potent and concentration-dependent inhibition of EGFR autophosphorylation was observed (Fig. 3A). Compounds **8l**, **9n**, **9o**, **9q**

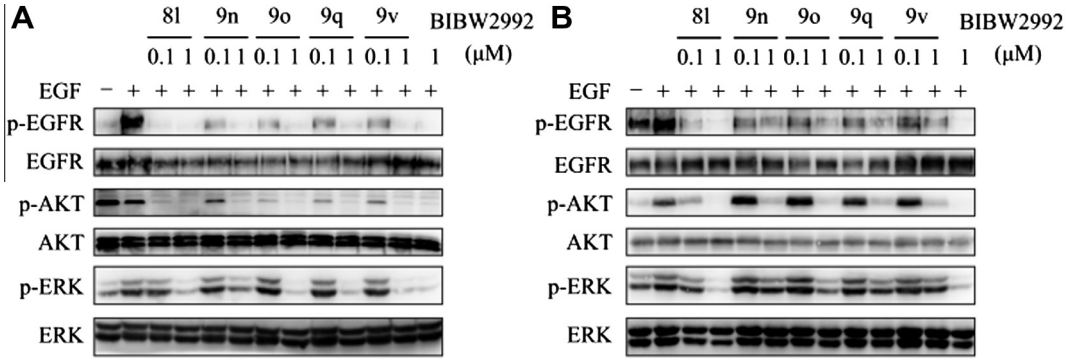


Figure 3. Compounds **8l**, **9n**, **9o**, **9q** and **9v** block EGFR phosphorylation and downstream signaling. (A) Western blot analysis for EGFR inhibition by **8l**, **9n**, **9o**, **9q** and **9v** in the A431 cell line. Analysis was done using monoclonal antibodies directed to *p*-Tyr1068 (see the Section 6). Total EGFR is shown as the loading control. (B) Western blot analysis for EGFR inhibition by Compounds **8l**, **9n**, **9o**, **9q** and **9v** in the NCI-H1975 cell line.

and **9v** at 0.1 μmol/L almost completely blocked the autophosphorylation of EGFR wt in A431 cells. In addition, they dramatically inhibited EGF-induced phosphorylation of ERK1/2 and AKT, two key signaling molecules downstream of EGFR. Similarly, compounds **8l**, **9n**, **9o**, **9q** and **9v** dose-dependently blocked EGFR T790M/L858R phosphorylation and downstream signaling in NCI-H1975 cells (Fig. 3B). Moreover, compound **8l** showed a stronger inhibitory effect on EGFR T790M/L858R at 0.1 μmol/L in NCI-H1975 than that of compounds **9n**, **9o**, **9q** and **9v**.

4.5. Irreversible inhibition

To ascertain if compound **8l** could inhibit EGFR activity by irreversible binding, we used a dilution method to assess the intrinsic property of compound **8l**.[28] In this assay, EGFR kinase was pre-incubated with excess compound **8l** at a final concentration of 100-fold IC_{50} under room temperature for 30 min, then the mixture was diluted 100-fold in reaction buffer with ATP and peptide, and EGFR kinase activity measured continuously. Phosphorylation of the peptide reflected enzyme activity according to the rate of dissociation of the enzyme. In general, after incubation of different types of inhibitors with EGFR, the recovery curve of reversible inhibitors, such as erlotinib (Fig. 4A), coincided with that of the non pre-incubation control, whereas the recovery of irreversible inhibitors, such as BIBW2992, was suppressed dramatically (Fig. 4B). As shown in Fig. 4C, when the kinases were pre-incubated with compound **8l**, the recovery was obviously decreased compared with the non pre-incubation control, suggesting that compound **8l** could irreversibly bind to EGFR protein (Fig. 4C) and thus inhibit kinase activity.

4.6. Kinase profiling

To assess further the selectivity of compounds **8l** and **9o**, we tested their activities on other members of the EGFR family ErbB2, ErbB4, as well as 11 types of tyrosine kinases using an *in vitro* kinase assay. As shown in Table 3, compounds **8l** and **9o** not only inhibited the kinase activities of EGFR wt and EGFR T790M/L858R presented in Tables 1 and 2, they also potently inhibited the activity of ErbB2 and ErbB4 with IC_{50} values ranging from 0.004 to 0.040 μM (Table 3). In addition, compound **8l**, but not compound **9o**, also possessed weak inhibitory activity on the RET tyrosine kinase domain (IC_{50} = 1.626 μM) with an IC_{50} 100-fold higher than that for EGFR (IC_{50} = 0.016 μM). Moreover, compounds **8l** and **9o** showed little effect on the other tyrosine kinases tested, including ABL, Flt-1, KDR, c-Kit, PDGFR-α, PDGFR-β, EPH-A2, IGF1R and FGFR1. These data demonstrated that these compounds are selective inhibitors of members of the EGFR family.

Table 3
Selectivity profile of compounds **8l** and **9o** against 15 kinases[a]

Kinase	**8l** (IC_{50}, μM)	**9o** (IC_{50}, μM)
EGFR wt	0.016 ± 0.004	0.0009 ± 0.0003
EGFR T790M/858R	0.024 ± 0.001	0.004 ± 0.004
ErbB2	0.040 ± 0.001	0.030 ± 0.002
ErbB4	0.004 ± 0.002	0.037 ± 0.006
RET	1.626 ± 0.096	>10.000
Flt-1	>10.000	>10.000
KDR	>10.000	>10.000
c-Kit	>10.000	>10.000
PDGFR-α	>10.000	>10.000
PDGFR-β	>10.000	>10.000
ABL	>10.000	>10.000
EPH-A2	>10.000	>10.000
RON	>10.000	>10.000
IGF1R	>10.000	>10.000
FGFR1	>10.000	>10.000

[a] The kinase profiling assay was conducted as described in the Experimental section by using ELISA kinases assay. IC_{50} values were obtained by Logit method based on the data obtained from three separate experiments and expressed as means ± SD.

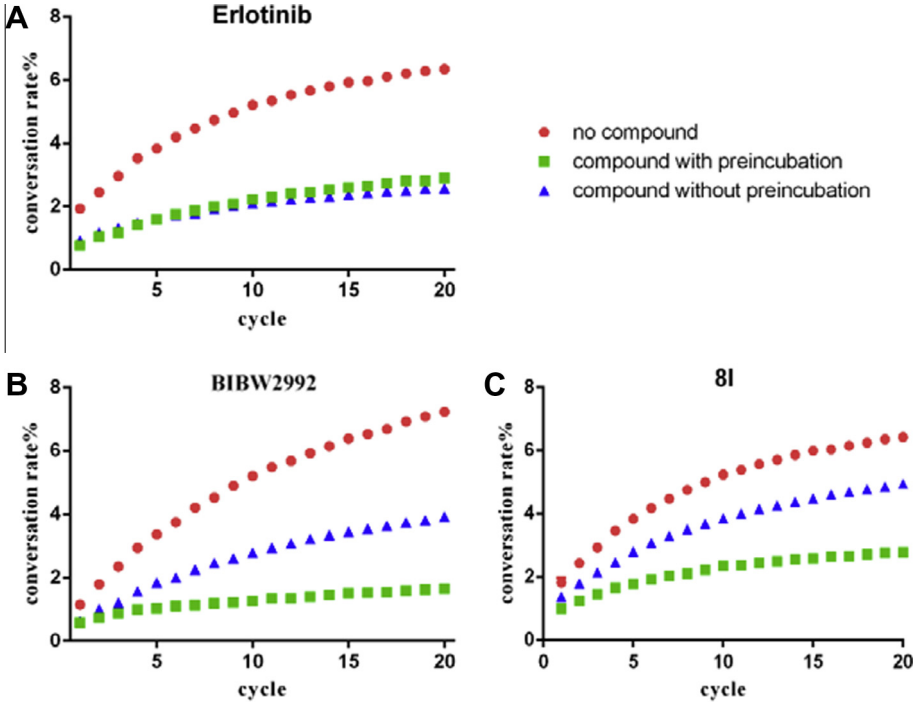


Figure 4. Compound **8l** irreversibly binds EGFR. Using Caliper EZ Reader to assay the enzyme activity of EGFR under three different conditions: without compound, with compound and preincubated with compound, and the enzyme activity was assessed by the percent of converge of substrate peptide (5-FAM-EEPLYWSFPAKKK-CONH2).

5. Conclusions

A novel series of 6-alkenylamide derivatives of 4-anilinothieno[2,3-*d*]pyrimidines was designed, synthesized and evaluated as EGFR inhibitors. Compound **8l** showed good inhibitory activities against EGFR wt and EGFR T790M/L858R *in vitro*, and almost completely blocked the phosphorylation of EGFR in the A431 cell line at 1 μM. Furthermore, the IC_{50} values of 10 compounds against EGFR T790M/L858R were less than 0.005 μM. Compounds **8l**, **9n**, **9q**, **9o** and **9v** blocked the autophosphorylation of EGFR in NCI-H1975 cells at high concentration (1 μM). Compound **8l** was confirmed to be an irreversible inhibitor through the dilution method.

6. Experimental section

Chemistry: The reagents (chemicals) were purchased and used without further purification. Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker AMX-400 (IS as TMS). Chemical shifts were reported in parts per million (ppm, δ) downfield from tetramethylsilane. Proton coupling patterns were described as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad (br). Low- and high-resolution mass spectra (LRMS and HRMS) were given with electric, electrospray, and matrix-assisted laser desorption ionization (EI, and ESI) produced by a Finnigan MAT-95, LCQ-DECA spectrometer and IonSpec 4.7 T.

6.1. General method to synthesize compounds

*General method to synthesize compounds **8a–f** and **8l–o**:* To a solution of compound **16** (0.10 mol) in THF was added different groups substituted acetyl chloride (0.15 mol) and TEA (0.20 mol) at 0 °C. Then the mixture was stirred at room temperature overnight. After the start material was completed, water was added to quench the reaction and extracted with EA, washed by water, $NaHCO_3$ solution, NaCl solution and dried by Na_2SO_4, concentrated and purified by flash silica gel column to obtain desired compounds **8a–f** and **8l–o**.

*General method to synthesize compounds **8g–k***: To a solution of compound **8o** (0.10 mol) in THF (5 mL) was added different amines (0.15 mol). Then the mixture was stirred at 60 °C for 4 h. After the start material was completed, water was added to quench the reaction and extracted with EA, washed by water, $NaHCO_3$ solution, NaCl solution and dried by Na_2SO_4, concentrated and purified by flash silica gel column to obtain desired compounds **8g–k**.

*General method to synthesize compounds **9a–z**:* To a solution of compound **20** (0.10 mol) in DMF (0.5 mL) was added different amines (0.15 mol) and NaI (0.15 mol) at 0 °C. Then the mixture was stirred at room temperature overnight. After the start material was completed, water was added to quench the reaction and extracted with EA, washed by water, $NaHCO_3$ solution, NaCl solution and dried by Na_2SO_4, concentrated and purified by flash silica gel column to obtain desired compounds **9a–z**.

6.1.1. Thieno[2,3-*d*]pyrimidin-4-ol (12)

A mixture of compound **10** (20 g, 0.127 mol) and formimidamide acetate **11** (16 g, 0.153 mol) was reacted in condition of microwave for 4–5 min. After cooled for 3 min, water was added and the solid was precipitate, then filtered, and the cake was washed with water, dried under infrared light to obtain the desired compound **12** as white solid in 89% yield. ESI-MS *m*/*z* 153 $[M+H]^+$.

6.1.2. 6-Nitrothieno[2,3-*d*]pyrimidin-4-ol (13)

To a mixture of H_2SO_4 (20 mL) and HNO_3 (20 mL) was added compound **12** (20 g) slowly under ice-bath condition. After the start material was added, the reaction mixture was heated for 2 h. After the start material was completed, the mixture was poured into ice-water carefully, and the yellow solid was precipitate. Then filtered, the cake was washed by water and dried under infrared light to obtain the desired compound **13** as yellow solid in a 80% yield. ESI-MS *m*/*z* 197 $[M+H]^+$.

6.1.3. 4-Chloro-6-nitrothieno[2,3-*d*]pyrimidine (14)

To a solution of compound **13** (10 g, 0.05 mol) in CH_3CN (100 mL) was added $POCl_3$ (14 mL, 0.15 mol) and TEA (21 mL, 0.15 mol). Then the mixture was heated to 70 °C for 3 h. After the start material was completed, the mixture was poured into ice-water, and extracted with EA, washed by $NaHCO_3$ solution, brine, dried by Na_2SO_4, concentrated and purified by flash silica gel column (0–100% EA in PE gradient) to obtain desired compound **14** in 75% yield. ESI-MS *m*/*z* 216 $[M+H]^+$.

6.1.4. *N*-(3-Chloro-4-fluorophenyl)-6-nitrothieno[2,3-*d*]pyrimidin-4-amine (15)

To a solution of compound **14** (10 g, 0.46 mol) in *i*-PrOH was added 3-chloro-4-fluoroaniline (7.43 g, 0.51 mol) at room temperature. Then the reaction mixture was heated to 80 °C for 2 h. After the start material was completed, the solvent was removed by vacuum. Then the crude product was suspended in EA, filtered to obtain desired compound **15** as yellow solid in a 95% yield. ESI-MS *m*/*z* 325 $[M+H]^+$.

6.1.5. N^4-(3-Chloro-4-fluorophenyl)thieno[2,3-*d*]pyrimidine-4,6-diamine (16)

To a solution of compound **15** (10 g) in ethanol (50 mL) was added iron powder (catalytic amount) and NH_4Cl solution (20 mL). Then the mixture was stirred at 45 °C for 1 h. After the start material was completed, the mixture was filtered through celite, and the cake was washed by ethanol. And 100 mL water was added to the filtrate, the yellow white solid was occurred. Then filtered and dried to obtain the desired compound **16** as yellow white solid in 70% yield. ESI-MS *m*/*z* 295 $[M+H]^+$.

6.1.6. (*E*)-4-Bromobut-2-enoic acid (18)

To a solution of (*E*)-but-2-enoic acid (10 g, 0.116 mol) in CCl_4 was added NBS (31 g, 0.174 mol) and benzoyl peroxide (0.263 g, 1.16 mmol). Then the mixture reaction was stirred at 77 °C for 4 h. After the start material was completed, the solvent was removed and *n*-hexane was added. The precipitate was formed, filtered and dried to obtain desired compound **18** as yellow white solid in 93% yield. ESI-MS *m*/*z* 166 $[M+H]^+$.

6.1.7. (*E*)-4-Bromobut-2-enoyl chloride or (*E*)-4-chlorobut-2-enoyl chloride (19)

To a solution of compound **18** (5 g, 0.03 mol) in DCM was added $SOCl_2$ (3.3 mL, 0.45 mol) dropwise at ice-bath condition. Then the mixture was stirred overnight. After the start material was finished, the solvent was removed to obtain desired compound **19** as yellow oil without further purification.

6.1.8. (*E*)-4-Bromo(chloro)-*N*-(4-((3-chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)but-2-enamide (20)

To a solution of compound **16** (8 g, 0.27 mol) in THF was added compound **19** (7.47 g, 0.41 mol) and pyridine (5.96 mL, 0.54 mol) at 0 °C. Then the mixture was stirred at room temperature overnight. After the start material was completed, water was added to quench the reaction and extracted with EA, washed by water, $NaHCO_3$ solution, NaCl solution and dried by Na_2SO_4, concentrated and purified by flash silica gel column (0–50% EA in PE gradient) to

obtain desired compound **20** as brown solid in 60% yield. ESI-MS *m*/*z* 442 or 398 $[M+H]^+$.

6.1.9. *N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)pentanamide (8a)

According to general method to synthesize compounds **8a**–**f** and **8l**–**o**, compound **8a** was synthesized in 61% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.90 (t, J = 5.7 Hz, 3H), 1.29–1.38 (m, 2H), 1.57–1.64 (m, 2H), 7.28 (s, 1H), 7.38–7.43 (m, 1H), 7.77–7.81 (m, 1H), 8.44 (s, 1H), 9.60 (s, 1H), 11.54 (s, 1H). ESI-MS *m*/*z* 379 $[M+H]^+$.

6.1.10. *N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-2-ethylbutanamide (8b)

According to general method to synthesize compounds **8a**–**f** and **8l**–**o**, compound **8b** was synthesized in 57% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.85 (t, J = 5.4 Hz, 6H), 1.47–1.63 (m, 4H), 2.33–2.35 (m, 1H), 7.34 (s, 1H), 7.38–7.43 (m, 1H), 7.77–7.80 (m, 1H), 8.18–8.20 (m, 1H), 8.45 (s, 1H), 9.62 (s, 1H), 11.54 (s, 1H). ESI-MS *m*/*z* 393 $[M+H]^+$.

6.1.11. *N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)cyclopentanecarboxamide (8c)

According to general method to synthesize compounds **8a**–**f** and **8l**–**o**, compound **8c** was synthesized in 56% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.57–1.62 (m, 2H), 1.65–1.79 (m, 4H), 1.89–1.91 (m, 2H), 2.87–2.90 (m, 1H), 7.30 (s, 1H), 7.39–7.44 (m, 1H), 7.77–7.81 (m, 1H), 8.17–8.20 (m, 1H), 8.44 (s, 1H), 9.62 (s, 1H), 11.57 (s, 1H). ESI-MS *m*/*z* 391 $[M+H]^+$.

6.1.12. Methyl 3-((4-((3-chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)amino)-3-oxopropanoate (8d)

According to general method to synthesize compounds **8a**–**f** and **8l**–**o**, compound **8d** was synthesized in 66% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.62 (s, 3H), 3.68 (s, 3H), 7.36 (s, 1H), 7.40–7.44 (m, 1H), 7.76–7.80 (m, 1H), 8.17–8.19 (m, 1H), 8.46 (s, 1H), 9.64 (s, 1H), 11.85 (s, 1H). ESI-MS *m*/*z* 395 $[M+H]^+$.

6.1.13. Methyl 5-((4-((3-chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)amino)-5-oxopentanoate (8e)

According to general method to synthesize compounds **8a**–**f** and **8l**–**o**, compound **8e** was synthesized in 69% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.84–1.91 (m, 2H), 2.40 (t, J = 5.7 Hz, 2H), 2.49 (t, J = 4.8 Hz, 2H), 3.60 (s, 3H), 7.28 (s, 1H), 7.38–7.42 (m, 1H), 7.77–7.80 (m, 1H), 8.17–8.19 (m, 1H), 8.44 (s, 1H), 9.61 (s, 1H), 11.70 (s, 1H). ESI-MS *m*/*z* 423 $[M+H]^+$.

6.1.14. *N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-(dimethylamino)butanamide (8f)

According to general method to synthesize compounds **8a**–**f** and **8l**–**o**, compound **8f** was synthesized in 57% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.00–2.05 (m, 2H), 2.59–2.64 (m, 2H), 2.75 (s, 6H), 3.07–3.18 (m, 4H), 7.38–7.44 (m, 1H), 7.52 (s, 1H), 7.87–7.92 (m, 1H), 8.25–8.28 (m, 1H), 8.44 (s, 1H), 9.98 (s, 1H), 12.16 (s, 1H). ESI-MS *m*/*z* 408 $[M+H]^+$.

6.1.15. *N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-2-((2-(dimethylamino)ethyl)(methyl)amino)acetamide (8g)

According to general method to synthesize compounds **8g**–**k**, compound **8g** was synthesized in 81% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 2.41 (s, 3H), 2.61 (s, 6H), 2.73–2.77 (m, 2H), 2.84–2.88 (m, 2H), 3.40 (s, 2H), 7.18–7.28 (m, 2H), 7.61–7.67 (m, 1H), 8.00–8.03 (m, 1H), 8.38 (s, 1H). ESI-MS *m*/*z* 437 $[M+H]^+$.

6.1.16. *N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-2-((3-(dimethylamino)propyl)(methyl)amino)acetamide (8h)

According to general method to synthesize compounds **8g**–**k**, compound **8h** was synthesized in 83% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 1.90–1.94 (m, 2H), 2.43 (s, 3H), 2.63–2.68 (m, 2H), 2.92 (s, 6H), 2.92–3.28 (m, 2H), 3.44 (s, 2H), 7.13–7.19 (m, 1H), 7.25 (s, 1H), 7.61–7.67 (m, 1H), 8.03–8.06 (m, 1H), 8.35 (s, 1H). ESI-MS *m*/*z* 451 $[M+H]^+$.

6.1.17. *N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-2-(4-(2-hydroxyethyl)piperidin-1-yl)acetamide (8i)

According to general method to synthesize compounds **8g**–**k**, compound **8i** was synthesized in 83% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 1.35–1.45 (m, 2H), 1.48–1.51 (m, 3H), 1.72–1.76 (m, 2H), 2.20–2.28 (m, 2H), 2.95–2.98 (m, 2H), 3.27 (s, 2H), 3.59–3.65 (m, 2H), 7.17–7.23 (m, 2H), 7.61–7.66 (m, 1H), 8.01–8.04 (m, 1H), 8.37 (s, 1H). ESI-MS *m*/*z* 464 $[M+H]^+$.

6.1.18. *N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-2-(4-methylpiperazin-1-yl)acetamide (8j)

According to general method to synthesize compounds **8g**–**k**, compound **8j** was synthesized in 79% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 2.51 (s, 3H), 2.75–2.84 (m, 8H), 3.34 (s, 2H), 7.15–7.21 (m, 2H), 7.59–7.65 (m, 1H), 8.00–8.03 (m, 1H), 8.35 (s, 1H). ESI-MS *m*/*z* 435 $[M+H]^+$.

6.1.19. *N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-2-(4-(2-hydroxyethyl)piperazin-1-yl)acetamide (8k)

According to general method to synthesize compounds **8g**–**k**, compound **8k** was synthesized in 84% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 2.74–2.78 (m, 6H), 2.86 (br s, 4H), 3.29–3.30 (m, 2H), 3.72–3.76 (m, 2H), 7.11–7.18 (m, 2H), 7.58–7.63 (m, 1H), 7.99–8.02 (m, 1H), 8.32 (s, 1H). ESI-MS *m*/*z* 465 $[M+H]^+$.

6.1.20. *N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)acrylamide (8l)

According to general method to synthesize compounds **8a**–**f** and **8l**–**o**, compound **8l** was synthesized in 62% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 5.88–5.92 (m, 1H), 6.35–6.39 (m, 1H), 6.46–6.53 (m, 1H), 7.39–7.43 (m, 2H), 7.76–7.80 (m, 1H), 8.17–8.19 (m, 1H), 8.46 (s, 1H), 9.66 (s, 1H), 11.86 (s, 1H). ESI-MS *m*/*z* 349 $[M+H]^+$.

6.1.21. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-2-methylbut-2-enamide (8m)

According to general method to synthesize compounds **8a**–**f** and **8l**–**o**, compound **8m** was synthesized in 72% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.82 (d, J = 4.8 Hz, 3H), 1.89 (s, 3H), 6.61–6.63 (m, 1H), 7.39–7.43 (m, 2H), 7.77–7.81 (m, 1H), 8.17–8.20 (m, 1H), 8.44 (s, 1H), 9.67 (s, 1H), 11.45(s, 1H). ESI-MS *m*/*z* 377 $[M+H]^+$.

6.1.22. Allyl (4-((3-chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)carbamate (8n)

According to general method to synthesize compounds **8a**–**f** and **8l**–**o**, compound **8n** was synthesized in 60% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.69 (d, J = 3.9 Hz, 2H),5.27–5.30 (m, 1H), 5.38–5.43 (m, 1H), 7.24 (s, 1H), 7.38–7.42 (m, 1H), 7.77–7.8 1 (m, 1H), 8.16–8.18 (m, 1H), 8.43 (s, 1H), 9.62 (s, 1H), 11.36(s, 1H). ESI-MS *m*/*z* 379 $[M+H]^+$.

6.1.23. 2-chloro-*N*-(4-((3-chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)acetamide (8o)

According to general method to synthesize compounds **8a**–**f** and **8l**–**o**, compound **8o** was synthesized in 75% yield. ^{1}H NMR

(300 MHz, DMSO-d_6) δ 4.40 (s, 2H), 7.40–7.44 (m, 2H), 7.76–7.80 (m, 1H), 8.16–8.19 (m, 1H), 8.47 (s, 1H), 9.69 (s, 1H), 12.0 (s, 1H). ESI-MS m/z 371 $[M+H]^+$

6.1.24. (*E*)-*N*-(4-((3-chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-((2-methoxyethyl)amino)but-2-enamide (9a)

According to general method to synthesize compounds **9a**–**z**, compound **9a** was synthesized in 51% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.28 (s, 1H), 8.46 (s, 1H), 8.40 (dd, J_1 = 8.0 Hz, J_2 = 4.0 Hz, 1H), 7.98–8.05 (m, 1H), 7.87 (s, 1H), 7.38 (t, J = 8.0 Hz, 1H), 4.06–4.10 (m, 1H), 3.72 (d, J = 8.0 Hz, 1H), 3.64 (br, 1H), 3.24 (s, 3H), 2.86 (dd, J_1= 8.0 Hz, J_2 = 8.0 Hz, 1H), 2.73 (d, J = 8.0 Hz, 2H). ESI-MS m/z 436 $[M+H]^+$

6.1.25. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-((3-(dimethylamino)propyl)amino)but-2-enamide (9b)

According to general method to synthesize compounds **9a**–**z**, compound **9b** was synthesized in 55% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.19 (s, 1H), 9.85 (s, 1H), 8.45 (s, 1H), 8.21 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.83–7.86 (m, 1H), 7.48 (s, 1H), 7.41 (t, J = 8.0 Hz, 1H), 6.61 (d, J = 12.0 Hz, 1H), 4.61–4.66 (m, 3H), 4.38–4.41 (m, 2H), 4.26 (s, 1H), 3.79–3.85 (m, 2H), 2.72–2.98 (m, 2H). ESI-MS m/z 448 $[M+H]^+$.

6.1.26. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-((2-(methylsulfonyl)ethyl)amino)but-2-enamide (9c)

According to general method to synthesize compounds **9a**–**z**, **9c** was synthesized in 65% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.92 (s, 1H), 9.77 (s, 1H), 8.44 (s, 1H), 8.20 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.81–7.87 (m, 1H), 7.39–7.48 (m, 2H), 6.64 (d, J = 8.0 Hz, 1H), 4.50(d, J = 4.0 Hz, 1H), 3.43–3.48 (m, 2H), 1.21–1.29 (m, 8H). ESI-MS m/z 462 $[M+H]^+$.

6.1.27. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-((oxetan-3-ylmethyl)amino)but-2-enamide (9d)

According to general method to synthesize compounds **9a**–**z**, compound **9d** was synthesized in 58% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.13 (s, 1H), 9.94 (s, 1H), 8.48 (s, 1H), 8.30 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.66 (s, 1H), 7.43 (t, J = 8.0 Hz, 1H), 6.41 (d, J = 4.0 Hz, 1H), 4.71 (s, 1H), 4.20–4.33 (m, 2H), 3.18–3.21 (m, 2H), 2.75 (s, 6H), 1.98–2.03 (m, 2H), 0.83–0.91 (m, 2H). ESI-MS m/z 463 $[M+H]^+$.

6.1.28. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-((tetrahydro-2*H*-pyran-4-yl)amino)but-2-enamide (9e)

According to general method to synthesize compounds **9a**–**z**, compound **9e** was synthesized in 70% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.65 (s, 1H), 9.71 (s, 1H), 8.93 (s, 1H), 8.70–8.81 (m, 2H), 8.43 (s, 1H), 8.18 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.74–7.81 (m, 1H), 7.25–7.41 (m, 2H), 4.85 (s, 1H), 3.95–4.00 (m, 2H), 2.93–2.98 (m, 2H). ESI-MS m/z 470 $[M+H]^+$.

6.1.29. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-((pyrimidin-2-ylmethyl)amino)but-2-enamide (9f)

According to general method to synthesize compounds **9a**–**z**, compound **9f** was synthesized in 65% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.81 (s, 1H), 9.66 (s, 1H), 8.45 (s, 1H), 8.18 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.77–7.81 (m, 1H), 7.42 (t, J = 8.0 Hz, 1H), 7.37 (s, 1H), 6.90–6.97 (m, 1H), 6.35 (d, J = 16.0 Hz, 1H), 3.43–3.44 (m, 2H), 3.28 (t, J = 8.0 Hz, 2H), 3.04 (s, 3H), 2.95–3.01 (m, 4H). ESI-MS m/z 484 $[M+H]^+$.

6.1.30. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-(dimethylamino)but-2-enamide (9g)

According to general method to synthesize compounds **9a**–**z**, compound **9g** was synthesized in 70% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.34 (s, 1H), 9.79 (s, 1H), 8.44 (s, 1H), 8.31 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.89–7.98 (m, 1H), 7.57 (s, 1H), 7.38 (t, J = 8.0 Hz, 1H), 6.82–6.85 (m, 1H), 6.52 (d, J = 16.0 Hz, 1H), 2.48–2.52 (m, 2H), 2.28 (s, 6H). ESI-MS m/z 406 $[M+H]^+$.

6.1.31. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-(diethylamino)but-2-enamide (9h)

According to general method to synthesize compounds **9a**–**z**, compound **9h** was synthesized in 66% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.71 (s, 1H), 10.12 (s, 1H), 8.43 (s, 1H), 8.25 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.88–7.92 (m, 1H), 7.69 (s, 1H), 7.38 (t, J = 8.0 Hz, 1H), 6.95–7.02 (m, 1H), 6.78 (d, J = 16.0 Hz, 1H), 3.93 (s, 1H), 2.98–3.08 (m, 6H), 1.23 (t, J = 4.0 Hz, 6H), 1.18 (t, J = 8.0 Hz, 3H). ESI-MS m/z 434 $[M+H]^+$.

6.1.32. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-((2,2-dimethoxyethyl)(methyl)amino)but-2-enamide (9i)

According to general method to synthesize compounds **9a**–**z**, compound **9i** was synthesized in 65% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.76 (s, 1H), 9.64 (s, 1H), 8.45 (s, 1H), 8.18 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.77–7.81 (m, 1H), 7.41 (t, J = 8.0 Hz, 1H), 7.36 (s, 1H), 6.85–6.91 (m, 1H), 6.35 (d, J = 16.0 Hz, 1H), 3.36–3.42 (m, 6H), 3.23 (s, 6H), 2.66 (t, J = 8.0 Hz, 4H). ESI-MS m/z 494 $[M+H]^+$.

6.1.33. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-((2-(diethylamino)ethyl)(methyl)amino)but-2-enamide (9j)

According to general method to synthesize compounds **9a**–**z**, compound **9j** was synthesized in 59% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.74 (s, 1H), 8.49 (s, 1H), 8.21 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.81–7.83 (m, 1H), 7.56 (s, 1H), 7.42 (t, J = 8.0 Hz, 1H), 6.64 (d, J = 12.0 Hz, 1H), 5.72–5.76 (m, 1H), 2.87 (s, 6H), 2.77–2.82 (m, 6H), 2.71 (s, 3H). ESI-MS m/z 492 $[M+H]^+$.

6.1.34. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-((3-(dimethylamino)propyl)(methyl)amino)but-2-enamide (9k)

According to general method to synthesize compounds **9a**–**z**, compound **9k** was synthesized in 60% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.13 (s, 1H), 9.94 (s, 1H), 8.48 (s, 1H), 8.30 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.66 (s, 1H), 7.43 (t, J = 8.0 Hz, 1H), 6.41 (d, J = 4.0 Hz, 1H), 4.71 (s, 1H), 4.20–4.33 (m, 2H), 3.18–3.21 (m, 2H), 2.75 (s, 6H), 1.98–2.03 (m, 2H), 0.83-0.91 (m, 2H). ESI-MS m/z 463 $[M+H]^+$.

6.1.35. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-(3-hydroxyazetidin-1-yl)but-2-enamide (9l)

According to general method to synthesize compounds **9a**–**z**, compound **9l** was synthesized in 71% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.84 (s, 1H), 9.70 (s, 1H), 8.44 (s, 1H), 8.18 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.78–7.82 (m, 1H), 7.35–7.42 (m, 2H), 6.63 (d, J = 8.0 Hz, 1H), 6.47–6.53 (m, 1H), 4,48 (d, J = 4.0 Hz, 1H), 3.41–3.46 (m, 2H), 1.14–1.27 (m, 4H). ESI-MS m/z 434 $[M+H]^+$.

6.1.36. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-(3,3-difluoropyrrolidin-1-yl)but-2-enamide (9m)

According to general method to synthesize compounds **9a**–**z**, compound **9m** was synthesized in 67% yield. ^{1}H NMR (400 MHz,

DMSO-d_6) δ 11.79 (s, 1H), 9.65 (s, 1H), 8.45 (s, 1H), 8.18 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.77–7.80 (m, 1H), 7.41 (t, J = 8.0 Hz, 1H), 7.35 (s, 1H), 6.83–6.90 (m, 1H), 6.36 (d, J = 16.0 Hz, 1H), 2.95 (t, J = 12.0 Hz, 2H), 2.77 (t, J = 8.0 Hz, 2H), 2.19–2.34 (m, 2H), 1.20–1.29 (m, 2H). ESI-MS m/z 468 $[M+H]^+$.

6.1.37. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-(piperidin-1-yl)but-2-enamide (9n)

According to general method to synthesize compounds **9a**–**z**, compound **9n** was synthesized in 50% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.35 (s, 1H), 9.87 (s, 1H), 8.44 (s, 1H), 8.21 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.81–7.86 (m, 1H), 7.53 (s, 1H), 7.39 (t, J = 4.0 Hz, 1H), 6.90–6.96 (m, 1H), 6.59 (d, J = 16.0 Hz, 1H), 3.79 (s, 2H), 2.96 (s, 4H), 1.52–1.72 (m, 6H). ESI-MS m/z 446 $[M+H]^+$.

6.1.38. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-(6-oxo-2-azabicyclo[2.2.2]octan-2-yl)but-2-enamide (9o)

According to general method to synthesize compounds **9a**–**z**, compound **9o** was synthesized in 60% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.86 (s, 1H), 9.71 (s, 1H), 8.44 (s, 1H), 8.18 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.78–7.81 (m, 1H), 7.38–7.42 (m, 1H), 6.90–6.96 (m, 1H), 6.48 (d, J = 12.0 Hz, 1H), 3.48 (s, 2H), 3.44 (d, J = 4.0 Hz, 2H), 2.65 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 2H), 2.08 (d, J = 16.0 Hz, 2H), 1.98 (t, J = 4.0 Hz, 2H), 1.49 (q, J = 8.0 Hz, 2H), 1.20 (s, 1H). ESI-MS m/z 486 $[M+H]^+$.

6.1.39. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-(4-fluoropiperidin-1-yl)but-2-enamide (9p)

According to general method to synthesize compounds **9a**–**z**, compound **9p** was synthesized in 71% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.34 (s, 1H), 10.01 (s, 1H), 8.42 (s, 1H), 8.24 (d, J = 8.0 Hz, 1H), 7.86–7.89 (m, 1H), 7.59 (s, 1H), 7.38 (t, J = 8.0 Hz, 1H), 6.85–6.88 (m, 1H), 6.55 (d, J = 16.0 Hz, 1H), 4.68 (d, J = 16.0 Hz, 1H), 2.32–2.52 (m, 6H), 1.78–1.98 (m, 4H). ESI-MS m/z 484 $[M+H]^+$.

6.1.40. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-(4,4-difluoropiperidin-1-yl)but-2-enamide (9q)

According to general method to synthesize compounds **9a**–**z**, compound **9q** was synthesized in 57% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.82 (s, 1H), 9.67 (s, 1H), 8.44 (s, 1H), 8.17 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.73–7.82 (m, 1H), 7.40 (t, J = 8.0 Hz, 1H), 7.37 (s, 1H), 6.81–6.88 (m, 1H), 6.36 (d, J = 12.0 Hz, 1H), 2.52–2.66 (m, 4H), 1.91–2.01 (m, 6H). ESI-MS m/z 482 $[M+H]^+$.

6.1.41. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-(4-(methylthio)piperidin-1-yl)but-2-enamide (9r)

According to general method to synthesize compounds **9a**–**z**, compound **9r** was synthesized in 54% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.85 (s, 1H), 9.71 (s, 1H), 8.45 (s, 1H), 8.20 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.80–7.85 (m, 1H), 7.36–7.44 (m, 2H), 6.65 (d, J = 8.0 Hz, 1H), 4.50 (d, J = 4.0 Hz, 1H), 3.43–3.48 (m, 2H), 1.99–2.03 (m, 1H), 1.20 (s, 8H). ESI-MS m/z 493 $[M+H]^+$.

6.1.42. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-(4-methylpiperazin-1-yl)but-2-enamide (9s)

According to general method to synthesize compounds **9a**–**z**, compound **9s** was synthesized in 70% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.25 (s, 1H), 10.15 (s, 1H), 8.41 (s, 1H), 8.30 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.93–7.97 (m, 1H), 7.69 (s, 1H), 7.37 (t, J = 8.0 Hz, 1H), 6.90 (d, J = 16.0 Hz, 1H), 3.37 (s, 8H), 2.80–2.98 (m, 3H), 2.66 (s, 3H). ESI-MS m/z 461 $[M+H]^+$.

6.1.43. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-(4-ethylpiperazin-1-yl)but-2-enamide (9t)

According to general method to synthesize compounds **9a**–**z**, compound **9t** was synthesized in 71% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.09 (s, 1H), 9.84 (s, 1H), 8.45 (s, 1H), 8.22 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.82–7.86 (m, 1H), 7.48 (s, 1H), 7.42 (t, J = 8.0 Hz, 1H), 6.80–6.87 (m, 1 H), 6.47 (d, J = 16.0 Hz, 1H), 3.35 (s, 10H), 3.24 (d, J = 4.0 Hz, 2H), 1.16 (t, J = 8.0 Hz, 3H). ESI-MS m/z 475 $[M+H]^+$.

6.1.44. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-(4-isopropylpiperazin-1-yl)but-2-enamide (9u)

According to general method to synthesize compounds **9a**–**z**, compound **9u** was synthesized in 68% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.13 (s, 1H), 9.86 (s, 1H), 8.45 (s, 1H), 8.21(dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.82–7.86 (m, 1H), 7.48 (s, 1H), 7.39 (t, J = 8.0 Hz, 1H), 6.78–6.83 (m, 1 H), 6.48 (d, J = 16.0 Hz, 1H), 3.35 (s, 8H), 3.24 (d, J = 4.0 Hz, 2H), 2.80–2.98 (m, 1H), 1.16 (s, 6H). ESI-MS m/z 490 $[M+H]^+$.

6.1.45. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-morpholinobut-2-enamide (9v)

According to general method to synthesize compounds **9a**–**z**, compound **9v** was synthesized in 67% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.82 (s, 1H), 9.68 (s, 1H), 8.44 (s, 1H), 8.17 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.77–7.81 (m, 1H), 7.40 (s, 1H), 6.80–6.87 (m, 1H), 6.36 (d, J = 16.0 Hz, 1H), 3.59 (t, J = 16.0 Hz, 4H), 3.15 (d, J = 8.0 Hz, 2H), 2.39 (t, J = 8.0 Hz, 4H). ESI-MS m/z 448 $[M+H]^+$.

6.1.46. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-(1,1-dioxidothiomorpholino)but-2-enamide (9w)

According to general method to synthesize compounds **9a**–**z**, compound **9w** was synthesized in 56% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.01 (s, 1H), 9.76 (s, 1H), 8.44 (s, 1H), 8.18 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.79–7.81 (m, 1H), 7.38–7.43 (m, 1H), 6.82–6.86 (m, 1H), 6.45 (d, J = 16.0 Hz, 1H), 3.22 (s, 6H), 3.05 (s, 5H). ESI-MS m/z 496 $[M+H]^+$.

6.1.47. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-(2-oxa-6-azaspiro[3.3]heptan-6-yl)but-2-enamide (9x)

According to general method to synthesize compounds **9a**–**z**, compound **9x** was synthesized in 55% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.01 (s, 1H), 9.72 (s, 1H), 8.46 (s, 1H), 8.19 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.78–7.82 (m, 1H), 7.42 (t, J = 8.0 Hz, 2H), 6.95–7.05 (m, 1H), 6.50 (d, J = 16.0 Hz, 1H), 4.50 (d, J = 4.0 Hz, 2H), 3.71 (s, 1H), 1.22–1.29 (m, 8H). ESI-MS m/z 460 $[M+H]^+$.

6.1.48. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-(7-oxa-2-azaspiro[3.5]nonan-2-yl)but-2-enamide (9y)

According to general method to synthesize compounds **9a**–**z**, compound **9y** was synthesized in 65% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.85 (s, 1H), 8.49 (s, 1H), 8.29 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.86–7.90 (m, 1H), 7.50 (s, 1H), 7.43 (t, J = 8.0 Hz, 1H), 3.98–4.01 (m, 1H), 3.48 (t, J = 8.0 Hz, 4H), 3.03 (t, J = 8.0 Hz, 4H), 2.77–2.83 (q, J = 4.0 Hz, 1H), 2.26 (d, J = 16.0 Hz, 1H), 1.63 (t, J = 8.0 Hz, 4H). ESI-MS m/z 488 $[M+H]^+$.

6.1.49. (*E*)-*N*-(4-((3-Chloro-4-fluorophenyl)amino)thieno[2,3-*d*]pyrimidin-6-yl)-4-(1,4-dioxa-8-azaspiro[4.5]decan-8-yl)but-2-enamide (9z)

According to general method to synthesize compounds **9a**–**z**, compound **9z** was synthesized in 72% yield. ^{1}H NMR (400 MHz,

DMSO-d_6) δ 11.76 (s, 1H), 9.64 (s, 1H), 8.45 (s, 1H), 8.19 (dd, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 1H), 7.77–7.81 (m, 1H), 7.41 (t, J = 8.0 Hz, 1H), 7.35 (s, 1H), 6.83–7.00 (m, 1H), 6.34 (d, J = 16.0 Hz, 1H), 3.86 (s, 4H), 3.18 (d, J = 4.0 Hz, 2H), 1.64 (t, J = 8.0 Hz, 4H), 1.19–1.29 (m, 4H). ESI-MS m/z 504 $[M+H]^+$.

6.2. Biological evaluations

6.2.1. ELISA kinase assay

The kinases domain of EGFR (WT), EGFR (T790M/L858R), ErbB2, IGF1R, FGFR1, KDR was expressed using the Bac-to-Bac™ baculovirus expression system (Invitrogen, Carlsbad, CA, USA) and purified on Ni-NTA columns (QIAGEN Inc., Valencia, CA, USA). Recombinant ErbB4, RET, RON, ABL, flt-1, c-kit, EPH-A2, PDGFRα and PDGFRβ proteins were obtained from Upstate Biotechnology. The screening of tyrosine kinase inhibitors was based on enzyme-linked-immunosorbent assay (ELISA). Briefly, 20 μg/mL Poly (Glu, Tyr)$_{4:1}$ (Sigma, St. Louis, MO) was precoated in 96-well ELISA plates as substrate. Each well was treated with 50 μL of 10 μmol/L ATP solution which was diluted in kinase reaction buffer (50 mM HEPES pH 7.4, 20 mM $MgCl_2$, 0.1 mM $MnCl_2$, 0.2 mM Na_3VO_4, 1 mM DTT). Then 1 μL of various concentrations of test compounds or reference compound dissolved in DMSO were added to each reaction well. Experiments at each concentration were performed in duplicate. The reaction was initiated by tyrosine kinase diluted in kinase reaction buffer. After incubation at 37 °C for 60 min, the wells were washed three times with phosphate buffered saline (PBS) containing 0.1% Tween 20 (T-PBS). Next, 100 μL anti-phosphotyrosine (PY99) antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in T-PBS containing 5 mg/mL BSA was added and the plate was incubated at 37 °C for 30 min. After the plate was washed three times, 100 μL horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000, Calbiochem, SanDiego, CA) diluted in T-PBS containing 5 mg/mL BSA was added and the plate was incubated at 37 °C for 30 min. The plate was washed, then 100 μL citrate buffer (0.1 M, pH 5.5) containing 0.03% H_2O_2 and 2 mg/mL *o*-phenylenediamine was added and samples were incubated at room temperature until color emerged. The reaction was terminated by adding 50 μL of 2 M H_2SO_4, and the plate was read using a multiwell spectrophotometer (VERSAmax™, Molecular Devices, Sunnyvale, CA, USA) at 492 nm. The inhibitory rate (%) was calculated with the formula: $[1-(A_{492}$ treated/A_{492} control$)] \times 100\%$. IC_{50} values were calculated from the inhibitory curves.

6.2.2. Cell proliferation assay

Cell proliferation was evaluated using the SRB (Sulforhodamine B) assay as previously described. Briefly, cells were seeded into 96-well plates and grown for 24 h. The cells were then treated with various concentrations of test compounds and grown for a further 72 h. The medium remained unchanged until the completion of the experiment. The cells were then fixed with 10% precooled trichloroacetic acid (TCA) for 1 h at 4 °C and stained for 15 min at room temperature with 100 μL of 4 mg/mL SRB solution (Sigma) in 1% acetic acid. The SRB was then removed, and the cells were quickly rinsed five times with 1% acetic acid. After cells were air-dried, protein-bound dye was dissolved in 150 μL of 10 mmol/L Tris base for 5 min and measured at 515 nm using a multiwell spectrophotometer (VERSAmax, Molecular Devices). The inhibition rate on cell proliferation was calculated as $[1-(A_{515}$ treated/A_{515} control$)] \times 100\%$. The IC_{50} value was obtained by the Logit method.

6.2.3. Western blot analysis

Cells were grown to half confluence in six-well plates, starved in serum-free medium for 24 h, and then exposed to indicated concentrations of compounds for 2 h. Cells were stimulated with 50 ng/ml EGF(R&D Systems, Minneapolis, MN, USA) for 10 min and lysed in 1× SDS sample buffer. Western blot analyses were subsequently performed with standard procedures. Antibodies against the following were used: p-EGFR (Tyr1068), EGFR, phospho-ERK, ERK, p-AKT (Ser473), AKT were obtained from (Cell Signaling Technologies, Cambridge, MA), and GAPDH was from (Santa Cruz Biotechnology, Santa Cruz, CA).

6.2.4. Irreversible assay

Rapid dilution experiment was used to demonstrate reversible binding of **8l** to EGFR. EGFR kinase (800 nM) was pre-incubated with excessive **8l** at a final concentration of 100-fold IC_{50} under room temperature for 30 min, then the mix solution were diluted with reaction solution containing substrate peptide (5-FAM-EEPLYWSFPAKKK-CONH2) and ATP (2.3 μM) to a hundred times. Then the enzyme activity of this mixture was assayed by the EZ Reader (Caliper Life Sciences, MA). Wells were repeatedly read for 2 h.

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