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Design, synthesis and biological activities of Nilotinib derivates as antitumor agents

Xiaoyan Pan, Fang Wang, Yanmin Zhang, Hongping Gao, Zhigang Hu, Sicen Wang, Jie Zhang *

School of Medicine, Xi'an Jiaotong University, No. 76, Yanta West Road, Xi'an, Shaanxi Province 710061, PR China

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ABSTRACT

A novel class of Nilotinib derivatives, **B1–B20**, were synthesized in high yields using various substituted anilines. All the title compounds were evaluated for their inhibitory activities against Bcr-Abl and antiproliferative effects on human leukemia cell (K562). The pharmacological results indicated that some compounds exhibited promising anticancer activity. In particular, compound **B14** containing tertiary amine side chain exhibited Bcr-Abl inhibitory activity similar to that of Nilotinib. It was suggested that the introduction of the tertiary amine moiety could improve Bcr-Abl inhibitory activity and antitumor effects.

1. Introduction

CML is a haematological malignancy caused by a chromosomal rearrangement which generates a fusion protein (Bcr-Abl kinase).[1,2] It arises from the juxtaposition of Abelson gene (Abl) on chromosome 9 to the break point cluster region (Bcr) gene on chromosome 22.[3] This fusion gene encodes a chimeric Bcr-Abl protein, in which the tyrosine kinase activity of Abl is constitutively activated.[4] Therefore, Bcr-Abl kinase plays an important role in the pathogenesis of CML. It is becoming an attractive target for CML targeted therapy.[5]

Nilotinib is a phenylamino-pyrimidine derivative which was rationally designed as second generation Bcr-Abl inhibitor.[6] It was approved to treat adult patients in all phases of CML with resistance to Imatinib. However, nilotibib could not suppress the proliferation of leukemia cells harboring some Bcr/Abl mutants (T315I).[7–9] Crystallographic studies have revealed that Nilotinib bound to Bcr-Abl through four key interactions (Fig. 1).[10–12]

Nilotinib was designed to fit into the ATP binding site of Bcr-Abl which was quite conserved. The allosteric binding region was considered as selectivity site. Therefore, the pharmacophore bound with allosteric region was optimized so as to improve the selectivity and activity. Similar to Nilotinib, we attempted to design novel derivatives which bind to Bcr-Abl more tightly, thereby enhancing the inhibitory activity. Aniline containing halogen substituents is useful for anticancer agents design. Halogen introduction can enhance the persistence and lipid solubility.[13] Therefore, various halogen-substituted anilines were introduced to develop novel Bcr-Abl inhibitors. Moreover, some heterocyclic amines or anilines containing tertiary amine side chain were used to afford new compounds with structural diversity.

2. Chemistry

In the present study, the facile total synthesis of Nilotinib from commercial available reagents was firstly performed.[14] In this study, the synthesis of one key intermediate 4-methoxyl-3-[[4-(3-pyridyl)-2-pyrimidinyl]amino] benzoic acid was similar to that of Nilotinib. The key intermediate (**5**) was prepared in five steps. At first, carboxylate group of 3-amino-4-methoxyl benzoic acid (**1**) was protected by esterizing.[15] Then refluxing of (**2**) and acetonitrile in the presence of concentrated HCl yielded 3-guanidino-4-methoxyl benzoic acid ethyl ester nitrate (**3**).[16,17] Reaction of (**3**) with 3-dimethylamino-1-(3-pyridyl)-2-propen-1-one afforded (**4**).[18,19] This reaction was carried out in the presence of NaOH. The hydrolysis of 4-methoxyl-3-[[4-(3-pyridyl)-2-pyrimidinyl] amino] benzoic acid ethyl ester (**4**) in NaOH aqueous afforded intermediate (**5**).[20] Finally, compound (**5**) condensed with various anilines to yield title compounds using active ester method.[21]

Twenty pyrimidin-2-ylamino-benzamides (**B1–B20**) were prepared as Bcr-Abl inhibitors. The synthetic route was shown in Scheme 1. Synthesis of **B1–B9** was similar to that of Nilotinib. Various heterocyclic amines were used in the synthesis of

* Corresponding author. Address: College of Medicine, Xi'an Jiaotong University, No. 76, Yanta West Road, Xi'an, Shaanxi Province 710061, PR China. Tel.: +86 29 82657740; fax: +86 29 82655451.
E-mail address: zhj8623@mail.xjtu.edu.cn (J. Zhang).

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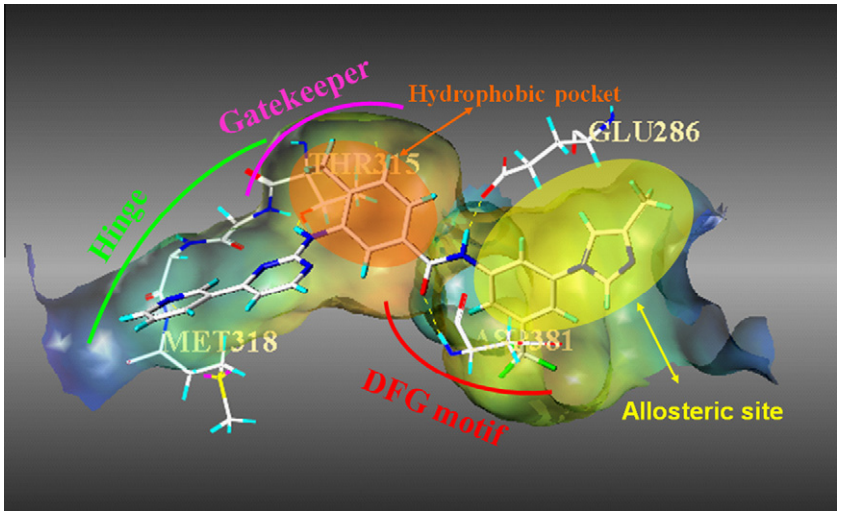
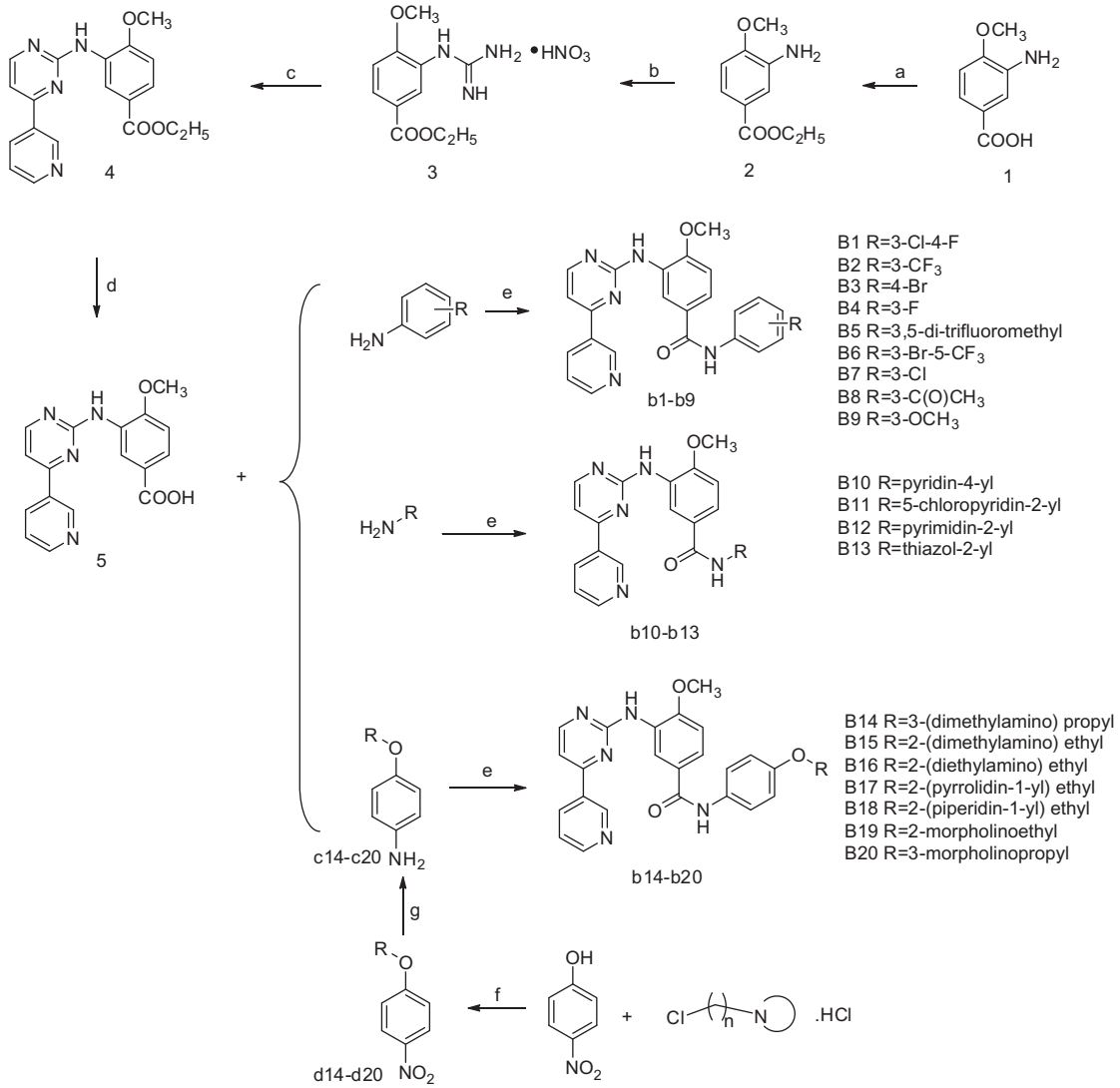


Figure 1. The interactions between Nilotinib and Bcr-Abl kinase.



Scheme 1. Reagents and conditions: (a) abs EtOH, concd H_2SO_4, reflux, 8 h; (b) H_2NCN, concd HCl, EtOH, reflux, 15 h, NH_4NO_3(aq); (c) 3-(dimethyl amino)-1-(3-pyridinyl)prop-2-en-1-one, NaOH, *n*-BuOH, reflux, 72 h; (d) 2 M NaOH, EtOH/H_2O(v:v = 1:1), 50 °C, 2 h; (e) isobutyl chloroformate, 4-methylmorpholine, 0 °C, 30 min; substituted benzamides or substituted heterocyclic amines or compound **c14**–**c20**, 4-methylmorpholine, rt, overnight; (f) Cs_2CO_3, DMF, 100 °C, 2–4 h; (g) Pd/C, MeOH, 4 h.

B10–B13. Intermediates **c14–c20** were firstly prepared to yield **B14**–**B20**. 4-Nitrophenol reacted with different chloro-substituted tertiary amines in DMF at 100 °C followed by the reduction of nitro to afford **c14**–**c20**.[22–24] The last procedure was completed using active ester method.

3. Results and discussion

All the title compounds were evaluated for their ability to inhibit Bcr-Abl with Nilotinib as positive control.[25] The results were described in Tables 1–3. Noticeably, most of them exhibited reasonable inhibitory activity against Bcr-Abl. Compound **B14** was the most potent with IC_{50} value of 0.52 nM. Moreover, some compounds (**B1**, **B3**, **B4**, **B12**, **B14**, **B16** and **B20**) were also potent Bcr-Abl inhibitors with IC_{50} values ranging from 1.48 to 6.34 nM. These compounds are considered as promising leading compounds for the development of novel Bcr-Abl inhibitors as anticancer agents.

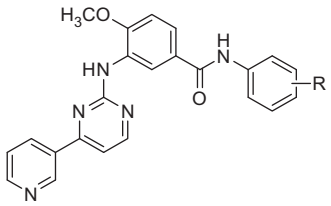
Some title compounds were also tested for antiproliferative activity on Bcr-Abl positive leukemia cell (K562). As shown in Tables 1–3, several compounds displayed strong growth suppression in K562 cells. In particular, **B9** and **B10** exhibited potent activities with IC_{50} values of 93.07 and 94.48 μM, respectively. Meanwhile, four compounds (**B5**, **B14**, **B17**, **B18**), were also more potent than Nilotinib.

It was found from Table 1 that compounds containing halogen substituted anilines were more potent than that without halogen. Many halogen substituted compounds exhibited potent antiproliferative activity in K562 cells. These results indicated that halogen introduction was essential for Bcr-Abl inhibitory activity. Among those compounds **B1**, **B4** and **B5** were more potent than the others.

The biological activities of **B10**–**B13** were listed in Table 2. The replacement of anilines with heterocyclic amines led to the reduction in activities. Compound **B12** displayed potent Bcr-Abl inhibitory activity. It was considered to be a novel Bcr-Abl inhibitor for further optimization.

As shown in Table 3, **B14**–**B20** showed potent Bcr-Abl inhibitory activity. Three of them exhibited potent Bcr-Abl kinase inhibitory activity with good K562 growth inhibitory activity. The results suggested that introduction of anilines containing tertiary amine side chain was benefit for antitumor activity. **B14** was potent inhibitors both on Bcr-Abl kinase and K562 cell, and it was a good Bcr-Abl inhibitor hit.

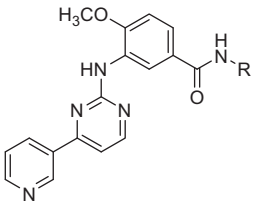
Table 1
Structure and activity of **B1**–**B9** towards Bcr-Abl and K562 cells in vitro



Compounds	R	Bcr-Abl IC_{50}[a] (nM)	K562 Cells IC_{50}[b] (μM)
B1	3-Cl-4-F	4.73	ND
B2	3-CF_3	4125.31	ND
B3	4-Br	4.79	ND
B4	3-F	3.21	ND
B5	3,5-di-CF_3	ND[c]	4.84
B6	3-Br-5-CF_3	ND	214.82
B7	3-Cl	34.77	237.47
B8	3-$COCH_3$	ND	237.47
B9	3-OCH_3	ND	93.07
Nilotinib	—	0.33	89.13

[a] ABL activity assays were performed using TTRF KinEASE-TK assay formats.
[b] K562 cells assays were performed using MTT assays.
[c] ND is not determined.

Table 2
Structure and activity of **B10**–**B13** towards Bcr-Abl and K562 cells in vitro



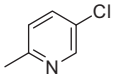
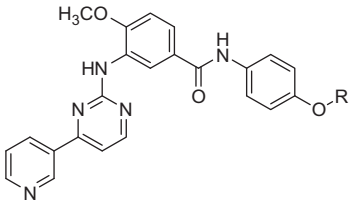
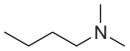
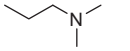
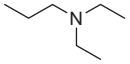
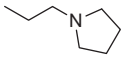
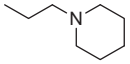
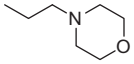
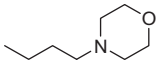
Compounds	R	Bcr-Abl IC_{50} (nM)	K562 Cells IC_{50} (μM)
B10		2754.90	94.48
B11		ND	ND
B12		3.78	ND
B13		294.34	ND
Nilotinib	—	0.33	89.13

Table 3
Structure and activity of **B14**–**B20** towards Bcr-Abl and K562 cells in vitro



Compounds	R	Bcr-Abl IC_{50} (nM)	K562 Cells IC_{50} (μM)
B14		0.52	38.49
B15		2.34	ND
B16		1.48	ND
B17		350.08	38.61
B18		1110.42	5.15
B19		63.31	ND
B20		6.34	ND
Nilotinib	—	0.33	89.13

4. Docking and QSAR studies

4.1. Docking

Molecular docking study was performed to investigate the binding mode of title compounds with Bcr-Abl. Docking was carried out using Surflex-Dock Module of Sybyl-X 2.0. The small molecules and the X-ray crystal structure of Bcr-Abl in complex with Nilotinib (PDB code: 3CS9[10]) were imported, and Nilotinib was used to define the binding cavity. All compounds were docked into

the active site of Bcr-Abl. The binding model of **B14** with Bcr-Abl was shown in Figure 2. It was found that **B14** bound to ATP pocket of Bcr-Abl in a similar fashion to that of Nilotinib. It binds to the kinase domain by making four hydrogen bond interactions involving the pyridyl-N and the backbone NH of Met-318, the anilino-NH and the side chain OH of Thr-315, the amido-NH and side chain carboxylate of Glu-286 and the amido carbonyl with the backbone NH of the Asp-381.

4.2. QSAR studies

Sybyl-X 2.0 program was used to carry out QSAR studies of these novel Bcr-Abl inhibitors. QSAR studies were performed with the CoMFA module. The test set consisted of Nilotinib, **B1**, **B3**, **B13**, **B14** and **B18**, another 15 compounds (**B1**–**B3**, **B7**, **B10**, **B12**, **B13**, and **B14**–**B20**) composed of the training set. The IC_{50} values were converted into pIC_{50} according to the formula: $pIC_{50} = \log_{10}IC_{50}$.

The docking conformation generated from training set with Bcr-Abl (PDB code: 3CS9) were used. Based on the docking results, the template molecule Nilotinib was taken and the rest of the molecules were aligned to it using structure A as scaffold by DATABASE ALIGNMENT method. The aligned molecules are shown in Figure 3.

The steric and electrostatic fields were calculated at each lattice intersection of a regularly spaced grid of 2.0 Å in all three dimensions within defined region. An sp^3 carbon atom with +1.00 charge was used as probe atom. The steric and electrostatic fields were truncated at +30.00 kcal mol^{-1}, and the electrostatic fields were ignored at the lattice points with maximal steric interactions.

PLS method was used to linearly correlate CoMFA fields to the inhibitory activity values. The cross-validation analysis was performed using the leave one out (LOO) method in which one compound is removed from the dataset and its activity is predicted using the model derived from the rest of the dataset. The cross-validated q^2 (0.3) that resulted in optimum number of components ($n = 8$) and lowest standard error of prediction were considered for further analysis. We have evaluated different filter value σ and at least selected σ as 2.00 kcal mol^{-1} to speed up the analysis and reduce noise.

The non cross-validated r^2 for the model established by the study is 0.999. The value of the variance ratio F ($n_1 = 8$, $n_2 = 7$) is 874.585 and standard error of the estimate (SEE) is 0.061. The contribution of electrostatic and steric is 53.8% and 46.2%, respectively. It was found from Figure 4b that the CoMFA model could predict test set well.

5. Conclusions

In summary, a series of novel Bcr-Abl inhibitors were prepared and evaluated for their biological activities. Most of them exhibited potent Bcr-Abl inhibitory activity and antiproliferation effect on K562 cells. Compounds containing anilines were more potent than that containing heterocyclic amines. The results revealed that the interaction between this moiety and Bcr-Abl could mainly be hydrophobic interaction. Moreover, the introduction of halogen might be beneficial for Bcr-Abl inhibitory activity. Furthermore, compounds containing tertiary amines side chain especially **B14** could be considered as a novel lead compound for further optimization.

6. Experimental

6.1. Cell growth inhibition assays

Growth inhibitory activities were evaluated on K562 leukemia cancer cell lines. The effects of the compounds on cell viability were evaluated using the MTT assay. Exponentially growing cells were harvested and plated in 96-well plates at a concentration of 1×10^4 cells/well, and incubated for 24 h at 37 °C. The cells in the wells were, respectively, treated with target compounds at various concentrations for 48 h. Then, 20 mL MTT (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. After the supernatant was discarded, 150 mL DMSO was added to each well, and the absorbance values were determined by a microplate reader (Bio-Rad Instruments) at 490 nm.

6.2. Kinase assays

The kinase inhibition assay and IC_{50} determinations for wild type Abl were measured with the homogeneous time-resolved fluorescence (HTRF) KinEASE-TK assay from Cisbio according to the manufacturer's instructions. Wild type Abl was purchased from

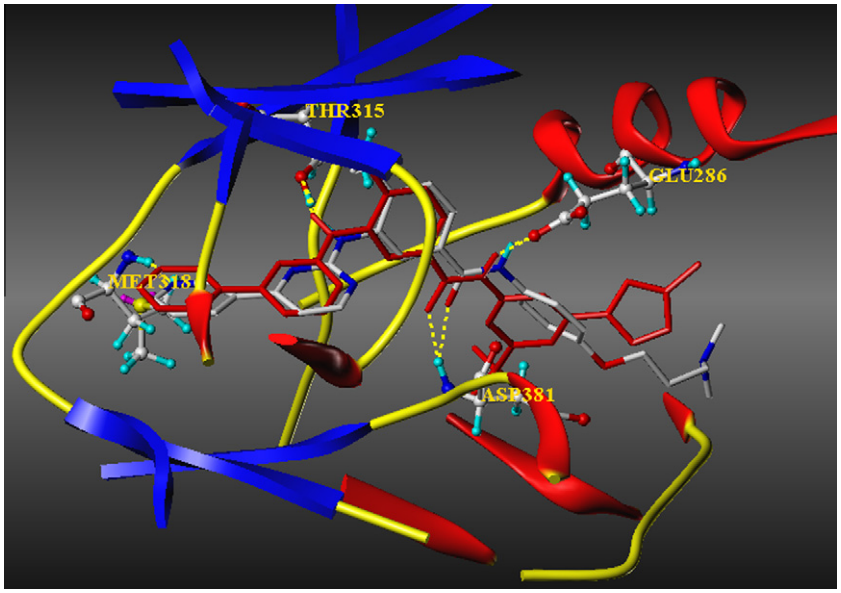


Figure 2. Binding mode of Nilotinib (red) and **B14** with the Bcr/Abl kinase domain.

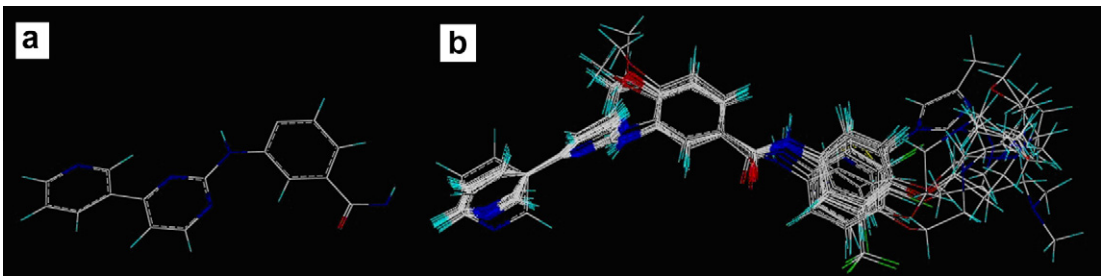


Figure 3. (a) Structure A; (b) superposition of 18 inhibitors for CoMFA construction.

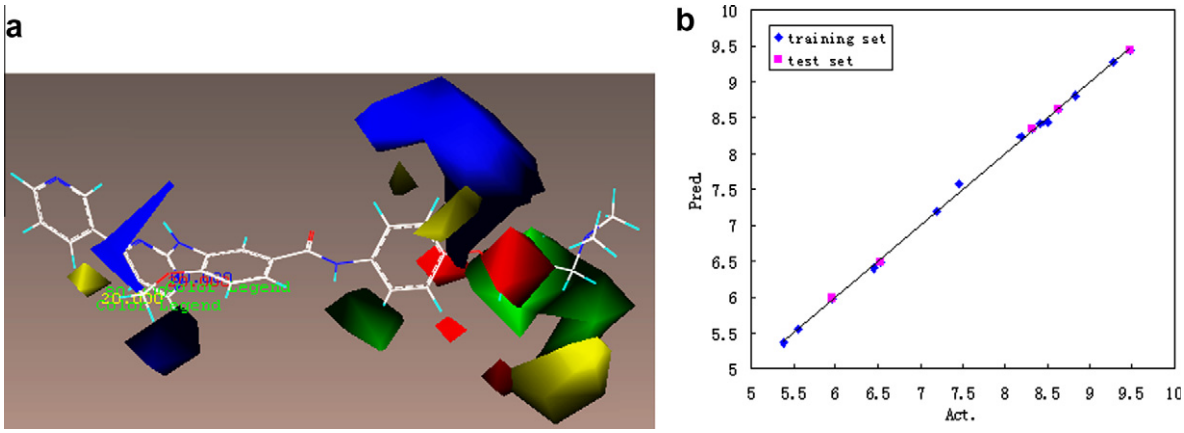


Figure 4. (a) The most active molecule **B14** is shown in the background. Red color represents the negative charge region, blue is the positive charge region, green is the more bulky region, yellow is the less bulky region. (b) The predictability of the CoMFA model.

Carna Biosciences and 0.04 ng/μL kinase were used for test. ATP concentration was set at its K_m values (6.021 μM), and 457.7 nM substrate was used. Kinase, substrate peptide and inhibitors were added in 384 well plate, and then reaction was started by addition of ATP. After completion of the reaction (30 min later), an anti-phosphotyrosine antibody labeled with europium cryptate and streptavidin labeled with the fluorophore XL665 were added. The FRET between europium cryptate and XL665 was measured to quantify the phosphorylation of the substrate peptide. A Tecan i-control infinite 500 was used to measure the fluorescence of the samples at 620 nm (Eu-labeled antibody) and 665 nm (XL665 labeled streptavidin) 500 μs after excition at 320 nm. The quotient of both intensities for reactions made with six different inhibitor concentrations (0.032 nM–3.2 μM, including no inhibitor) was plotted against inhibitor concentrations to determine IC_{50} values. Each reaction was performed in duplicate, and at least two independent determinations of each IC_{50} were made.

6.3. Chemistry: general procedures

All solvents and reagents were purified by standard techniques. Petroleum ether (PE) used refers to the fraction boiling in the range 60–90 °C. All reactions except those in aqueous media were carried out by standard techniques for moisture exclusion. Anhydrous reactions were carried out over dried glassware under nitrogen atmosphere. Reactions were monitored by TLC on 0.25 mm silica gel plates ($60GF_{254}$) and visualized with ultraviolet light. Melting points were obtained on electrothermal meliting point apparatus and are uncorrected. ^{1}H NMR spectra was recorded with a Bruker Advance 400 MHz instrument. Mass spectra were obtained on a Shimadzu GC–MS-QP2010 instrument.

6.3.1. 3-Amino-4-methoxyl-benzoic acid ethyl ester (2)

3-Amino-4-methoxyl benzoic acid (6.01 g, 36 mmol) was dissolved in 100 mL dehydrated ethanol. Under 0 °C, 2 mL concentrated H_2SO_4 was dropped slowly to the above solution, and then the solution was heated to reflux for 12 h. After the reaction was completed, ethanol was removed by reduced pressure distillation. Under 0 °C, 100 mL water was added, and the solution was adjusted to pH 6–7 with sodium bicarbonate. Then the aqueous was extracted with ethyl acetate (50 mL × 2). The organic phase was combined, and was washed by water (30 mL × 2), saturated sodium chloride (30 mL). The organic layer was dried over Na_2SO_4, filtered, and distilled under vacuum to give the crude product. The crude product was purified by chromatography (petroleum ether/ethyl acetate = 1:1), giving a white solid (6.87 g). The total yield was 98%, mp 55–57 °C. EI-MS (*m/z*): 195.1 ($[M]^+$). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.39 (t, *J* = 6.0 Hz, 3H), 3.92 (s, 3H), 4.34 (q, *J* = 6.0 Hz, 14.00 Hz, 2H), 6.81 (d, *J* = 8.0 Hz, 1H), 7.42 (s, 1H), 7.50 (d, *J* = 8.0 Hz, 1H).

6.3.2. 3-Guanidino-4-methoxyl benzoic acid ethyl ester nitrate (3)

In 100 mL round bottom flask, 3-amino-4-methoxyl-benzoic acid ethyl ester (**2**) (3.00 g, 16.56 mmol), and cyanamide (1.59 g, 38.09 mmol) were added to ethanol (20 mL). While being stirred, concentrated HCl (2.1 mL, 24.84 mmol) was dropped slowly into the mixture. The mixture was heated to reflux for 15 h. Ethanol was removed by reduced pressure distillation, and water (20 mL) was added into the residue. Under 0 °C, NH_4NO_3 (2.64 g, 33.12 mmol) solution was dropped during 30 min. Then the mixture was stirred for 30 min, and filtered, giving the crude product. The crude product need to be purified by being washed with ether,

and finally a white solid (4.01 g) was maintained. The total yield was 84.7%, mp 172–174 °C. EI-MS (*m/z*): 237.1 ($[M]^+-HNO_3$). ^1H NMR (400 MHz, D_2O): δ = 1.22 (t, *J* = 8.0 Hz, 3H), 3.81 (s, 3H), 4.21 (q, *J* = 6.0 Hz, 14.00 Hz, 2H), 7.09 (d, *J* = 12.0 Hz, 1H), 7.80 (s, 1H), 7.93 (d, *J* = 8.0 Hz, 1H).

6.3.3. 4-Methoxyl-3-[[4-(3-pyridyl)-2-pyrimidinyl] amino] benzoic acid ethyl ester (4)

In a round-bottom flask, 3-guanidino-4-methoxyl nitrate ethyl benzoate (**3**) (3.00 g, 10 mmol), 3-dimethylamino-1-(3-pyridyl)-2-propen-1-one (1.76 g, 10 mmol), sodium hydroxide (0.48 g, 12 mmol) was dissolved in *n*-butanol (20 mL). The mixture was stirred and heated to reflux for 48 h. Then *n*-butanol was removed completely by reduced pressure distillation. EtOAc (50 mL) and water (30 mL) were added into the residue, and the mixture was stirred for 20 min. The organic phase was separated from the aqueous phase, and the aqueous phase was extracted by EtOAc (20 mL × 2). The combined organic phase was washed by water (15 mL), saturated sodium chloride (15 mL). The organic layer was dried over Na_2SO_4, filtered, and distilled under vacuum to give the crude product. The crude product was purified by recrystallization from ether and EtOAc, giving a white solid (2.57 g). The total yield was 73.4%, mp 99–100 °C. EI-MS (*m/z*): 350.1 $[M]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ = 1.34 (t, *J* = 8.0 Hz, 3H), 3.96 (s, 3H), 4.34 (q, *J* = 8.0 Hz, 16.0 Hz, 2H), 7.19 (d, *J* = 8.0 Hz, 1H), 7.57–7.59 (m, 1H), 7.61 (d, *J* = 4.0 Hz, 1H), 7.71 (d, *J* = 8.0 Hz, 1H), 8.37 (s, 1H), 8.55 (d, *J* = 8.0 Hz, 1H), 8.64 (d, *J* = 8.0 Hz, 1H), 8.75 (d, *J* = 8.0 Hz, 1H), 9.03 (s, 1H).

6.3.4. 4-Methoxyl-3-[[4-(3-pyridyl)-2-pyrimidinyl] amino] benzoic acid (5)

In a 250 mL flask, 4-methyl-3-[[4-(3-pyridyl-2-pyrimidinyl) amino] ethyl benzoate (**4**) (6.70 g, 19.14 mmol) was dissolved into the mixed solution of ethanol/water (60/60 mL). While being stirred, NaOH (2 mol/L, 25 mL) was drooped in the mixture. Then the mixture was heated to 45–50 °C and was maintained at this temperature overnight. Ethanol was removed by reduced pressure distillation, and then EtOAc (20 mL) was added. Then the organic phase was abandoned. The aqueous phase was adjusted pH to 7–8 using HCl (2 mol/L), and yellow solid was precipitated. The suspension was filtered, giving the crude product (5.36 g). The total yield was 87%, mp 340–342 °C. EI-MS (*m/z*): 305.1 $[M-OH]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ = 3.86 (s, 3H), 6.96 (d, *J* = 8.0 Hz, 1H), 7.50 (d, *J* = 4.0 Hz, 1H), 7.55–7.58 (m, 1H), 7.62 (d, *J* = 8.0 Hz, 1H), 8.24 (s, 1H), 8.57 (d, *J* = 8.0 Hz, 1H), 8.60 (d, *J* = 8.0 Hz, 1H), 8.68 (s, 1H), 8.72 (d, *J* = 4.0 Hz, 1H).

6.3.5. *N*-(3-Chloro-4-fluorophenyl)-4-methoxyl-3-(4-(pyridin-3-yl)pyrimidin-2-ylamino) benzamide (B1)

In a 100 ml flask, 4-methoxyl-3-[[4-(3-pyridyl)-2-pyrimidinyl] amino] benzoic acid (**5**) (1.00 g, 3.10 mmol) and 4-methylmorpholine (1 mL, 9.3 mmol) were added in $CHCl_2$ (15 mL). Under 0 °C, the CH_2Cl_2 (8 mL) solution of isobutyl chloroformate (0.6 mL, 4.65 mmol) was dropped slowly into the above suspension. Then the mixture was reacted under 0 °C for 30 min.

After that, the CH_2Cl_2 (10 mL) solution of 3-chloro-4-fluoro aniline (0.54 g, 3.10 mmol), isobutyl chloroformate (0.6 mL, 4.65 mmol) was dropped slowly into the above suspension. Then the ice bath was removed and the mixture was reacted at rt overnight. The mixture was diluted with CH_2Cl_2 (20 mL), and was washed with saturated water (10 mL × 2), saturated Na_2CO_3 solution (10 mL × 3), NaCl solution (10 mL). Then the organic phase was dried by Na_2SO_4, and filtered, giving the crude product. The crude product was purified by chromatography (ethyl acetate/methanol = 1:1), giving a solid (0.69 g). The total yield was 34%, mp 226–227 °C, EI-MS (*m/z*): 449.0$[M]^+$, ^1H NMR (400 MHz, $CDCl_3$): δ = 4.04 (s, 3H), 7.02 (d, *J* = 8.0 Hz, 1H), 7.16 (t, *J* = 8.0 Hz, 1H), 7.26 (d, *J* = 8.0 Hz, 1H), 7.45 (d, *J* = 4.0 Hz, 1H), 7.56–7.58 (m, 1H), 7.63 (d, *J* = 8.0 Hz, 1H), 7.85 (d, *J* = 4.0 Hz, 1H), 7.99 (d, *J* = 12.0 Hz, 2H), 8.47 (d, *J* = 8.0 Hz, 1H), 8.60 (d, *J* = 4.0 Hz, 1H), 8.77 (s, 1H).

Compounds **B2**–**B13** were prepared by using the general procedure described above.

6.3.6. 4-Methoxyl-3-(4-(pyridin-3-yl) pyrimidin-2-ylamino)-*N*-(3-(trifluoro methyl) phenyl) benzamide (B2)

Mp 244–246 °C, EI-MS(*m/z*): 465.0$[M]^+$, ^1H NMR (400 MHz, $CDCl_3$): δ = 4.04 (s, 3H), 7.02 (d, *J* = 8.0 Hz, 1H), 7.26 (d, *J* = 8.0 Hz, 1H), 7.43–7.46 (m, 1H), 7.50 (d, *J* = 8.0 Hz, 2H), 7.61 (d, *J* = 8.0 Hz, 2H), 7.63 (d, *J* = 4.0 Hz, 1H), 7.97 (s, 2H), 8.50 (d, *J* = 8.0 Hz, 1H), 8.60 (d, *J* = 4.0 Hz, 1H), 8.76 (d, *J* = 4.0 Hz, 1H).

6.3.7. *N*-(4-Bromophenyl)-4-methoxyl-3-(4-(pyridin-3-yl)pyrimidin-2-ylamino) benzamide (B3)

Mp 220–223 °C, EI-MS (*m/z*): 474.9$[M-1]^+$, ^1H NMR (400 MHz, $CDCl_3$): δ = 4.05 (s, 3H), 7.04 (d, *J* = 12.0 Hz, 1H), 7.26 (s, 1H), 7.42–7.46 (m, 2H), 7.52 (t, *J* = 8.0 Hz, 1H), 7.66 (d, *J* = 8.0 Hz, 1H), 7.96 (s, 2H), 7.99 (s, 1H), 8.10 (s, 1H), 8.50 (d, *J* = 8.0 Hz, 1H), 8.61 (d, *J* = 4.0 Hz, 1H), 8.76 (d, *J* = 8.0 Hz, 1H).

6.3.8. *N*-(3-Fluorophenyl)-4-methoxyl-3-(4-(pyridin-3-yl)pyrimidin-2-yl amino) benzamide (B4)

Mp 220–221 °C, EI-MS (*m/z*): 415.1$[M]^+$, ^1H NMR (400 MHz, $CDCl_3$): δ = 4.03 (s, 3H), 6.85–6.88 (m, 1H), 7.00 (d, *J* = 8.0 Hz, 1H), 7.25 (d, *J* = 8.0 Hz, 1H), 7.32 (s, 2H), 7.42–7.45 (m, 1H), 7.62 (d, *J* = 8.0 Hz, 1H), 7.67 (d, *J* = 8.0 Hz, 1H), 8.08 (s, 1H), 8.50 (d, *J* = 8.0 Hz, 1H), 8.59 (d, *J* = 8.0 Hz, 1H), 8.76 (d, *J* = 4.0 Hz, 1H).

6.3.9. *N*-(3,5-Bis(trifluoromethyl)phenyl)-4-methoxyl-3-(4-(pyridin-3-yl) pyrimidin-2-ylamino) benzamide (B5)

Mp 241–243 °C, EI-MS (*m/z*): 533.1$[M]^+$, ^1H NMR (400 MHz, $CDCl_3$): δ = 4.06 (s, 3H), 7.05 (d, *J* = 8.0 Hz, 1H), 7.44–7.47 (m, 1H), 7.66 (s, 1H), 7.68–7.72(m, 1H), 8.01 (d, *J* = 8.0 Hz, 1H), 8.19 (s, 1H), 8.27(s, 2H), 8.34 (s, 1H), 8.46 (d, *J* = 8.0 Hz, 1H), 8.62 (d, *J* = 8.0 Hz, 1H), 8.76 (s, 1H).

6.3.10. *N*-(3-Bromo-5-(trifluoromethyl)phenyl)-4-methoxyl-3-(4-(pyridin-3-yl) pyrimidin-2-ylamino) benzamide (B6)

Mp 227–230 °C, EI-MS (*m/z*): 544.0$[M]^+$, ^1H NMR (400 MHz, $CDCl_3$): δ = 4.05 (s, 3H), 7.04 (d, *J* = 8.0 Hz, 1H), 7.27 (s, 1H), 7.44–7.47 (m, 1H), 7.55 (s, 1H), 7.67 (d, *J* = 8.0 Hz, 1H), 7.98 (d, *J* = 12.0 Hz, 2H), 8.20 (d, *J* = 4.0 Hz, 2H), 8.46 (d, *J* = 8.0 Hz, 1H), 8.62 (d, *J* = 4.0 Hz, 1H), 8.80 (s, 1H).

6.3.11. *N*-(3-Chlorophenyl)-4-methoxyl-3-(4-(pyridin-3-yl)pyrimidin-2-ylamino) benzamide (B7)

Mp 211–213 °C, EI-MS (*m/z*): 431.0$[M]^+$, ^1H NMR (400 MHz, $CDCl_3$): δ = 4.04 (s, 3H), 7.03 (d, *J* = 8.0 Hz, 1H), 715 (d, *J* = 8.0 Hz, 1H), 7.27 (d, *J* = 8.0 Hz, 1H), 7.30–7.34 (m, 1H), 7.44–7.47 (m, 1H), 7.60 (d, *J* = 8.0 Hz, 1H), 7.64 (d, *J* = 8.0 Hz, 1H), 7.77 (s, 1H), 7.99 (d, *J* = 4.0 Hz, 2H), 8.51 (d, *J* = 8.0 Hz, 1H), 8.62 (d, *J* = 4.0 Hz, 1H), 8.78 (d, *J* = 4.0 Hz, 1H).

6.3.12. *N*-(3-Acetylphenyl)-4-methxol-3-(4-(pyridin-3-yl)pyrimidin-2-ylamino) benzamide (B8)

Mp 191–195 °C, EI-MS (*m/z*): 439.1$[M]^+$, ^1H NMR (400 MHz, DMSO-d_6): δ = 2.60 (s, 3H), 3.97 (s, 3H), 7.23 (d, *J* = 8.0 Hz, 1H), 7.50–7.53 (m, 2H), 7.59 (d, *J* = 8.0 Hz, 1H), 7.72 (d, *J* = 8.0 Hz, 1H), 7.81 (d, *J* = 8.oHz, 1H), 8.12 (d, *J* = 8.0 Hz, 1H), 8.39 (s, 1H), 8.49 (s, 1H), 8.58 (d, *J* = 8.0 Hz, 1H), 8.64 (d, *J* = 4.0 Hz, 1H), 8.71 (d, *J* = 8.0 Hz,), 8.91 (s, 1H).

6.3.13. *N*-(3-Methoxyphenyl)-4-methoxyl-3-(4-(pyridin-3-yl) pyrimidin-2-yl amino) benzamide (B9)

Mp 208–210 °C, EI-MS (*m/z*): 427.0[M]$^+$, ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.85 (s, 3H), 4.02 (s, 3H), 6.91–6.95 (m, 2H), 7.00 (d, *J* = 8.0 Hz, 1H), 7.25 (d, *J* = 4.0 Hz, 1H), 7.40–7.43 (m, 1H), 7.50 (d, *J* = 12.0 Hz, 1H), 7.58 (d, *J* = 12.0 Hz, 1H), 7.61–7.65 (m, 1H), 7.90 (s, 1H), 7.96 (d, *J* = 8.0 Hz, 1H), 8.51 (d, *J* = 8.0 Hz, 1H), 8.59 (d, *J* = 8.0 Hz, 1H), 8.74 (s, 1H).

6.3.14. 4-Methoxyl-3-(4-(pyridin-3-yl) pyrimidin-2-ylamino)-*N*-(pyridin-4-yl) benzamide (B10)

Mp 241–243 °C, EI-MS (*m/z*): 398.1[M]$^+$, ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.98 (s, 3H), 7.24 (d, *J* = 8.0 Hz, 1H), 7.50–7.55 (m, 1H), 7.59 (d, *J* = 8.0 Hz, 1H), 7.79 (d, *J* = 8.0 Hz, 1H), 7.82 (d, *J* = 8.0 Hz, 2H), 8.47 (s, 3H), 8.57 (d, *J* = 8.0 Hz, 1H), 8.64 (d, *J* = 8.0 Hz, 1H), 8.72 (d, *J* = 8.0 Hz, 1H), 8.93 (s, 1H).

6.3.15. *N*-(5-Chloropyridin-2-yl)-4-methoxyl-3-(4-(pyridin-3-yl)pyrimidin-2-ylbamino) benzamide (B11)

Mp 216–218 °C, EI-MS (*m/z*): 432.0[M]$^+$, ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.97 (s, 3H), 7.19 (d, *J* = 8.0 Hz, 1H), 7.53–7.56 (m, 1H), 7.60 (d, *J* = 8.0 Hz, 1H), 7.69 (d, *J* = 8.0 Hz, 1H), 7.88 (d, *J* = 8.0 Hz, 1H), 7.98 (d, *J* = 8.0 Hz, 1H), 8.28 (d, *J* = 12.0 Hz, 1H), 8.45 (d, *J* = 8.0 Hz, 1H), 8.59 (d, *J* = 8.0 Hz, 1H), 8.64 (t, *J* = 4.0 Hz, 1H), 8.73 (t, *J* = 6.0 Hz, 1H), 8.97 (d, *J* = 8.0 Hz, 1H).

6.3.16. 4-Methoxyl-3-(4-(pyridin-3-yl)pyrimidin-2-ylamino)-*N*-(pyrimidin-2-yl) benzamide (B12)

Mp 187–190 °C, EI-MS (*m/z*): 399.1[M]$^+$, ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.96 (s, 3H), 7.19 (d, *J* = 12.0 Hz, 1H), 7.6 (m, 1H), 7.51–7.54 (m, 1H), 7.59 (d, *J* = 4.0 Hz, 1H), 7.79 (d, *J* = 8.0 Hz, 1H), 8.47 (s, 1H), 8.59 (d, *J* = 8.0 Hz, 1H), 8.63 (d, *J* = 8.0 Hz, 1H), 8.72 (d, *J* = 8.0 Hz, 1H), 8.75 (d, *J* = 4.0 Hz, 2H), 8.89 (s, 1H).

6.3.17. 4-Methoxyl-3-(4-(pyridin-3-yl) pyrimidin-2-ylamino)-*N*-(thiazol-2-yl) benzamide (B13)

Mp 205–208 °C, EI-MS (*m/z*): 403.9[M]$^+$, ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.97 (s, 3H), 7.22 (d, *J* = 8.0 Hz, 1H), 7.28 (d, *J* = 4.0 Hz, 1H), 7.58 (d, *J* = 4.0 Hz, 2H), 7.60 (d, *J* = 4.0 Hz, 1H), 7.96 (d, *J* = 8.0 Hz, 1H), 8.45 (s, 1H), 8.60 (d, *J* = 8.0 Hz, 1H), 8.65 (d, *J* = 4.0 Hz, 1H), 8.74 (d, *J* = 4.0 Hz, 1H), 9.03 (s, 1H).

6.3.18. *N*,*N*-Dimethyl-3-(4-nitrophenoxy) propan-1-amine (d14)

In 100 mL flask, 4-nitrophenol (1.39 g, 10 mmol) was dissolved in 20 mL anhydro-DMF. Then 3-chloro- *N*,*N*-dimethylpropan-1-amine hydrochloride (1.57 g, 10 mmol) and Cs_2CO_3 (5.29 g, 15 mmol) were added to the solution. Under N_2, the mixture was heated to 100 °C, and was reacted for 2.5 h. The mixture was filtered, and the filtrate was poured to ice water (200 mL). The aqueous phase was extracted by EtOAc (30 mL × 3). The combined organic phase was washed by Na_2CO_3 (10 mL × 4), water (10 mL × 2), saturated sodium chloride (15 mL). The organic layer was dried over Na_2SO_4, filtered, and distilled under vacuum to give the crude product.

6.3.19. 4-(3-(Dimethylamino) propoxy) benzenamine (c14)

N,*N*-Dimethyl-3-(4-nitrophenoxy) propan-1-amine (**d14**) (3.92 mmol) was dissolved in 30 mL anhydrous methanol, and then 0.1 g Pd/C (5%) was added to this solution. After that, the mixture was reacted at room temperature under N_2 for 4 h. The mixture was filtered, and Pd/C was washed with methanol for 3–4 times. The organic phases were combined and methanol was removed by reduced pressure distillation to give the crude product. The residue was reserved for the next step.

Compounds **c14**–**c20** were prepared by using the general procedure described above.

6.3.20. *N*-(4-(3-(Dimethylamino) propoxy) phenyl)-4-methoxyl-3-(4-(pyridin-3-yl)pyrimidin-2-ylamino)benzamide (B14)

In a 100 mL flask, 4-methoxyl-3-[[4-(3-pyridyl)-2-pyrimidinyl] amino] benzoic acid (**5**) (1.00 g, 3.10 mmol) and 4-methylmorpholine (1 mL, 9.3 mmol) were added in $CHCl_2$ (15 mL). Under 0 °C, the CH_2Cl_2 (8 mL) solution of isobutyl chloroformate (0.6 mL, 4.65 mmol) was dropped slowly into the above suspension. Then the mixture was reacted under 0 °C for 30 min.

After that, the CH_2Cl_2 (10 mL) solution of 4-(3-(dimethylamino)propoxy)benzenamine (**c14**) (3.92 mmol), isobutyl chloroformate (0.6 mL, 4.65 mmol) was dropped slowly into the above suspension. Then the ice bath was removed and the mixture was reacted at rt overnight. The mixture was diluted with CH_2Cl_2 (20 mL), and was washed with saturated water (10 mL × 2), saturated Na_2CO_3 solution (10 mL × 3), NaCl solution (10 mL). Then the organic phase was dried by Na_2SO_4, and filtered, giving the crude product. The crude product was purified by chromatography (ethyl acetate/methanol = 1:1), giving a solid (0.4 g). The total yield was 26%, mp 133–136 °C, EI-MS (*m/z*): 498.3[M]$^+$, ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.83–1.90 (m, 2H), 2.21 (s, 6H), 2.43 (t, *J* = 8.0 Hz, 2H), 3.95 (s, 3H), 3.99 (t, *J* = 6.0 Hz, 2H), 6.92 (d, *J* = 8.0 Hz, 2H), 7.20 (d, *J* = 8.0 Hz, 1H), 7.50–7.53 (m, 1H), 7.58 (d, *J* = 8.0 Hz, 1H), 7.68 (d, *J* = 8.0 Hz, 2H), 7.74 (d, *J* = 12.0 Hz, 1H), 8.47 (s, 1H), 8.57 (d, *J* = 8.0 Hz, 1H), 8.63 (d, *J* = 4.0 Hz, 1H), 8.72 (d, *J* = 4.0 Hz, 1H), 8.85 (s, 1H).

Compounds **B15**–**B20** were prepared by using the general procedure described above.

6.3.21. *N*-(4-(2-(Dimethylamino)ethoxy)phenyl)-4-methoxyl-3-(4-(pyridin-3-yl)pyrimidin-2-ylamino)benzamide (B15)

Mp 149–151 °C, EI-MS (*m/z*): 484.2[M]$^+$, ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.22 (s, 6H), 2.62 (t, *J* = 6.0 Hz, 2H), 3.95 (s, 3H), 4.04 (t, *J* = 6.0 Hz, 2H), 6.94 (d, *J* = 8.0 Hz, 2H), 7.20 (d, *J* = 8.0 Hz, 1H), 7.50–7.53 (m, 1H), 7.58 (d, *J* = 8.0 Hz, 1H), 7.68 (d, *J* = 8.0 Hz, 2H), 7.74 (d, *J* = 8.0 Hz, 1H), 8.47 (s, 1H), 8.57 (d, *J* = 8.0 Hz, 1H), 8.63 (d, *J* = 4.0 Hz, 1H), 8.72 (d, *J* = 4.0 Hz, 1H), 8.85 (s, 1H).

6.3.22. *N*-(4-(2-(Diethylamino)ethoxy)phenyl)-4-methoxyl-3-(4-(pyridin-3-yl)pyrimidin-2-ylamino)benzamide (B16)

Mp 149–152 °C, EI-MS (*m/z*): 512.3[M]$^+$, ^{1}H NMR (400 MHz, DMSO-d_6): δ = 0.98 (t, *d* = 8.0 Hz, 6H), 2.55 (q, *J* = 6 Hz, 14 Hz, 4H), 2.77 (t, *J* = 6.0 Hz, 2H), 3.95 (s, 3H), 4.00 (t, *J* = 6.0 Hz, 2H), 6.93 (d, *J* = 8.0 Hz, 2H), 7.20 (d, *J* = 8.0 Hz, 1H), 7.50–7.53 (m, 1H), 7.58 (d, *J* = 8.0 Hz, 1H), 7.68 (d, *J* = 8.0 Hz, 2H), 7.74 (d, *J* = 8.0 Hz, 1H), 8.47 (s, 1H), 8.57 (d, *J* = 8.0 Hz, 1H), 8.63 (d, *J* = 4.0 Hz, 1H), 8.72 (d, *J* = 4.0 Hz, 1H), 8.85 (s, 1H).

6.3.23. 4-Methoxyl-3-(4-(pyridin-3-yl)pyrimidin-2-ylamino)-*N*-(4-(2-(pyrrolidin-1-yl) ethoxy) phenyl) benzamide (B17)

Mp 118–121 °C, EI-MS (*m/z*): 510.2[M]$^+$, ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.70 (m, 4H), 2.55 (m, 4H), 2.82 (t, *J* = 6.0 Hz, 2H), 3.95 (s, 3H), 4.06 (t, *J* = 6.0 Hz, 2H), 6.94 (d, *J* = 8.0 Hz, 2H), 7.20 (d, *J* = 8.0 Hz, 1H), 7.50–7.53 (m, 1H), 7.58 (d, *J* = 8.0 Hz, 1H), 7.68 (d, *J* = 8.0 Hz, 2H), 7.75 (d, *J* = 8.0 Hz, 1H), 8.47 (s, 1H), 8.57 (d, *J* = 8.0 Hz, 1H), 8.63 (d, *J* = 4.0 Hz, 1H), 8.72 (d, *J* = 4.0 Hz, 1H), 8.86 (s, 1H).

6.3.24. 4-Methoxyl-*N*-(4-(2-(piperidin-1-yl)ethoxy) phenyl)-3-(4-(pyridin-3-yl) pyrimidin-2-ylamino) benzamide (B18)

Mp 141–144 °C, EI-MS (*m/z*): 524.2[M]$^+$, ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.10 (m, 2H), 2.60 (m, 8H), 3.06 (t, *J* = 4.0 Hz, 2H), 4.03 (s, 3H), 4.27 (t, *J* = 4.0 Hz, 2H), 6.95 (d, *J* = 8.0 Hz, 2H), 7.01 (d, *J* = 8.0 Hz, 1H), 7.41–7.45 (m, 1H), 7.59 (d, *J* = 8.0 Hz, 2H), 7.63 (d, *J* = 8.0 Hz, 1H), 7.90 (s, 1H), 7.98 (s, 1H), 8.51 (d, *J* = 8.0 Hz, 1H), 8.60 (d, *J* = 4.0 Hz, 1H), 8.76 (s,1H).

6.3.25. 4-Methoxyl-*N*-(4-(2-morpholinoethoxy) phenyl)-3-(4-(pyridin-3-yl) pyrimidin-2-ylamino) benzamide (B19)

Mp 149–153 °C, EI-MS (*m/z*): 439.1[M−C_4H_8NO]$^+$, ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.71 (m, 2H), 3.60 (m, 4H), 3.5 (s, 3H), 4.08 (t, *J* = 6.0 Hz, 2H), 6.94 (d, *J* = 12.0 Hz, 2H), 7.20 (d, *J* = 8.0 Hz, 1H), 7.25 (m, 1H), 7.50–7.53 (m, 1H), 7.58 (d, *J* = 4.0 Hz, 1H), 7.68 (d, *J* = 12.0 Hz, 2H), 7.75 (d, *J* = 12.0 Hz, 1H), 8.47 (s, 1H), 8.57 (d, *J* = 8.0 Hz, 1H), 8.63 (d, *J* = 4.0 Hz, 1H), 8.72 (d, *J* = 4.0 Hz, 1H), 8.85 (s, 1H).

6.3.26. 4-Methoxyl-*N*-(4-(3-morpholinopropoxy) phenyl)-3-(4-(pyridin-3-yl) pyrimidin-2-ylamino) benzamide (B20)

mp 177–180 °C, EI-MS (*m/z*): 540.2[M]$^+$, ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.04–2.07 (m, 2H), 2.58 (m, 4H), 2.63 (t, *J* = 8.0 Hz, 2H), 3.0 (t, *J* = 4.0 Hz, 4H), 4.03 (s, 3H), 4.06 (t, *J* = 6.0 Hz, 2H), 6.93 (d, *J* = 8.0 Hz, 2H), 7.00 (d, *J* = 12.0 Hz, 1H), 7.25 (d, *J* = 8.0 Hz, 1H), 7.40–7.44 (m, 1H), 7.58 (d, *J* = 12.0 Hz, 2H), 7.62 (d, *J* = 8.0 Hz, 1H), 7.90 (s, 1H), 7.95 (s, 1H), 8.50 (d, *J* = 8.0 Hz, 1H), 8.60 (d, *J* = 4.0 Hz, 1H), 8.74 (d, *J* = 4.0 Hz, 1H).

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Supplementary data

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