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DEVELOPMENT OF 5-TRIFLUOROMETHYLPYRIMIDINE DERIVATIVES AS DUAL INHIBITORS OF EGFR AND SRC FOR CANCER THERAPY

Qin Wang,[1] Nian Rao,[1] Li Liu,[1,2]* Yi Le,[1,2] and Longjia Yan[1,2]*

[1] School of Pharmaceutical Sciences, Guizhou University, Guiyang 550025, China; [2] Guizhou Engineering Laboratory for Synthetic Drugs, Guiyang 550025, China; E-mail: lliu2@gzu.edu.cn (Li Liu); ylj1089@163.com (Longjia Yan).

Abstract – In this paper, we reported a new series of 5-trifluoromethylpyrimidine derivatives (**4a-4f**, **6a-6j**) for cancer therapy. They were tested for antitumor activity *in vitro* on four human cancer cell lines including A549, K562, HepG2, MCF-7 and two kinase including wild type epidermal growth factor receptor tyrosine kinase ($EGFR^{wt}$-TK) and c-Src. The results suggested that some of the compounds (**4a**, **4b**, **4c**, **4e**, **6b**, **6d**, **6e**, **6f**, **6g**, **6h**) performed well activities. Especially 2-((2-((4-((2-(cyclohexylamino)-3,4-dioxocyclobut-1-en-1-yl)amino-phenyl)amino)-5-(trifluoromethyl)pyrimidin-4-yl)amino)-*N*-methylbenzamide (**6g**) showed high antitumor activities against four cancer cell lines with 1.08 μM, 2.06 μM, 1.24 μM and 2.57 μM, respectively. Furthermore, compound **6g** inhibited $EGFR^{wt}$ and Src at the values of 0.75 μM and 0.15 μM.

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase (RTK), which plays an important role in regulating cell growth, proliferation and differentiation and other physiological activities of cancer cells. And also it is an important target for new anti-cancer drug research.[1-4] As shown in **Figure 1**, lots of EGFR inhibitors such as Gefitinib, Erlotinib, and Lapatinib have been approved in market, which significantly improve the clinical treatment of cancer patients.[5] However, the resistance problems of EGFR inhibitors in clinic have been made it urgent to find novel generation of EGFR kinase inhibitors.[6,7]

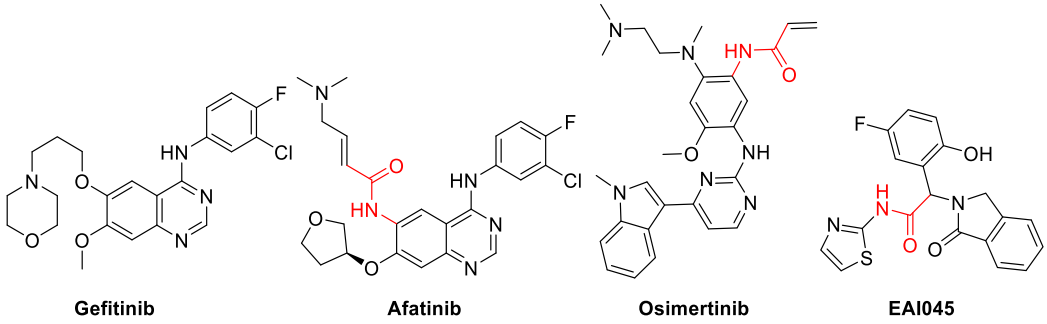


Figure 1. EGFR inhibitors in market

Src is a kind of non-receptor tyrosine protein kinases (NTKs), which play a crucial role in maintaining the normal physiological functions of the body.[8] Src kinase is precisely regulated in normal cells and tissues, but it is highly expressed in a variety of tumor cells. Src kinase also plays a key role in many tumor cell regulation processes, including affecting cell adhesion, invasion, proliferation and angiogenesis.[9-11] Therefore, the study of Src kinase as an anti-tumor drug target has received extensive attention.[12-14] In recent years, many Src kinase inhibitors such as Dasatinib, Bosutinib, Vandetanib, and Ponatinib (**Figure 2**) have been approved for marketing. And also a variety of Src inhibitors are still in clinical research.[15,16]

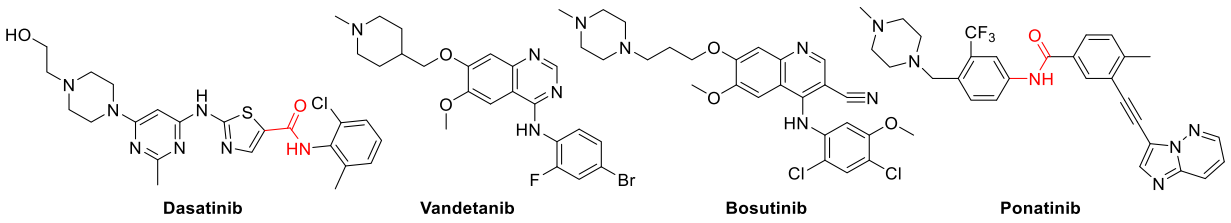


Figure 2. Src inhibitors in market

Multi-target drugs can regulate multiple functions in the disease network system, and produce a synergistic effect on the effects of each target.[17] In addition, these drugs can achieve better therapeutic effects, and reduce the risk of drug resistance, toxic and side effects. So multi-target new drug discovery has become a hotspot.[18,19] In 2017, Jiang and co-authors reported EGFR/Src dual-target tyrosine kinase inhibitors.[20] In 2019, Abbas and co-authors reported 1,3-oxadiazole and 1,4-oxadiazole derivatives as EGFR and Src multi-target kinase inhibitors.[21] Besides, the multi-target inhibitors of EGFR or Src are still underway in lots of labrotories. Researchers are trying to solve the current problem of targeted drug resistance with the strategy of multi-target.[22-24]

Our group has been focused on the development of kinase inhibitor for several years.[25,26] As shown in **Figure 3**, we previously found compound **A** was a novel Src inhibitor with IC_{50} value of 0.21 μM. Based

on the priority of EGFR/Src protein interaction, we are interested in design and synthesis new EGFR/Src inhibitors with the lead of compound **A**. In this paper, we will introduce our progress in dual inhibitors of EGFR/Src.

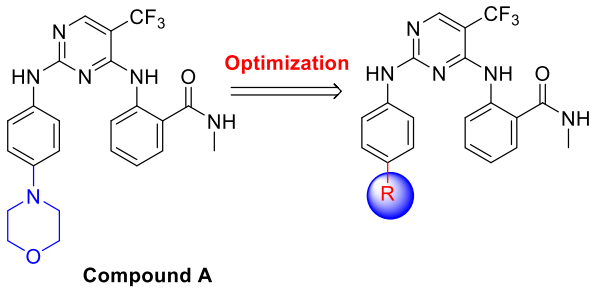
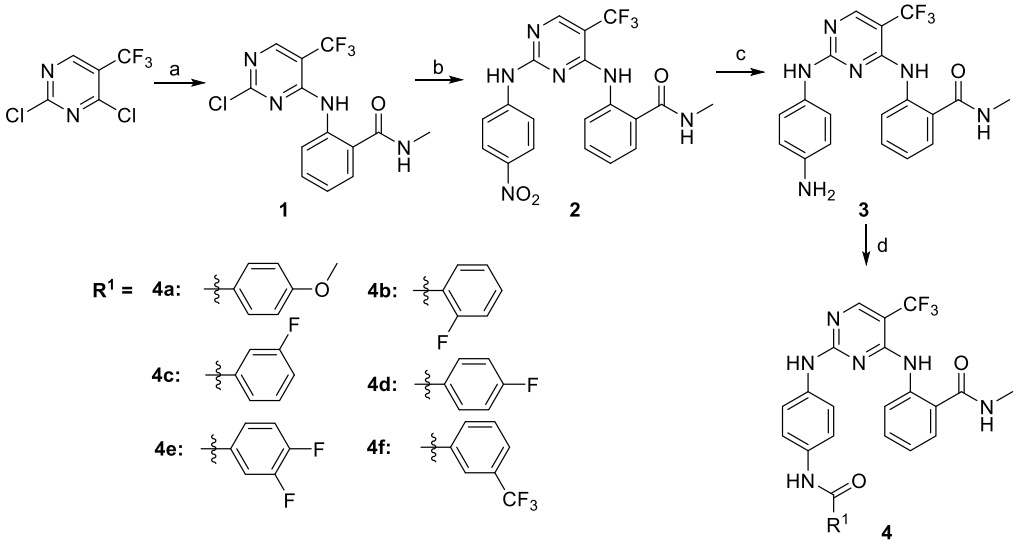


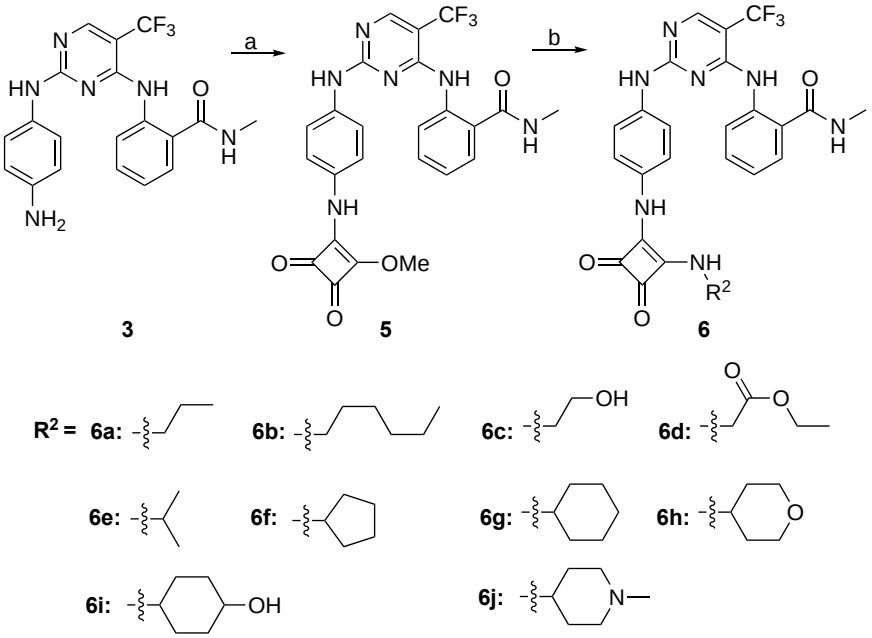
Figure 3. Design strategy of EGFR/Src inhibitors

The synthetic method of target compounds **4a-4f** was shown in **Scheme 1**. The nucleophilic substitution reaction of 2,4-dichloro-5-trifluoromethylpyrimidine with 2-aminobenzamide was carried out, and compound **1** was obtained in 55% yield, which was consistent with NMR spectrum as the previous report in literature.[26] And then, compound **1** reacted with *p*-nitroaniline under the condition of trifluoroacetic acid in the solvent of trifluoroethanol to obtain compound **2** with 49% yield. Subsequently, the nitro group of **2** was reduced to amino group under the condition of Pd/C. Compound **3** was obtained with 76% yield. At last, compound **3** reacted with different substituted carboxylic acids to obtain the compounds **4a-4f** in 67% - 81% yield.



Scheme 1. Synthetic route of target compounds **4a-4f**. Reagents and conditions: a) 2-amino-*N*-methylbenzamide, $NaHCO_3$, EtOH, rt, overnight; b) 4-nitroaniline, TFA, TFE, reflux, overnight; c) Pd/C, MeOH, H_2, rt, 24 h; d) corresponding acid, HATU, DIEA, DMF, rt, 12 h.

Cyclobutene diketone has been proved as a kind of important drug fragments. Many small molecules in market have existed cyclobutene diketone structure.[27,28] In consideration of the widely biological activity of cyclobutene diketone, we introduced it into our molecular of the target compounds. As shown in **Scheme 2**, compound **3** in **Scheme 1** as the starting material successfully reacted with 3,4-dimethoxy-3-cyclobutene-1,2-dione to obtain intermediate **5** in 62% yield. Fortunately, compound **5** reacted with different substituted amines to give the compounds **6a-6j** in 42% - 49% yields.



Scheme 2. Synthetic route of target compounds **6a-6j**. Reagents and conditions: a) dimethyl squarate, DIEA, DMF, rt, 12 h; b) corresponding amine, DIEA, DMF, 80 ℃, 12 h.

Four tumor cell lines (A549, K562, HepG2 and MCF-7) were used to evaluate the antiproliferative activities of the final targets under the method of methyl thiazolyl tetrazolium colorimetric assay (MTT). Gefitinib (EGFR inhibitor) and Dasatinib (Src inhibitor) were performed as positive control. The IC_{50} values of all compounds were listed in **Table 1**. The results indicated that some compounds exhibited well activities for all cancer cell lines. Against A549 cells, most compounds were more potent than Getitinib (IC_{50} = 8.48 μM), while compounds **4d**, **6c** and **6i** were less than Gefitinib. Against K562 cells, most compounds were more potent than Gefitinib (IC_{50} = 9.73 μM), while Dasatinib (IC_{50} = 0.012 μM) was less than all compounds. Against HepG2 cells, most compounds were more potent than Gefitinib (IC_{50} = 15.86 μM) and Dasatinib (IC_{50} = 10.83 μM). Especially, the IC_{50} value of compound **4c** reached to 0.06 μM for HepG2 cell. Against MCF-7 cells, compounds **4a**, **4b**, **4c**, **6d**, **6e**, **6g**, **6h**, and **6j** were more

active than Gefitinib (IC_{50} = 7.12 μM) and Dasatinib (IC_{50} = 10.08 μM). In general, the antiproliferative activities of compounds **4a**, **4b**, **4c**, **4e**, **6b**, **6d**, **6e**, **6f**, **6g**, and **6h** against all the tumor cells were higher than the other compounds.

Table 1. IC_{50} values for cancer cell lines[a]

Comp.	IC_{50} (μM) [a]			
	A549	K562	HepG2	MCF-7
4a	2.07 ± 0.45	1.67 ± 0.081	1.01 ± 0.049	1.12 ± 0.58
4b	3.13 ± 0.89	5.80 ± 0.34	1.12 ± 0.084	2.56 ± 0.91
4c	1.05 ± 0.42	5.48 ± 0.16	0.06 ± 0.012	0.75 ± 0.34
4d	>20	>20	>20	>20
4e	4.15 ± 0.47	2.44 ± 0.093	3.26 ± 0.15	7.44 ± 2.45
4f	5.58 ± 0.67	3.62 ± 0.094	5.53 ± 0.38	8.19 ± 1.27
5	4.81 ± 0.77	10.78 ± 0.98	6.10 ± 0.88	8.28 ± 0.92
6a	4.33 ± 0.48	10.55 ± 0.88	6.89 ± 0.46	10.07 ± 2.19
6b	4.45 ± 0.61	5.40 ± 0.44	6.98 ± 0.26	9.18 ± 2.56
6c	11.54 ± 1.87	>20	0.66 ± 0.096	8.18 ± 0.75
6d	5.71 ± 0.79	7.35 ± 0.45	6.15 ± 0.52	6.92 ± 0.41
6e	5.54 ± 0.71	7.63 ± 0.49	6.00 ± 0.66	6.40 ± 0.75
6f	6.28 ± 0.58	7.36 ± 0.67	7.71 ± 0.56	7.89 ± 0.38
6g	1.08 ± 0.15	2.06 ± 0.18	1.24 ± 0.091	2.57 ± 0.33
6h	3.15 ± 0.69	7.31 ± 0.73	4.32 ± 0.31	5.89 ± 0.08
6i	>20	>20	>20	>20
6j	4.78 ± 0.34	>20	2.18 ± 0.11	6.05 ± 0.29
Gefitinib	8.48 ± 0.55	9.73 ± 0.82	15.86 ± 0.86	7.12 ± 1.02
Dasatinib	14.38 ± 0.52	0.012 ± 0.005	10.83 ± 0.49	10.08 ± 1.33

[a] The values are mean ± SD of three replicates

Due to the suitable anti-tumor activity of the designed compounds (**4a**, **4b**, **4c**, **4e**, **6b**, **6d**, **6e**, **6f**, **6g**, and **6h**), $EGFR^{wt}$ and Src kinase inhibition capability was also evaluated for further studies. As shown in

Table 2, Gefitinib was 6.1 nM and Dasatinib was 1 nM, which were consisted with the former report.[29] However, for our tested compounds, only compound **6g** was lower than 1 μM against the two kinases, which were 0.75 μM and 0.15 μM respectively. These results suggested that compound **6g** was good candidate for further studies.

Table 2. IC_{50} values for $EGFR^{wt}$ and Src[a]

Entry	Comp.	IC_{50} (μM)	
		$EGFR^{wt}$-TK	c-Src
1	**4a**	1.02 ± 0.13	0.48 ± 0.054
2	**4b**	1.78 ± 0.35	2.51 ± 0.29
3	**4c**	4.82 ± 0.32	0.31 ± 0.047
5	**4e**	2.02 ± 0.17	0.52 ± 0.18
6	**6b**	4.79 ± 0.53	2.61 ± 0.44
7	**6d**	3.88 ± 0.42	2.79 ± 0.53
8	**6e**	3.99 ± 0.28	3.01 ± 0.11
9	**6f**	4.15 ± 0.32	3.17 ± 0.14
10	**6g**	0.75 ± 0.19	0.15 ± 0.049
11	**6h**	1.87 ± 0.29	2.64 ± 0.087
12	Gefitinib	0.0061 ± 0.0003	-
13	Dasatinib	-	0.001 ± 0.0001

[a] The values are mean ± SD of three replicates

To explain the activities of EGFR and Src with compound **6g**, the possible binding modes were performed in molecular docking studies. Therefore, X-ray crystal structures of $EGFR^{wt}$ (PDB entry 1M17) and Src (PDB entry 3G6H) were used for identifying candidate binding modes. For EGFR kinase, as shown in **Figure 4(a)**, compound **6g** formed hydrogen bonds with multiple amino acids, including Gln767 (hydrogen bond length 2.2 Å), Thr766 (hydrogen bond length 1.6 Å), and Asp831 (hydrogen bond length 1.8 Å). In addition, compound **6g** interacted with Lys721 to form p-π cation interaction with pyrimidine ring. For Src kinase, as shown in **Figure 4(b)**, compound **6g** mainly formed two hydrogen bonds with Met341 (hydrogen bond length 2.2 Å) and Asp348 (hydrogen bond length 2.1 Å). Besides,

compound **6g** also weakly interacted with Ile338, Ala293, Lys295, Val281, Ala403, Leu393, Phe405, Leu273, Gly274, Gly344, Ser345 and Asp348. These results indicate that compound **6g** was likely to be potential EGFR and Src inhibitor.

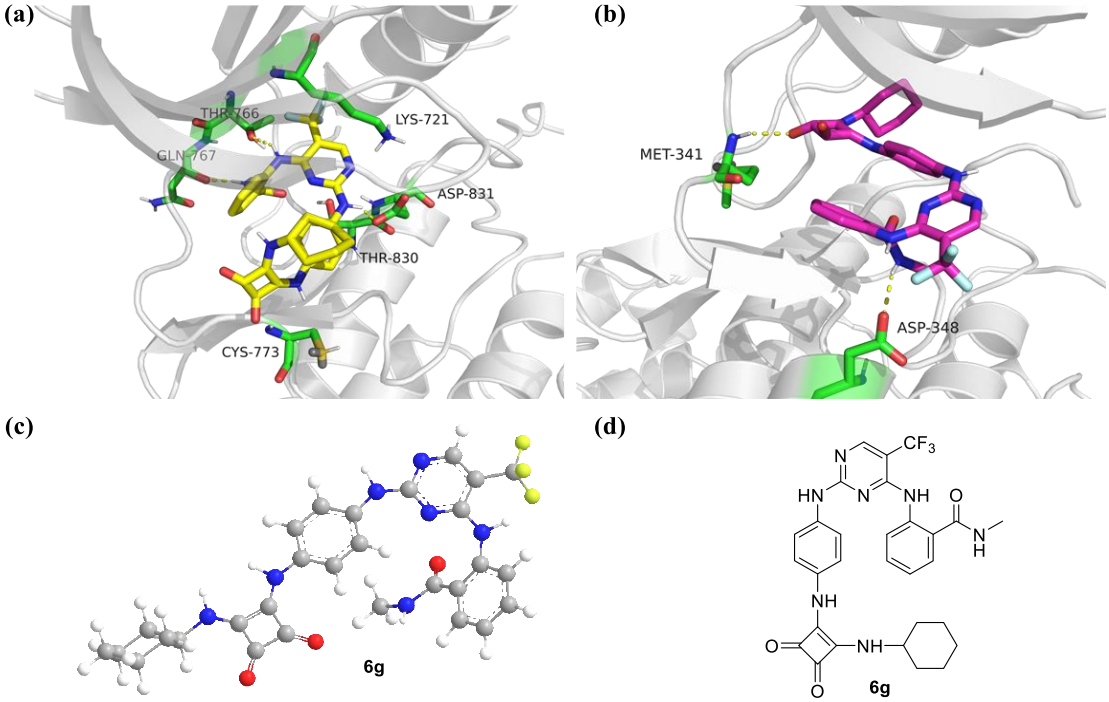


Figure 4. Docking structures of compound **6g** (a) Binding configurations of compound **6g** with EGFR (PDB: 1M17); (b) Binding configurations of compound **6g** with Src (PDB: 3G6H); (c) The 3D model of compound **6g**; (d) The 2D model of compound **6g**.

CONCLUSION

Generally, sixteen novel 5-trifluoromethylpyrimidine derivatives were designed and synthesized for development of dual inhibitors of EGFR and Src. After *in vitro* evaluation in four human cancer cell lines including A549, K562, HepG2, and MCF-7, the result was shown that compounds **4a**, **4b**, **4c**, **4e**, **6b**, **6d**, **6e**, **6f**, **6g**, and **6h** have good antiproliferative activity. Next, they were selected for studying the inhibition against EGFR and Src. Interestingly, compound **6g** was the strongest activities with IC_{50} values of 0.75 μM and 0.15 μM for EGFR and Src, respectively. Further studies on compounds **6g** are still going on in our lab.

EXPERIMENTAL SECTION

All commercial materials were used without further purification. Melting points were determined with X-4X digital display micro melting point analyzer (uncorrected, Shanghai Microelectronics Technology

Co., Ltd.). Analytical thin-layer chromatography was performed on precoated 250 μm layer thickness silica gel 60 F254 plates and visualized with UV light. Column chromatography was performed silica gel 300-400 mesh. ^{1}H NMR and ^{13}C NMR spectroscopic data were recorded with Bruker 400 MHz NMR spectrometer and JEOL-ECX 500 NMR spectrometer in DMSO-d_6 solution, with TMS serving as the internal standard. The high-resolution mass spectrometer (HRMS) was tested in TSQ 8000 and AB SCIEX X500R QTOF. FT-IR was recorded in a Shimadzu FT-IR-8400S spectrometer as KBr pellets.

2-(2-Chloro-5-trifluoromethylpyrimidin-4-ylamino)-*N*-methylbenzamide (1)

2,4-Dichloro-5-trifluoromethylpyrimidine (8 mmol) was added to a stirred solution of 2-amino-*N*-methylbenzamide (8.8 mmol) and $NaHCO_3$ (8.8 mmol) in anhydrous EtOH (10 mL) at room temperature. The resulted mixture was heated to reflux and stirred overnight before cooled to room temperature. The precipitate was filtered out, washed with EtOH to give the title compound as yellow solid (1.452 g; 55% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.06 (s, 1H), 8.86 (q, J = 4.4 Hz, 1H), 8.68 (s, 1H), 8.38 (d, J = 8.4 Hz, 1H), 7.78 (dd, J = 8.0, 1.2 Hz, 1H), 7.59 (td, J = 8.0, 1.2 Hz, 1H), 7.26 (td, J = 8.0, 1.2 Hz, 1H), 2.34 (d, J = 4.4 Hz, 3H). Spectral properties were in accordance with the literature.[26]

***N*-Methyl-2-((2-((4-nitrophenyl)amino)-5-(trifluoromethyl)pyrimidin-4-yl)amino)benzamide (2)**

To a solution of compound **1** (5 mmol) in TFE (2,2,2-trifluoroethanol, 20 mL) were added 4-nitroaniline (6 mmol) and TFA (trifluoroacetic acid, 15 mmol). The resulted mixture was heated to reflux under nitrogen atmosphere and stirred overnight before cooled to room temperature. The mixture was added EtOAc (100 mL) and washed with saturated aq. $NaHCO_3$ (50 mL). The organic layer was dried over magnesium sulfate, filtered, and concentrated in vacuo to afford the crude compound. The residue was purified by silica-gel column using DCM/MeOH = 30/1 to give the product. 1.058 g yellow solid; 49% yield; mp > 250 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.06 (s, 1H), 8.86 (q, J = 4.4 Hz, 1H), 8.68 (s, 1H), 8.38 (d, J = 8.4 Hz, 1H), 7.78 (dd, J = 8.0, 1.2 Hz, 1H), 7.59 (m, 1H), 7.26 (m, 1H), 2.34 (d, J = 4.4 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.1, 160.6, 156.8, 156.4, 146.7, 141.5, 138.7, 131.7, 128.5, 126.0, 125.1, 123.9, 123.8, 123.4, 123.3, 119.3 (q, J = 5.2 Hz), 26.7; ESI-HRMS $C_{19}H_{15}F_3N_6O_3$ ([M+Na]$^+$): calcd 455.1056, found 455.1037.

2-((2-((4-Aminophenyl)amino)-5-(trifluoromethyl)pyrimidin-4-yl)amino)-*N*-methylbenzamide (3)

To a solution of compound **2** (864 mg, 2 mmol) in MeOH (10 mL) was added Pd/C (86 mg). The mixture was stirred at room temperature under hydrogen atmosphere for 24 h. The solution was filtered with celite and the filtration was evaporated under vacuum. The crude solid was recrystallized with MeOH to afford

compound **3**. 0.611 g yellow solid; 76% yield; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.30 (s, 1H), 9.42 (s, 1H), 8.72 (d, *J* = 4.4 Hz, 1H), 8.36 (m, 2H), 7.70 (d, *J* = 7.6 Hz, 1H), 7.47 – 7.07 (m, 4H), 6.51 (d, *J* = 8.8 Hz, 2H), 4.89 (s, 2H), 2.78 (d, *J* = 4.4 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.3, 161.9, 160.2, 156.3, 145.5, 139.3, 132.0, 128.23, 126.6, 125.7, 123.7, 122.6, 118.8 (q, *J* = 5.2 Hz), 116.1, 114.7, 114.2, 26.7; ESI-HRMS $C_{19}H_{17}F_3N_6O$ ([M+H]$^+$): calcd 403.1488, found 403.1478 .

A mixture of compound **3** (201 mg, 0.5 mmol), DMF (4 mL) and DIEA (129 mg, 1 mmol) was stirred at room temperature. And then the corresponding acid (0.5 mmol) and HATU (380 mg, 1 mmol) were added into the solution. The mixture was stirred at room temperature for 24 h. The solution was extracted with EtOAc (50 mL x 3) and the combined organic phase was washed with saturated brine (20 mL x 3). The organic layer was dried over magnesium sulfate, filtered, and concentrated in vacuo to afford the crude compound. The residue was purified by silica-gel column using DCM/MeOH = 30/1 to give the product **4a-4i**.

2-((2-((4-(4-Methoxybenzamido)phenyl)amino)-5-(trifluoromethyl)pyrimidin-4-yl)amino)-*N*-methylbenzamide (4a)

White solid, 75% yield; mp: 240.9-241.6 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.33 (s, 1H), 10.04 (s, 1H), 9.83 (s, 1H), 8.75 (m, 1H), 8.44 (s, 1H), 7.99 – 7.95 (m, 2H), 7.68 (m, 6H), 7.49 (t, *J* = 7.6 Hz, 1H), 7.19 (t, *J* = 7.6 Hz, 1H), 7.09 – 7.05 (m, 2H), 3.85 (s, 3H), 2.79 (d, *J* = 4.4 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 155.5, 152.1, 150.3, 149.9, 149.0, 145.4, 145.3, 145.2, 131.3, 128.4, 127.9, 125.23, 124.0, 122.7, 122.0, 121.0, 119.3 (q, *J* = 5.2 Hz), 118.5, 116.9, 116.9, 111.3, 64.7, 41.4; ESI-HRMS $C_{27}H_{23}F_3N_6O_3$ ([M+H]$^+$): calcd 537.1856, found 537.1856.

2-Fluoro-*N*-(4-((4-((2-(methylcarbamoyl)phenyl)amino)-5-(trifluoromethyl)pyrimidin-2-yl)amino)-phenyl)benzamide (4b)

White solid, 78% yield; mp: 239.5-240.6 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.32 (s, 1H), 10.34 (s, 1H), 9.85 (s, 1H), 8.77 – 8.73 (m, 1H), 8.44 (s, 1H), 7.73 (dd, *J* = 7.6, 1.2 Hz, 1H), 7.67 (td, *J* = 7.6, 1.2 Hz, 2H), 7.62 (s, 5H), 7.48 (t, *J* = 7.6 Hz, 1H), 7.35 (m, 2H), 7.18 (t, *J* = 7.6 Hz, 1H), 2.79 (d, *J* = 4.4 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 155.5, 150.4, 149.0, 148.3, 146.7, 145.2 , 128.7 (d, *J* = 6.8 Hz), 127.6 (d, *J* = 4.4 Hz), 126.4 (d, *J* = 6.4 Hz), 126.3 (d, *J* = 6.4 Hz), 125.3 (d, *J* = 6.4 Hz), 124.3 (d, *J* = 1.2 Hz), 123.3, 122.7, 120.5, 120.4, 120.1, 119.1 (q, *J* = 5.2 Hz), 118.5, 117.1, 116.4, 113.4, 113.26, 41.4; ESI-HRMS $C_{26}H_{20}F_4N_6O_2$ ([M+H]$^+$): calcd 525.1656, found 525.1657.

2-((2-((4-(3-Fluorobenzamido)phenyl)amino)-5-(trifluoromethyl)pyrimidin-4-yl)amino)-*N*-methyl-benzamide (4c)

White solid, 81% yield; mp >250 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.33 (s, 1H), 10.26 (s, 1H), 9.86 (s, 1H), 8.75 (dd, *J* = 8.4, 4.0 Hz, 1H), 8.45 (s, 1H), 7.86 – 7.76 (m, 3H), 7.73 (dd, *J* = 7.6, 1.2 Hz, 1H), 7.65 (m, 5H), 7.47 (m, 2H), 7.19 (t, *J* = 7.6 Hz, 1H), 2.79 (d, *J* = 4.4 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.3, 169.3, 164.3, 164.3, 163.6 (d, *J* = 3.6 Hz), 161.2, 156.4, 137.8, 137.7, 135.9 (d, *J* = 3.2 Hz), 134.3 (d, *J* = 3.6 Hz), 131.1, 131.0, 128.4, 124.5, 124.3 (d, *J* = 2.4 Hz), 123.1, 121.2, 118.9 (q, *J* = 5.2 Hz), 118.7 (d, *J* = 3.2 Hz), 114.9, 114.8 (d, *J* = 5.2 Hz), 106.1, 26.7; ESI-HRMS $C_{26}H_{20}F_4N_6O_2$ ([M+H]$^+$): calcd 525.1656, found 525.1656.

2-((2-((4-(4-Fluorobenzamido)phenyl)amino)-5-(trifluoromethyl)pyrimidin-4-yl)amino)-*N*-methyl-benzamide (4d)

White solid, 67% yield; mp >250 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.34 (s, 1H), 10.21 (s, 1H), 9.85 (s, 1H), 8.75 (d, *J* = 4.4 Hz, 1H), 8.44 (s, 1H), 8.08 – 8.02 (m, 2H), 7.73 (dd, *J* = 7.6, 1.2 Hz, 1H), 7.64 (q, *J* = 8.8 Hz, 5H), 7.49 (t, *J* = 7.6 Hz, 1H), 7.40 – 7.34 (m, 2H), 7.19 (t, *J* = 8.0 Hz, 1H), 2.79 (d, *J* = 4.4 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.3, 165.7 (d, *J* = 3.6 Hz), 164.6, 163.3, 161.2, 156.6, 146.4, 143.9 , 135.7, 134.5, 131.9, 131.9, 130.8 (d, *J* = 3.2 Hz), 130.7, 128.4 (d, *J* = 2.4 Hz), 123.1, 121.2, 121.1 (q, *J* = 5.2 Hz), 115.9, 115.7 (d, *J* = 2.4 Hz), 105.2, 26.7; ESI-HRMS $C_{26}H_{20}F_4N_6O_2$ ([M+H]$^+$): calcd 525.1656, found 525.1656.

3,4-Difluoro-*N*-(4-((4-((2-(methylcarbamoyl)phenyl)amino)-5-(trifluoromethyl)pyrimidin-2-yl)-amino)phenyl)benzamide (4e)

White solid, 72% yield; mp: 244.8-245.6 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.34 (s, 1H), 10.26 (s, 1H), 9.86 (s, 1H), 8.75 (d, *J* = 4.4 Hz, 1H), 8.45 (s, 1H), 8.08 – 7.86 (m, 3H), 7.76 – 7.63 (m, 6H), 7.49 (t, *J* = 7.6 Hz, 1H), 7.19 (t, *J* = 7.6 Hz, 1H), 2.79 (d, *J* = 4.4 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 155.5, 150.7 (t, *J* = 6.4 Hz), 149.0 (t, *J* = 7.6 Hz), 145.4, 145.2, 142.4, 140.8, 140.5, 138.9, 131.3 (t, *J* = 5.2 Hz), 128.8, 127.4, 126.3 (t, *J* = 3.2 Hz), 125.3 (t, *J* = 5.2 Hz), 122.7, 120.6, 119.3 (q, *J* = 5.2 Hz), 118.5, 117.0, 114.6, 114.5, 114.1 (t, *J* = 6.4 Hz), 114.0, 41.4; ESI-HRMS $C_{26}H_{19}F_5N_6O_2$ ([M+H]$^+$): calcd 543.1562, found 543.1562.

***N*-Methyl-2-((5-(trifluoromethyl)-2-((4-(3-(trifluoromethyl)benzamido)phenyl)amino)pyrimidin-4-yl)amino)benzamide (4f)**

White solid, 79% yield; mp >250 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.33 (s, 1H), 10.43 (s, 1H), 9.88 (s, 1H), 8.75 (q, *J* = 4.0 Hz, 1H), 8.45 (s, 1H), 8.33 – 8.27 (m , 2H), 7.97 (d, *J* = 7.6 Hz, 1H), 7.83 – 7.64 (m, 7H), 7.50 (t, *J* = 7.6 Hz, 1H), 7.19 (t, *J* = 8.0 Hz, 1H), 2.79 (d, *J* = 4.4 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 155.5, 151.4, 149.0, 145.4, 145.2, 129.1, 128.8 (q, *J* = 5.2 Hz), 127.4, 125.8, 124.2, 123.9, 123.7, 122.9, 122.8, 122.7, 120.9, 120.5, 119.9, 119.8, 119.3 (q, *J* = 5.2 Hz), 118.8, 118.5, 117.1, 116.9, 41.4; ESI-HRMS $C_{27}H_{20}F_6N_6O_2$ ([M+H]$^+$): calcd 575.1624, found 575.1624.

2-((2-((4-((2-Methoxy-3,4-dioxocyclobut-1-en-1-yl)amino)phenyl)amino)-5-(trifluoromethyl)-pyrimidin-4-yl)amino)-*N*-methylbenzamide (5)

To a solution of compound **3** (804 mg, 2 mmol) in DMF (10 mL) were added dimethyl squarate (284 mg, 2 mmol) and DIEA (258 mg, 2 mmol). The mixture was stirred at room temperature for 12 h. The mixture was extracted with EtOAc (100 mL x 3) and the combined organic phase was washed with saturated brine (50 mL x 3). The organic layer was dried over magnesium sulfate, filtered, and concentrated in vacuo to afford the crude compound. The residue was purified by silica-gel column using DCM/MeOH = 30/1 to give the product. White solid 635 mg; 62% yield; mp: 234.8-235.6 °C; IR (KBr pellet): v 3229.42, 1804.30, 1703.14, 1619.83, 1539.50, 1515.70, 1018.84, 828.43, 760.00; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.32 (s, 1H), 10.72 (s, 1H), 9.87 (s, 1H), 8.75 (s, 1H), 8.44 (s, 1H), 7.68 (m, 3H), 7.50 (t, *J* = 7.6 Hz, 1H), 7.31 - 7.16 (m, 4H), 4.39 (s, 3H), 2.79 (d, *J* = 4.4 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 186.6, 170.2, 166.8, 165.8, 155.4, 150.3, 148.9, 145.4, 145.2, 140.5, 131.2, 129.0, 125.3, 122.7, 121.0, 119.2 (q, *J* = 5.2 Hz), 118.5, 117.2, 116.4, 68.8, 41.4. ESI-HRMS $C_{24}H_{19}F_3N_6O_4$ ([M+H]$^+$): calcd 513.1492, found 513.1492.

To a solution of compound **5** (528 mg, 1 mmol) in DMF (10 mL) were added the corresponding aniline (1.2 mmol) and DIEA (129 mg, 1 mmol). The mixture was stirred at room temperature for 8 h. The mixture was extracted with EtOAc (50 mL x 3) and the combined organic phase was washed with saturated brine (20 mL x 3). The organic layer was dried over magnesium sulfate, filtered, and concentrated in vacuo to afford the crude compound. The residue was purified by silica-gel column using DCM/MeOH = 30/1 to give the product **6a-6j**.

2-((2-((4-((3,4-Dioxo-2-(propylamino)cyclobut-1-en-1-yl)amino)phenyl)amino)-5-(trifluoromethyl)-pyrimidin-4-yl)amino)-*N*-methylbenzamide (6a)

White solid, 45% yield; mp >250 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.31 (s, 1H), 9.83 (s, 1H), 9.63 (s, 1H), 8.74 (d, *J* = 4.4 Hz, 1H), 8.43 (s, 1H), 7.72 (d, *J* = 7.6 Hz, 1H), 7.58 (m, 5H), 7.35 (d, *J* = 8.4 Hz,

2H), 7.17 (t, *J* = 7.6 Hz, 1H), 3.57 (m, 2H), 2.79 (d, *J* = 4.4 Hz, 3H), 1.60 (m, 2H), 0.93 (t, *J* = 7.6 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 187.5, 171.3, 164.6, 155.7, 155.4, 154.1, 152.3, 151.1, 148.9, 145.1, 134.6, 131.3, 127.7, 125.4, 122.7, 121.0 (q, *J* = 5.2 Hz), 118.5, 117.6, 114.9, 56.7, 41.4, 39.6, 29.1; ESI-HRMS $C_{26}H_{24}F_3N_7O_3$ ([M+H]$^+$): calcd 540.1965, found 540.1964.

2-((2-((4-((2-(Hexylamino)-3,4-dioxocyclobut-1-en-1-yl)amino)phenyl)amino)-5-(trifluoromethyl)-pyrimidin-4-yl)amino)-*N*-methylbenzamide (6b)

White solid, 48% yield; mp >250 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.33 (s, 1H), 9.84 (s, 1H), 9.58 (s, 1H), 8.74 (d, *J* = 4.4 Hz, 1H), 8.43 (s, 1H), 7.72 (d, *J* = 7.6 Hz, 1H), 7.67 – 7.48 (m, 5H), 7.35 (d, *J* = 8.0 Hz, 2H), 7.17 (t, *J* = 7.6 Hz, 1H), 3.60 (d, *J* = 5.6 Hz, 2H), 2.79 (d, *J* = 4.4 Hz, 3H), 1.61 – 1.53 (m, 2H), 1.30 (d, *J* = 2.0 Hz, 6H), 0.88 (t, *J* = 6.0 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 187.2, 167.3, 164.6, 155.6, 155.5, 151.1, 149.1, 148.9, 145.4, 145.1, 131.3, 127.7, 125.4, 124.9, 122.7, 120.9 (q, *J* = 5.2 Hz), 118.5, 117.6, 114.9, 55.4, 45.1, 44.9, 41.4, 40.9, 38.1, 31.6; ESI-HRMS $C_{29}H_{30}F_3N_7O_3$ ([M+H]$^+$): calcd 582.2435, found 582.2432.

2-((2-((4-((2-((2-Hydroxyethyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)phenyl)amino)-5-(trifluoromethyl)pyrimidin-4-yl)amino)-*N*-methylbenzamide (6c)

Yellow solid, 46% yield; mp: 220.6-222.1 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.33 (s, 1H), 9.83 (s, 1H), 9.73 (s, 1H), 8.75 (d, *J* = 4.4 Hz, 1H), 8.43 (s, 1H), 7.82 – 7.70 (m, 2H), 7.66 – 7.50 (m, 4H), 7.37 (d, *J* = 8.4 Hz, 2H), 7.18 (t, *J* = 7.6 Hz, 1H), 5.03 (s, 1H), 3.68 (d, *J* = 4.4 Hz, 2H), 3.63 – 3.57 (m, 2H), 2.79 (d, *J* = 4.4 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 187.0, 170.2, 167.3, 164.7, 155.7, 155.5, 151.2, 149.4, 148.9, 145.4, 145.2, 127.9, 127.8, 125.4, 122.7, 119.3 (q, *J* = 5.2 Hz), 118.5, 117.7, 114.9, 68.9, 57.4, 41.4; ESI-HRMS $C_{25}H_{22}F_3N_7O_4$ ([M+H]$^+$): calcd 542.1758, found 542.1759.

Ethyl (2-((4-((4-((2-(methylcarbamoyl)phenyl)amino)-5-(trifluoromethyl)pyrimidin-2-yl)amino)-phenyl)amino)-3,4-dioxocyclobut-1-en-1-yl)glycinate (6d)

White solid, 49% yield; mp: 200.6-201.3 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.31 (s, 1H), 9.99 (s, 1H), 9.85 (d, *J* = 4.0 Hz, 1H), 8.75 (q, *J* = 4.0 Hz, 1H), 8.43 (s, 1H), 7.87 (s, 1H), 7.75 – 7.69 (m, 1H), 7.69 – 7.48 (m, 4H), 7.34 (d, *J* = 8.4 Hz, 2H), 7.18 (t, *J* = 7.2 Hz, 1H), 4.46 (d, *J* = 6.0 Hz, 2H), 4.18 (q, *J* = 7.2 Hz, 2H), 2.79 (d, *J* = 4.4 Hz, 3H), 1.23 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 186.8, 171.6, 167.3, 165.3, 156.2, 155.6, 155.5, 151.4, 150.3, 148.9, 145.1, 128.8, 127.5, 125.4, 122.7, 120.9, 119.3 (q, *J* = 5.2 Hz), 118.6, 117.6, 115.2, 69.2, 49.1, 41.4, 31.7; ESI-HRMS $C_{27}H_{24}F_3N_7O_5$ ([M+H]$^+$): calcd 584.1863, found 584.1864.

2-((2-((4-((2-(Isopropylamino)-3,4-dioxocyclobut-1-en-1-yl)amino)phenyl)amino)-5-(trifluoromethyl)pyrimidin-4-yl)amino)-*N*-methylbenzamide (6e)

White solid, 44% yield; mp >250 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.32 (s, 1H), 9.84 (s, 1H), 9.52 (s, 1H), 8.75 (d, *J* = 4.4 Hz, 1H), 8.43 (s, 1H), 7.73 (d, *J* = 7.6 Hz, 1H), 7.66 – 7.49 (m, 5H), 7.36 (d, *J* = 8.4 Hz, 2H), 7.18 (t, *J* = 7.6 Hz, 1H), 4.20 (m, 1H), 2.79 (d, *J* = 4.4 Hz, 3H), 1.27 (d, *J* = 6.4 Hz, 6H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 187.2, 169.9, 167.0, 164.5, 155.4, 155.0, 151.1, 150.3, 148.9, 145.1, 128.8, 128.0, 127.7, 125.4, 122.7, 120.9 (q, *J* = 5.2 Hz), 118.6, 117.6, 114.9, 49.0, 41.4, 39.4; ESI-HRMS $C_{26}H_{24}F_3N_7O_3$ ([M+H]$^+$): calcd 540.1965, found 540.1964.

2-((2-((4-((2-(Cyclopentylamino)-3,4-dioxocyclobut-1-en-1-yl)amino)phenyl)amino)-5-(trifluoromethyl)pyrimidin-4-yl)amino)-*N*-methylbenzamide (6f)

White solid, 47% yield; mp: 242.3-243.6 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.31 (s, 1H), 9.84 (s, 1H), 9.47 (s, 1H), 8.74 (d, *J* = 4.4 Hz, 1H), 8.43 (s, 1H), 7.70 (t, *J* = 12.8 Hz, 3H), 7.61 (d, *J* = 7.6 Hz, 2H), 7.53 (t, *J* = 7.6 Hz, 1H), 7.35 (d, *J* = 8.4 Hz, 2H), 7.17 (t, *J* = 7.6 Hz, 1H), 4.40 (m, 1H), 2.79 (d, *J* = 4.4 Hz, 3H), 1.98 (m, 2H), 1.77 – 1.70 (m, 2H), 1.64 – 1.54 (m, 4H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 167.1, 164.5, 155.4, 155.1, 151.1, 148.9, 145.4, 145.2, 138.9, 131.3, 128.0, 127.7, 125.4, 122.7, 120.9, 119.3 (q, *J* = 5.2 Hz), 118.5, 117.6, 114.9, 64.8, 47.5, 41.4, 38.9; ESI-HRMS $C_{28}H_{26}F_3N_7O_3$ ([M+H]$^+$): calcd 566.2122, found 566.2122.

2-((2-((4-((2-(Cyclohexylamino)-3,4-dioxocyclobut-1-en-1-yl)amino)phenyl)amino)-5-(trifluoromethyl)pyrimidin-4-yl)amino)-*N*-methylbenzamide (6g)

White solid, 42% yield; mp: 178.1-179.4 °C; IR (KBr pellet): v 3280.00, 2934.88, 2854.55, 1792.40, 1649.59, 1622.81, 1619.83, 1584.13, 1521.65, 1093.22, 828.43, 751.07. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.31 (s, 1H), 9.83 (s, 1H), 9.61 (s, 1H), 8.74 (m, 1H), 8.43 (s, 1H), 7.72 (d, *J* = 7.6 Hz, 2H), 7.65 – 7.50 (m, 4H), 7.37 (d, *J* = 8.4 Hz, 2H), 7.17 (t, *J* = 8.0 Hz, 1H), 3.88 (d, *J* = 6.4 Hz, 1H), 2.79 (d, *J* = 4.4 Hz, 3H), 1.95 (s, 2H), 1.65 (m, 4H), 1.34 (t, *J* = 9.2 Hz, 4H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 185.4, 170.3, 166.9, 164.5, 161.0, 155.5, 154.9, 151.2, 150.3, 148.9, 145.1, 127.9, 127.7, 125.4, 122.7, 120.9 (q, *J* = 5.2 Hz), 118.5, 117.6, 114.9, 62.4, 47.3, 41.4, 40.2, 39.6; ESI-HRMS $C_{29}H_{28}F_3N_7O_3$ ([M+H]$^+$): calcd 580.2278, found 580.2279.

2-((2-((4-((3,4-Dioxo-2-((tetrahydro-2H-pyran-4-yl)amino)cyclobut-1-en-1-yl)amino)phenyl)amino)-5-(trifluoromethyl)pyrimidin-4-yl)amino)-*N*-methylbenzamide (6h)

White solid, 45% yield; mp: 185.5-187.2 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.32 (s, 1H), 9.80 (m, 2H), 8.75 (d, *J* = 3.6 Hz, 1H), 8.43 (s, 1H), 7.94 (m, 2H), 7.72 (d, *J* = 7.6 Hz, 1H), 7.66 – 7.48 (m, 3H), 7.37 (d, *J* = 7.6 Hz, 2H), 7.18 (t, *J* = 6.0 Hz, 1H), 4.07 (s, 1H), 3.89 (m, 2H), 3.41 (m, 2H), 2.79 (d, *J* = 4.0 Hz, 3H), 1.94 (m, 2H), 1.63 – 1.53 (m, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 187.8, 166.9, 164.6, 161.6, 159.9, 155.5, 155.0, 151.3, 150.3, 148.9, 145.3, 145.1, 128.1, 127.7, 125.4, 122.7, 118.6 (q, *J* = 5.2 Hz), 117.6, 114.9, 72.7, 60.4, 45.0, 41.4; ESI-HRMS $C_{28}H_{26}F_3N_7O_4$ ([M+H]$^+$): calcd 582.2071, found 582.2071.

2-((2-((4-((2-((4-Hydroxycyclohexyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)phenyl)amino)-5-(trifluoromethyl)pyrimidin-4-yl)amino)-*N*-methylbenzamide (6i)

Yellow solid, 46% yield; mp: 204.5-206.1 °C; IR (KBr pellet): v 3235.37, 2931.90, 2696.86, 1789.42, 1670.41, 1584.13, 1539.50, 1096.20, 834.38, 751.87. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.31 (s, 1H), 10.65 (s, 1H), 9.80 (s, 1H), 9.01 (s, 1H), 8.63 (s, 1H), 8.42 (s, 1H), 7.73 (d, *J* = 7.6 Hz, 1H), 7.66 – 7.40 (m, 6H), 7.17 (t, *J* = 7.6 Hz, 1H), 4.61 (d, *J* = 4.4 Hz, 1H), 3.83 (d, *J* = 7.6 Hz, 1H), 3.16 – 3.09 (m, 1H), 2.79 (d, *J* = 4.4 Hz, 3H), 1.92 (m, 4H), 1.39 (m, 4H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 186.9, 170.2, 166.8, 164.3, 158.7, 155.5, 155.2, 151.3, 149.0, 145.1, 128.0, 127.8, 125.4, 122.7, 121.0, 119.3 (q, *J* = 5.2 Hz), 118.5, 117.7, 114.8, 62.2, 41.4, 34.8, 33.8, 30.3; ESI-HRMS $C_{29}H_{28}F_3N_7O_4$([M+H]$^+$): calcd 596.2227, found 596.2227.

***N*-Methyl-2-((2-((4-((2-((1-methylpiperidin-4-yl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)phenyl)-amino)-5-(trifluoromethyl)pyrimidin-4-yl)amino)benzamide (6j)**

White solid, 42% yield; mp: 188.9-189.5 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.31 (s, 1H), 9.83 (s, 1H), 9.65 (s, 1H), 8.74 (d, *J* = 4.4 Hz, 1H), 8.42 (s, 1H), 7.81 – 7.70 (m, 2H), 7.65 – 7.48 (m, 4H), 7.36 (d, *J* = 8.4 Hz, 2H), 7.17 (t, *J* = 7.6 Hz, 1H), 3.85 (m, 1H), 2.78 (d, *J* = 4.4 Hz, 3H), 2.72 (d, *J* = 11.2 Hz, 2H), 2.18 (s, 3H), 2.07 – 1.89 (m, 4H), 1.62 – 1.51 (m, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 187.5, 169.5, 166.9, 164.6, 162.1, 155.5, 155.0, 151.3, 148.9, 145.4, 145.1, 128.0, 127.7, 125.4, 122.7, 119.3 (q, *J* = 5.2 Hz), 118.5, 117.6, 114.9, 63.2, 60.9, 57.1, 46.7, 41.4; ESI-HRMS $C_{29}H_{29}F_3N_8O_3$ ([M+H]$^+$): calcd 595.2387, found 595.2387.

***In vitro* EGFR^{wt} and Src assay** Recombinant EGFR^{wt} and Src were purchased from Sino Biology Inc. Antiphosphotyrosine mouse mAb was purchased from PTM Bio. The effects of compounds on the activity of EGFR^{wt} and Src were determined by enzyme-linked immunosorbent assays (ELISAs) with recombinant EGFR^{wt} and Src according to reported methods.[29]

Cytotoxicity Evaluation (MTT Assay) A549 (Human non-small cell lung cancer cell line) cells, K562 (Human erythroleukemic cancer cell line) cells, HepG2 (Human hepatocellular carcinomas cell line) cells and MCF-7 (Human breast cancer cell line) cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. All cell lines were maintained in RPMI 1640 or DMEM complete medium. *In vitro* cytotoxicity of synthesized compounds against human tumor cell lines was determined by MTT assay described as our previous article.[30]

Molecular Docking X-Ray crystal structures of EGFR (PDB entry 1M17) and Src (PDB entry 3G6H) states were used for identifying candidate binding modes.[31] The possible binding modes of compound **6g** with EGFR and Src were predicted by molecular docking with Sybyl X-2.0 software from Tripos Inc. USA.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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