Bioorganic & Medicinal Chemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Structure-based design and synthesis of pyrimidine-4,6-diamine derivatives as Janus kinase 3 inhibitors

Ru-Nan Yu[a], Cheng-Juan Chen[b], Lei Shu[a], Yuan Yin[a], Zhi-Jian Wang[a], Tian-Tai Zhang[b,*], Da-Yong Zhang[a,*]

[a] College of Science, China Pharmaceutical University, Nanjing, PR China
[b] Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, PR China

ARTICLE INFO

Keywords:
Janus kinase
JAK3 inhibitors
Pyrimidine-4,6-diamine derivatives
Cys909

ABSTRACT

Janus kinases (JAKs) play a key role in the proliferation, apoptosis and differentiation of immune cells, and JAKs are considered as an attractive target for the treatment of inflammatory and autoimmune diseases. Here we show the design and optimization of pyrimidine-4,6-diamine derivatives as selectivity JAK3 inhibitors. Compound **11e**, which might interact with unique cysteine (Cys909) residue in JAK3, exhibited excellent JAK3 inhibitory activity (IC_{50} = 2.1 nM) and high JAK kinase selectivity. In cellular assay, **11e** showed moderate potency inhibiting IL-2-stimulated T cell proliferation. The data supports the further development of novel JAKs inhibitors.

1. Introduction

The Janus kinases (JAKs) as non-receptor tyrosine kinases consist of four known members in humans: JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2), which are critically important for signaling transduction pathways mediated by cytokines.[1] JAKs transduce cytokines signaling from membrane receptors to various signal transducer and activator of transcription proteins (STATs), and the JAK-STAT signaling pathway has been extensively studied.[2,3] Upon binding of cytokine to the receptors, JAKs become activated and phosphorylate tyrosine residues in the receptor cytoplasmic domains, which lead to recruitment and activation of downstream signaling proteins such as STATs.[4,5] STATs phosphorylation leads to their dimerization and translocation to the nucleus where they regulate expression of specific genes involved in proliferation, apoptosis and differentiation.[6,7]

In humans, JAKs are nearly ubiquitously expressed, with the exception of JAK3, which is mainly expressed in hematopoietic cells.[8] Another feature of JAK3 is that it specifically associates with the common gamma chain (γc) cytokines such as IL-2, IL-4, IL-7, IL-9, and IL-15, which play important roles in *T*-cell differentiation, proliferation and survival.[18] Genetic analysis of severe combined immunodeficiency (SCID) patients showed genetic mutations of JAK3 and decreased expression of JAK3 protein expression.[9] JAK3 knockout mice also exhibit immunodeficiency, consistent with the mutational consequences found in humans. On the other hand, JAK1 knockout mice were found to die shortly after birth and JAK2 deficiency in mice is embryonic lethal due to a lack of erythropoiesis.[10–12] TYK2 knockout mice are viable and fertile but showed increased susceptibility to infections, decreased responses to lipopolysaccharide triggering and development of collagen-induced rheumatoid arthritis (RA).[13]

From these insights in the role of JAK3 for the development and functions of the immune system and its restricted expression in hematopoietic tissues, JAK3 has been pursued as a target for the treatment of inflammation, autoimmune diseases, such as RA psoriasis, inflammatory bowel disease, and organ transplant rejection. Another feature of JAK3 inhibitors is that toxic and side effects are avoided observed with current therapies.[14] We therefore selected JAK3 as the target for our research, with an immediate goal of understanding the relationships between inhibitor structure and selectivity across the Janus associated kinases, JAK 1–3 and TYK2.

Due to the high degree of structural conservation of the JAK ATP binding pockets, it remains a challenging task, for medicinal chemists, to develop highly selective inhibitors as pharmacological probes and as clinical drugs. To date, only a few compounds with appropriate isoform selectivity for JAK3 have been reported. Tofacitinib (**1**, Fig. 1) was, at first, described as a selective JAK3 inhibitor and then subsequent studies revealed that it is a pan JAK inhibitor, and Ruxolitinib (**2**, Fig. 1) is accepted as a selective JAK1/2 inhibitor.[15,16] A US patent from Portola describes **4** is at least 100-fold selective for JAK3 over JAK1/2. In 2016, it was reported that **3** (**PF-06651600**) inhibited JAK3 kinase activity with an IC_{50} of 33.1 nm but without activity against JAK1, JAK2 and TYK2. Then they demonstrated that **PF-06651600** form an irreversible

* Corresponding authors. Tel.: +86 25 83271315 (D.-Y. Zhang), +86 10 63035779 (T.-T. Zhang).
E-mail addresses: ttzhang@imm.ac.cn (T.-T. Zhang), cpuzdy@163.com (D.-Y. Zhang).

https://doi.org/10.1016/j.bmc.2019.03.009
Received 9 January 2019; Received in revised form 28 February 2019; Accepted 3 March 2019

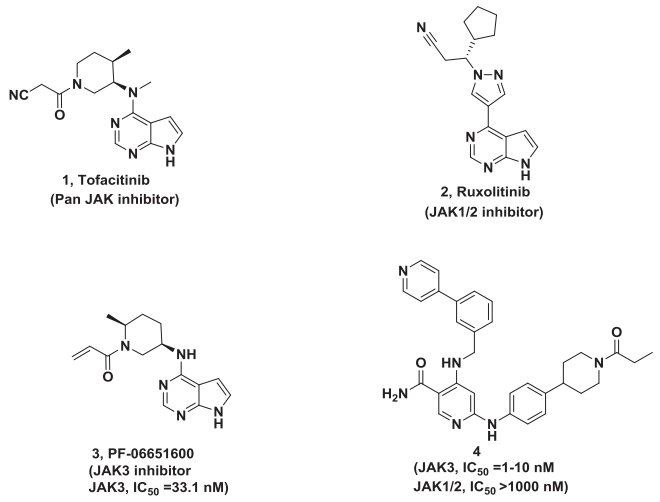


Fig. 1. Chemical structures of JAK inhibitors.

covalent bond with Cys909 in JAK3, a residue replaced by a serine in the other three JAK isoforms.[10,17] In 2017, Michael Forster[19] also reported that acrylamide could react with Cys909 covalently via 1,4-(conjugate) addition (sulfa-Michael addition).

Here we present our initial studies toward selective JAK3 inhibitors. In our laboratory, we aimed to exploit a compound which can interact with Cys909. Based on the structure comparison of **3** and **4**, compound **7**, a pyrimidine-4,6-diamine derivative, was designed with the strategy of scaffold hopping (Fig. 2). The molecular structure of pyrimidine-4,6-diamine derivatives consist of four parts: core, side chain, tail and the linkage between core and tail. The core was expected to interact with hinge residues by two hydrogen bonds in similar binding way to that of **3**. The side chain was considered to facilitate interaction with JAK3 at the hydrophobic cavity, and have opportunities to improve pharmacokinetic profile. And benzylamine links the core and the tail. The tail, as the key to both high isoform and kinome selectivity, tends to possess electrophilic group, such as Michael acceptors, which can form a covalent bond with the cysteine thiol group.[19] After further optimization, our studies showed that the compound **11e**, **11d** are potent JAK3 inhibitors. Here, we report the design, synthesis and structure-activity relationship (SAR) of pyrimidine-4,6-diamine JAK inhibitors.

2. Chemistry

A series of pyrimidine-4,6-diamine derivatives were synthesized according to pathways described in Schemes 1–4.

The key intermediates **10a–f** and **10a'–e'** were synthesized according to Scheme 1. Commercially available 4,6-dichloropyrimidine was treated with (3-nitrophenyl)methanamine or (4-nitrophenyl)methanamine to afford **8a** or **8a'**. Then **8a** or **8a'** was reacted with several kinds of amines to give the *N*, *N*-substituted pyrimidine-4,6-diamine compounds (**9a-f**, **9a'–e'**) under acidic conditions. Treatment of **9a–f** or **9a'–e'** with 85% hydrazine hydrate under nitrogen and Pd/C catalysis provided reduction products **10a–f** or **10a'–e'**.

The target compounds was prepared from **10a–f** or **10a'–e'** as shown in Scheme 2. Intermediates (**10a–f**, **10a'–e'**) was treated with a few kinds of acyl chlorides to give the desired compounds (**11a–f**, **11a'–e'**, **12a–e**, **12a'–e'**, **13a–e** and **13a'–e'**) under basic conditions.

Scheme 3 shows the synthesis of the other target compounds. Treatment of **10c-e** or **10e'** with cyanoacetic acid and 2-(7-Aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) gave **14c–e**, **14e'**. Intermediates (**10c–e** and **10e'**) were reacted with cyanogen bromide to give the targets **15c–e** and **15e'**. And **10d** or **10d'** were treated with trifluoroacetic anhydride to give **16d**, **16d'**.

Scheme 4 shows the synthesis of several amines. Commercially available 4-nitro-1*H*-pyrazole was treated with alkyl halide such as iodomethane, 2-bromoethanol, 1-bromo-2-methoxyethane to give **17a–c**, and followed by hydrogenation in the presence of Pd/C to give amines (**18a–c**). Commercially available 4-fluoronitrobenzene was reacted with morpholine or 1-methylpiperazine to provide **19d**, **19e**. Then 4-fluoronitrobenzene and **19d**, **19e** were, separately, treated with hydrogen under Pd/C catalysis to give several amines (**20d–f**).

3. Results and discussion

3.1. In vitro enzymatic inhibitor activities

We evaluated the inhibitory activity of newly synthesized compounds on human JAK1, JAK2 and JAK3 enzyme, and some compounds (JAK3 IC_{50} < 10 nM) on human TYK2 enzyme. After scaffold hopping, the chemotype of target compounds was divided to four moieties, core, side chain (R_1), tail (R_2) and the linkage between core and tail. Then side chain and tail were varied sequentially.

The first series of analogues focused on modification of the side chain (R_1) (Table 1). Compound **7** showed low inhibitory activity, and when the 4-position of the aniline was substituted by fluorine, **11f** had increased the potency against JAK3 (IC_{50} = 589 nM). In consideration of **4** possessing a long side chain, adding a morpholine ring at 4-position of the aniline dramatically improved the kinase selectivity and potency. Introduction of a piperazine ring **11e** exhibited a similar or higher JAK3 inhibitory activity than **11d** (**11d**, IC_{50} = 2.4 nM, **11e**, IC_{50} = 2.1 nM). Regarding the feature of JAK3-ATP binding site, side chains of **11d** and **11e** might effectively interact with hydrophobic cavity. To enhance affinity for JAK3 in the pocket of the hydrophobic region, we investigated the conversion of side chain to a pyrazole ring (**11a**, **11b**, **11c**, IC_{50} = 12.3 nM, 7.2 nM, 7.0 nM, respectively) showed higher

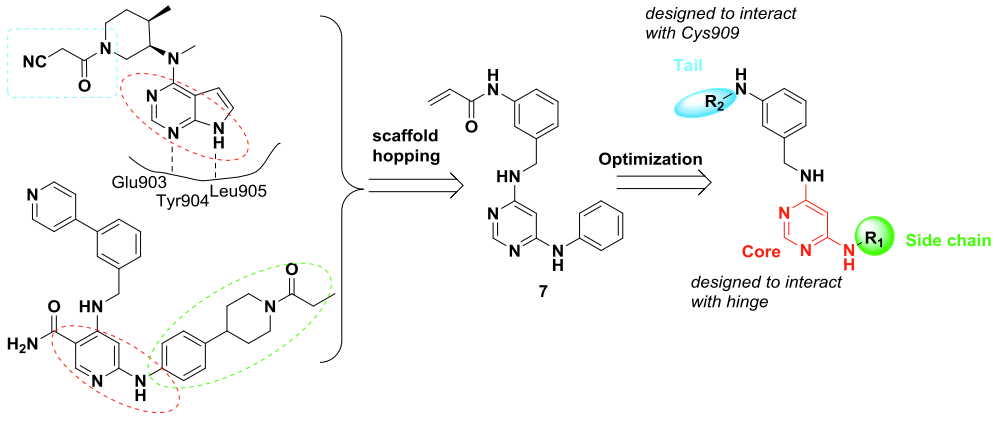
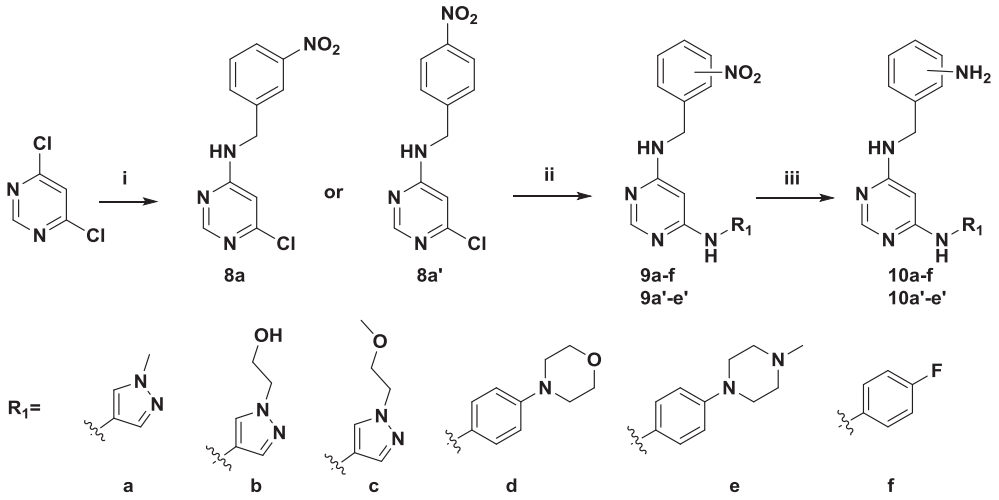


Fig. 2. Design of the target compound.



Scheme 1. Reagents and conditions: (i) Nitro-substituted benzyl amine, DIEA, Isopropanol, 82 °C, 6 h; (ii) amine, TFA, *n*-BuOH, 120 °C, 6 h; (iii) Pd/C, hydrazine hydrate, EtOH, 100 °C, 2 h.

activity than **11f**, and exhibited moderately selective JAK3 inhibition, with about 50-fold, 150-fold, 110-fold, respectively, higher IC_{50} against JAK1. Obviously the 2-methoxyethyl (**11c**) displayed more potent than 1-methyl (**11a**), which means that the length of side chain plays an important role in JAK3 inhibitory activity. In addition, some compounds with acrylamide, such as **11b**, **11c**, **11d**, **11e**, exhibited poor TYK2 inhibitory activity, which may be due to acrylamide not matching TYK2 active site.

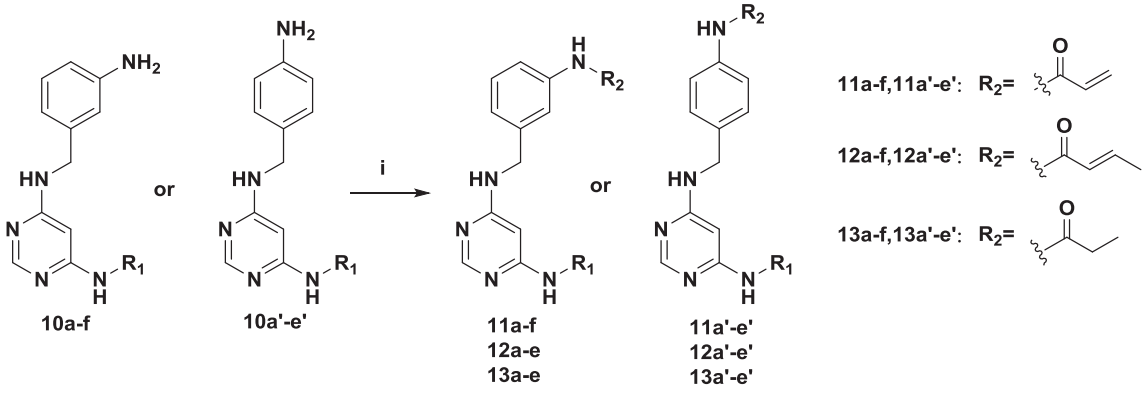
After optimization of the side chain, the SAR of the tail (R_2) was investigated (Tables 1 and 2). To investigate spatial tolerance around the tail, the acrylamide was switched from meta- to para- on the phenyl ring. The results (**11a'–11e'**) exhibited weaker both JAK3 inhibitory potency and selectivity than analogues with *meta*-acrylamide (**11a–11e**). As shown in Table 2, replacing the acrylamide with a crotonamide (**12d**, **12e**) or propionamide (**13d**, **13e**) resulted in over 40-fold or 100-fold decrease of potency against JAK3. Incorporation of cyanoacetamide (**14e**) maintained the potency with the JAK3 IC_{50} of 3.5 nM, while **14e'** did not, and as reported by H. Yamagishi et al.,[21,22] they showed poor selectivity against JAK3. Meanwhile cyanoacetamides showed higher TYK2 inhibitory activity than acrylamides. To our surprise, **15c**, **15d**, **15e**, with a cyan group, exhibited not JAK3 selectivity but JAK1 selectivity. And **16d**, **16d'**, with a trifluoroacetamide, exhibited JAK2 selectivity. In summary, the modification of R_2 failed to further improve our JAK3 inhibitors, but found some similar potent JAK3 inhibitors (**14e**, **14d**).

3.2. The docking study to human JAK3

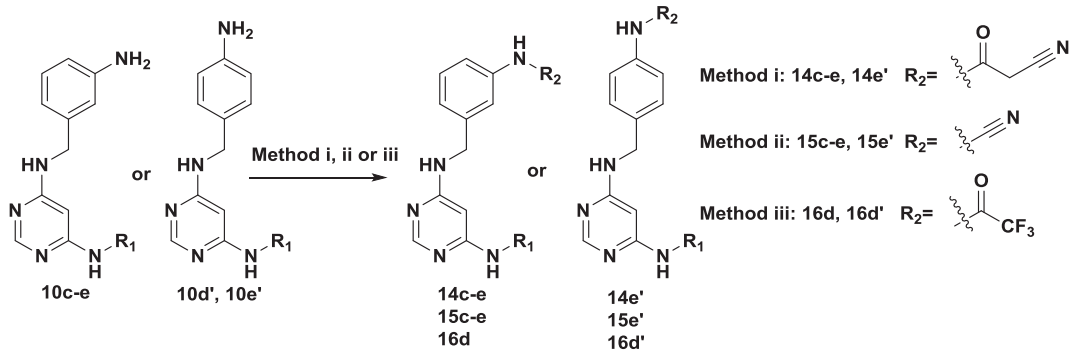
Docking study of the pyrimidine-4,6-diamine derivatives with JAK3 kinase was investigated. In Fig. 3, this study showed that the structure was found to be consistent with our expected model. The pyrilamine core makes the expected bidentate hinge hydrogen bonds with Leu905, and the unique Cys909 in human JAK3 have been successfully targeted by acrylamide, as the distance between the sulfur atom of Cys909 and acrylamide warhead in the **11e** is 1.948 Å. The side chain of **11e** extends toward the hydrophobic region. Furthermore, the phenyl group of **11e** is in van der Waals contact with Leu956 and Leu828 in the ATP-binding pocket. Moreover, the acrylamide forms a hydrogen bond with the carbonyl of Arg953.

3.3. Rat T cell proliferation

Due to its high kinase potency and selectivity, we selected compounds **11c**, **11d**, **11e**, **14e** and **14d** for further tests (Table 3). In the cellular assay of IL-2-stimulated T cell proliferation, **11e** exhibited excellent anti-proliferative activity of the T cell lines with IC_{50} value of 12 nM, and **11d** (IC_{50} = 15 nM) showed lower inhibitory compared to **11e**. Compounds **14d**, **14e** were demonstrated low potency for the T cell lines, despite the fact that they have moderate potency against JAK3. From this point of view, acrylamide plays a more important role in inhibitors than cyanoacetamide.



Scheme 2. Reagents and conditions: (i) acyl chlorides, THF, 0 °C to room temperature (RT), 30 min.



Scheme 3. Reagents and conditions: (i) Cyanoacetic acid, HATU, DIEA, THF, 16 h (for **14c–e**, **14e'**); (ii) cyanogen bromide, potassium acetate, THF, RT, 16 h (for **15c–e**, **15e'**); (iii) trifluoroacetic anhydride, THF, RT, 16 h (for **16d**, **16d'**).

4. Conclusion

We designed and synthesized pyrimidine-4,6-diamine derivatives as novel JAK3 inhibitors. Among these analogues, compound **11d**, **11e**, **14e** stood out with overall favorable inhibitory activity and selectivity. And **11e** is our most selective JAK3 inhibitor, with at least 400-fold over other JAKs in vitro enzymatic assays. Primary studies demonstrated that hinge hydrogen bonds, hydrophobic moiety and its specific length are responsible for **11e**'s interaction with JAK3. Furthermore, acrylamide, reacting covalently with Cys909 same as reported by others,[19,20] significantly enhance both activity and selectivity against JAK3. Our further studies will be aimed to exploiting the complicated SARs, and the studies about metabolic stability and oral bioavailability in rats will be planned in the future.

5. Experimental

5.1. Chemical synthesis

^{1}H NMR and ^{13}C NMR spectra were recorded with Bruker ARX-300 spectrometer with TMS as the internal standard in $CDCl_3$, DMSO-d_6. Peak multiplicities are abbreviated: singlet, s; doublet, d; triplet, t; quartet, q; multiplet, m; etc. ESI-MS was run on a Bruker micro-TOF-Q mass spectrometer. Most chemicals were purchased from Sigma–Aldrich and Fluka. All commercially available reagents were used without further purification. The solvents used were all AR grade and were redistilled under positive pressure of dry nitrogen atmosphere in the presence of proper desiccant when necessary. Silica gel (200–300 mesh, Qingdao city, China) was used for column chromatography. The progress of the reactions was monitored by analytical thin-layer chromatography (TLC) on HSGF254 precoated silica gel plates.

5.1.1. 6-Chloro-N-(3-nitrobenzyl)pyrimidin-4-amine (8a)

2,6-Dichloropyrimidine (0.1 g, 0.68 mmol), (3-nitrophenyl)methanamine (0.11 g, 0.74 mmol), and *N*, *N*-diisopropylethylamine (DIEA, 0.22, 1.69 mmol) were combined in isopropanol (5 mL) and stirred overnight at 82 °C. The mixture was then diluted with ethyl acetate and washed with water and brine, dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by column chromatography (hexane:ethyl acetate = 1:1) to yield 0.14 g (81%) of **8a** as a white solid. MS (ESI) *m/z* 265 $[M+H]^+$.

5.1.2. 6-Chloro-N-(4-nitrobenzyl)pyrimidin-4-amine (8a')

Compound **8a'** was prepared in 80% yield by a method similar to that described for **8a**. MS (ESI) *m/z* 265 $[M+H]^+$.

5.1.3. N_4-(1-Methyl-1H-pyrazol-4-yl)-N_6-(3-nitrobenzyl)pyrimidine-4,6-diamine (9a)

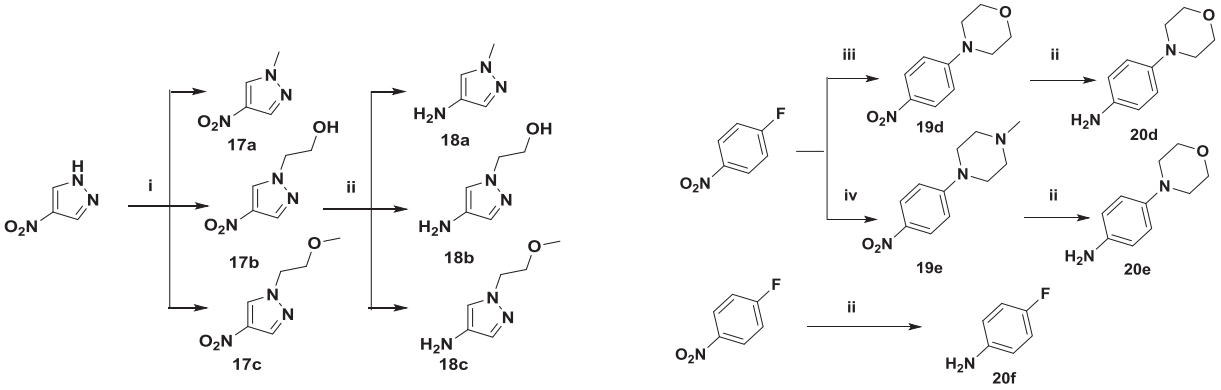
To **8a** (0.25 g, 0.95 mmol) and 1-methyl-1*H*-pyrazol-4-amine hydrochloride salt (0.14 g, 1.1 mmol) in *n*-butanol (5 mL) was added trifluoroacetic acid (0.27 g, 2.4 mmol), and the mixture was stirred overnight at 120 °C. The mixture was then concentrated, neutralized with ammonia in methanol, and purified by column chromatography (dichloromethane: methanol = 60:1) to yield 0.26 g (86.7%) of **9a** as a pale-yellow solid. MS (ESI) *m/z* 326 $[M+H]^+$.

5.1.4. 2-(4-((6-((3-Nitrobenzyl)amino)pyrimidin-4-yl)amino)-1H-pyrazol-1-yl)ethan-1-ol (9b)

Compound **9b** was prepared from **8a** in 79% yield by a method similar to that described for **9a**. MS (ESI) *m/z* 356 $[M+H]^+$.

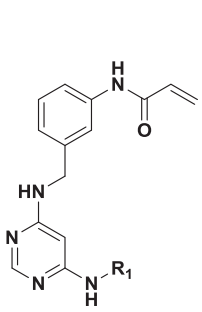
5.1.5. N_4-(1-(2-methoxyethyl)-1H-pyrazol-4-yl)-N_6-(3-nitrobenzyl) pyrimidine-4,6-diamine (9c)

Compound **9c** was prepared from **8a** in 83% yield by a method

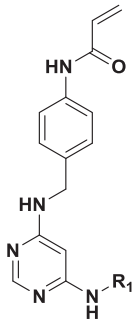


Scheme 4. Reagents and conditions: (i) alkyl halide, K_2CO_3, MeCN, ref., 6 h; (ii) Pd/C, H_2, RT, 3 h; (iii) morpholine, K_2CO_3, DMSO, 120 °C, 20 h (for **19d**); (iv) 1-methylpiperazine, K_2CO_3, DMSO, RT, 6 h(for **19e**).

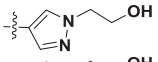
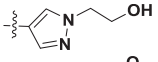
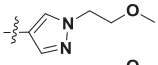
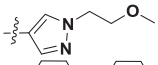
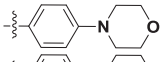
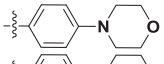
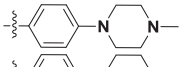
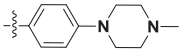
Table 1
SARs of side chains (R_1).



7, 11f, 11a-e



11a'-e'

Compd	R_1	IC_{50}[a](nM)				JAK1/JAK3[b]	JAK2/JAK3[c]	TYK2/JAK3[d]
		JAK3	JAK2	JAK1	TYK2			
7		730	> 3000	> 3000	ND[e]	–	–	–
11f		589	> 3000	> 3000	ND[e]	–	–	–
11a		12.3	> 3000	656	ND[e]	53.3	–	–
11a'		24.1	> 3000	643	ND[e]	26.7	–	–
11b		7.2	> 3000	1126	2561	156.4	–	156.4
11b'		9.4	2098	1240	2175	131.9	223.2	131.9
11c		7.0	2043	774	2290	110.6	291.8	327.1
11c'		9.3	ND[e]	852	2295	91.6	–	246.8
11d		2.4	1096	1286	> 3000	535.8	456.7	–
11d'		8.4	916	1159	> 3000	138.0	109	–
11e		2.1	945	> 3000	> 3000	–	450	–
11e'		7.9	> 3000	731	> 3000	92.5	–	–
Tofacitinib		0.7	0.5	1.4	32.3	2	0.7	46.1

[a] IC_{50} values are the average of duplicate experiments.
[b] Fold selectivity derived from JAK1/JAK3 enzyme IC_{50}.
[c] Fold selectivity derived from JAK2/JAK3 enzyme IC_{50}.
[d] Fold selectivity derived from TYK2/JAK3 enzyme IC_{50}.
[e] ND = not determined.

similar to that described for **9a**. MS (ESI) *m/z* 370 $[M+H]^+$.

5.1.6. N_4-(4-Morpholinophenyl)-N_6-(3-nitrobenzyl)pyrimidine-4,6-diamine (9d)

Compound **9d** was prepared from **8a** in 89% yield by a method similar to that described for **9a**. MS (ESI) *m/z* 407 $[M+H]^+$.

5.1.7. N_4-(4-(4-Methylpiperazin-1-yl)phenyl)-N_6-(3-nitrobenzyl) pyrimidine-4,6-diamine (9e)

Compound **9e** was prepared from **8a** in 81.5% yield by a method similar to that described for **9a**. MS (ESI) *m/z* 420 $[M+H]^+$.

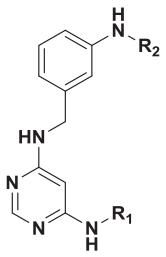
5.1.8. N_4-(4-fluorophenyl)-N_6-(3-nitrobenzyl)pyrimidine-4,6-diamine (9f)

Compound **9f** was prepared from **8a** in 82% yield by a method similar to that described for **9a**. MS (ESI) *m/z* 340 $[M+H]^+$.

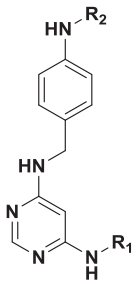
5.1.9. N_4-(1-Methyl-1H-pyrazol-4-yl)-N_6-(4-nitrobenzyl)pyrimidine-4,6-diamine (9a')

To **8a'** (0.25 g, 0.95 mmol) and 1-methyl-1*H*-pyrazol-4-amine hydrochloride salt (0.14 g, 1.1 mmol) in *n*-butanol (5 mL) was added trifluoroacetic acid (0.27 g, 2.4 mmol), and the mixture was stirred overnight at 120 °C. The mixture was then concentrated, neutralized with ammonia in methanol, and purified by column chromatography (dichloromethane: methanol = 60:1) to yield 0.26 g (83.0%) of **9a'** as a

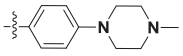
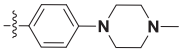
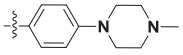
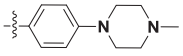
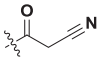
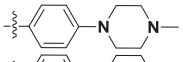
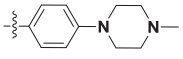
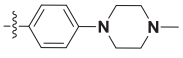
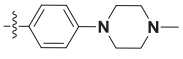
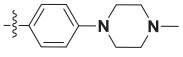
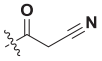
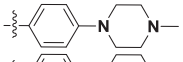
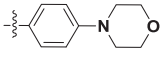
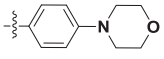
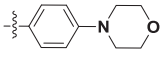
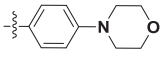
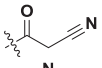
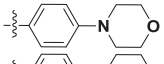
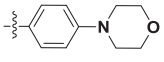
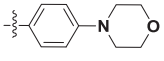
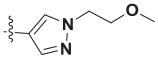
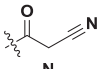
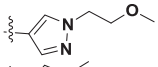
Table 2
SARs of tails (R_2).



11e, 12e, 13e, 14e, 15e, 11d, 12d, 13d, 14d, 15d, 16d, 14c, 15c, 12a



11e', 12e', 13e', 14e', 15e', 16d', 12a'

Compd	R_1	R_2	IC_{50}[a](nM)				JAK1/JAK3[b]	JAK2/JAK3[c]	TYK2/JAK3[d]
			JAK3	JAK2	JAK1	TYK2			
11e			2.1	945	> 3000	> 3000	–	450	–
12e			89	ND[e]	1821	ND[e]	20.46	–	–
13e			212	ND[e]	ND[e]	ND[e]	–	–	–
14e			3.5	1015	923	1532	263	290	437.7
15e			> 3000	350	90	ND[e]	–	–	–
11e'			7.9	> 3000	731	> 3000	92.5	–	–
12e'			101	ND[e]	2610	ND[e]	25.8	–	–
13e'			290	ND[e]	ND[e]	ND[e]	–	–	–
14e'			155	ND[e]	1242	ND[e]	8	–	–
15e'			> 3000	503	124	ND[e]	–	–	–
11d			2.4	1096	1286	> 3000	535.8	456.7	–
12d			99	ND[e]	2032	ND[e]	20.52	–	–
13d			278	ND[e]	ND[e]	ND[e]	–	–	–
14d			4.84	1444	920	1564	190	298	323.1
15d			> 3000	345	92	ND[e]	–	–	–
16d			> 3000	366	> 3000	ND[e]	–	–	–
16d'			> 3000	280	> 3000	ND[e]	–	–	–
14c			7.3	ND[e]	1262	1740	172.8	–	238.4
15c			> 3000	2790	285	ND[e]	–	–	–
12a			342	ND[e]	1319	ND[e]	3.9	–	–
12a'			389	ND[e]	1586	ND[e]	4.1	–	–

(*continued on next page*)

Table 2 (*continued*)

Compd	R_1	R_2	IC_{50}[a](nM)				JAK1/JAK3[b]	JAK2/JAK3[c]	TYK2/JAK3[d]
			JAK3	JAK2	JAK1	TYK2			
Tofacitinib			0.7	0.5	1.4	32.3	2	0.7	46.1

[a] IC_{50} values are the average of duplicate experiments.
[b] Fold selectivity derived from JAK1/JAK3 enzyme IC_{50}.
[c] Fold selectivity derived from JAK2/JAK3 enzyme IC_{50}.
[d] Fold selectivity derived from TYK2/JAK3 enzyme IC_{50}.
[e] ND = not determined.

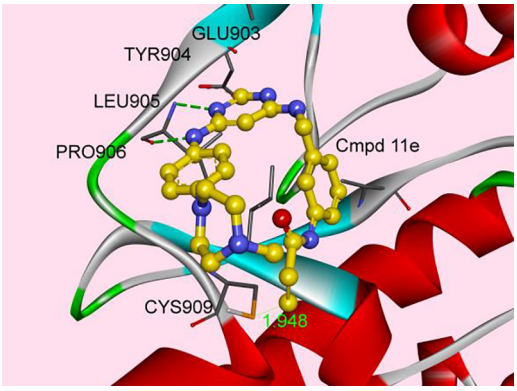


Fig. 3. The structure of JAK3 bound with compound **11e** (yellow sticks) in the ATP-binding site. Hydrogen bonds of **11e** with JAK3 are indicated by green dotted lines. The distance between the sulfur atom of Cys909 and acrylamide warhead in the **11e** is showed.

Table 3
Cellular assay of pyrimidine-4,6-diamine derivatives.

Compound	T cell proliferation IC_{50}[a](nM)
11c	41
11d	15
11e	12
14e	50
14d	64
Tofacitinib	2

[a] Inhibitory effect on IL-2-stimulated T cell proliferation using rats spleen cells.

pale-yellow solid. MS (ESI) *m/z* 326 $[M+H]^+$.

*5.1.10. 2-(4-((6-((3-Nitrobenzyl)amino)pyrimidin-4-yl)amino)-1H-pyrazol-1-yl)ethan-1-ol (**9b'**)*

Compound **9b'** was prepared from **8a'** in 82% yield by a method similar to that described for **9a'**. MS (ESI) *m/z* 356 $[M+H]^+$.

*5.1.11. N_4-(1-(2-methoxyethyl)-1H-pyrazol-4-yl)-N_6-(3-nitrobenzyl) pyrimidine-4,6-diamine (**9c'**)*

Compound **9c'** was prepared from **8a'** in 85% yield by a method similar to that described for **9a'**. MS (ESI) *m/z* 370 $[M+H]^+$.

*5.1.12. N_4-(4-Morpholinophenyl)-N_6-(3-nitrobenzyl)pyrimidine-4,6-diamine (**9d'**)*

Compound **9d'** was prepared from **8a'** in 80% yield by a method similar to that described for **9a'**. MS (ESI) *m/z* 407 $[M+H]^+$.

*5.1.13. N_4-(4-(4-Methylpiperazin-1-yl)phenyl)-N_6-(3-nitrobenzyl) pyrimidine-4,6-diamine (**9e'**)*

Compound **9e'** was prepared from **8a'** in 82% yield by a method similar to that described for **9a**. MS (ESI) *m/z* 420 $[M+H]^+$.

*5.1.14. N_4-(3-Aminobenzyl)-N_6-(1-methyl-1H-pyrazol-4-yl)pyrimidine-4,6-diamine (**10a**)*

To **9a** (100 mg, 0.30 mmol) and 10% Pd/C 20 mg in EtOH (20 mL) was added drop by drop 85% hydrazine hydrate (0.36 g, 6.1 mmol) at 100 °C. The reaction mixture was stirred for 2 h under nitrogen. The mixture was then filtered with Celite, and the filtrate was concentrated and dried under vacuum to yield 86 mg (95%) of **10a** as a white solid. MS (ESI) *m/z* 296 $[M+H]^+$.

*5.1.15. 2-(4-((6-((3-Aminobenzyl)amino)pyrimidin-4-yl)amino)-1H-pyrazol-1-yl)ethan-1-ol (**10b**)*

Compound **10b** was prepared from **9b** in 95% yield by a method similar to that described for **10a**. MS (ESI) *m/z* 326 $[M+H]^+$.

*5.1.16. N_4-(3-Aminobenzyl)-N_6-(1-(2-methoxyethyl)-1H-pyrazol-4-yl) pyrimidine-4,6-diamine (**10c**)*

Compound **10c** was prepared from **9c** in 96% yield by a method similar to that described for **10a**. MS (ESI) *m/z* 340 $[M+H]^+$.

*5.1.17. N4-(3-Aminobenzyl)-N6-(4-morpholinophenyl)pyrimidine-4,6-diamine (**10d**)*

Compound **10d** was prepared from **9d** in 93% yield by a method similar to that described for **10a**. MS (ESI) *m/z* 377 $[M+H]^+$.

*5.1.18. N4-(3-Aminobenzyl)-N6-(4-(4-methylpiperazin-1-yl)phenyl) pyrimidine-4,6-diamine (**10e**)*

Compound **10e** was prepared from **9e** in 98% yield by a method similar to that described for **10a**. MS (ESI) *m/z* 390 $[M+H]^+$.

*5.1.19. N_4-(3-Aminobenzyl)-N_6-(4-fluorophenyl)pyrimidine-4,6-diamine (**10f**)*

Compound **10f** was prepared from **9f** in 95% yield by a method similar to that described for **10a**. MS (ESI) *m/z* 310 $[M+H]^+$.

*5.1.20. N_4-(4-Aminobenzyl)-N_6-(1-methyl-1H-pyrazol-4-yl)pyrimidine-4,6-diamine (**10a'**)*

Compound **10a'** was prepared from **9a'** in 93% yield by a method similar to that described for **10a**. MS (ESI) *m/z* 296 $[M+H]^+$.

*5.1.21. 2-(4-((6-((4-Aminobenzyl)amino)pyrimidin-4-yl)amino)-1H-pyrazol-1-yl)ethan-1-ol (**10b'**)*

Compound **10b'** was prepared from **9b'** in 96% yield by a method similar to that described for **10a**. MS (ESI) *m/z* 326 $[M+H]^+$.

*5.1.22. N_4-(4-Aminobenzyl)-N_6-(1-(2-methoxyethyl)-1H-pyrazol-4-yl) pyrimidine-4,6-diamine (**10c'**)*

Compound **10c'** was prepared from **9c'** in 92% yield by a method similar to that described for **10a**. MS (ESI) *m/z* 340 $[M+H]^+$.

*5.1.23. N_4-(4-Aminobenzyl)-N_6-(4-morpholinophenyl)pyrimidine-4,6-diamine (**10d'**)*

Compound **10d'** was prepared from **9d'** in 97% yield by a method similar to that described for **10a**. MS (ESI) *m/z* 377 $[M+H]^+$.

*5.1.24. N_4-(4-Aminobenzyl)-N_6-(4-(4-methylpiperazin-1-yl)phenyl) pyrimidine-4,6-diamine (**10e'**)*

Compound **10e'** was prepared from **9e'** in 94% yield by a method similar to that described for **10a**. MS (ESI) *m/z* 390 $[M+H]^+$.

*5.1.25. N_4-(3-nitrobenzyl)-N_6-phenylpyrimidine-4,6-diamine (**5**)*

To **8a** (0.25 g, 0.95 mmol) and aniline (0.09 g, 1 mmol) in *n*-butanol (5 mL) was added trifluoroacetic acid (0.27 g, 2.4 mmol), and the mixture was stirred overnight at 120 °C. The mixture was then concentrated, neutralized with ammonia in methanol, and purified by column chromatography (DCM: MeOH = 150:1) to yield 0.25 g (83.3%) of **5** as a pale-yellow solid. MS (ESI) *m/z* 322 $[M+H]^+$.

*5.1.26. N_4-(3-aminobenzyl)-N_6-phenylpyrimidine-4,6-diamine (**6**)*

To **5** (100 mg, 0.31 mmol) and 10% Pd/C 20 mg in EtOH (20 mL) was added drop by drop 85% hydrazine hydrate (0.36 g, 6.1 mmol) at 100 °C. The reaction mixture was stirred for 2 h under nitrogen. The mixture was then filtered with Celite, and the filtrate was concentrated and dried under vacuum to yield 76 mg (84%) of **6** as a white solid. MS (ESI) *m/z* 292 $[M+H]^+$.

*5.1.27. N-(3-(((6-(phenylamino)pyrimidin-4-yl)amino)methyl)phenyl) acrylamide (**7**)*

To a solution of **6** (40 mg, 0.14 mmol) in DCM (5 mL) were added DIEA (17.7 mg, 0.14 mmol) and acryloyl chloride (12.4 mg, 0.14 mmol) at −5 °C. The resulting mixture was stirred for 30 min. Then it was quenched by MeOH (2 mL), concentrated, and purified by silica gel column chromatography (DCM: MeOH = 40:1) to afford the title compound **7** (24.6 mg, 52%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.28 (s, 1H), 8.78 (s, 1H), 8.23 (s, 1H), 8.07 (s, 1H), 7.60 (d, J = 6 Hz, 2H), 7.44 (t, J = 9 Hz, 1H), 7.34 (t, J = 9 Hz, 2H), 7.26 (d, J = 9 Hz, 1H), 7.06 (d, J = 9 Hz, 1H), 7.00(t, J = 6 Hz, 1H), 6.46(dd, J = 15 Hz, 12 Hz, 1H), 6.17(d, J = 12 Hz, 1H), 5.64 (s, 1H), 5.56(d, J = 12 Hz, 1H), 4.41(d, J = 6 Hz, 2H); ^{13}C NMR (300 M DMSO-d_6) δ 164.76, 161.72, 158.86, 156.75, 139.82, 138.95, 137.54, 129.91, 128.88, 128.01, 126.57, 124.25, 121.76, 119.28, 118.20, 117.75, 79.17, 44.75; MS (ESI) *m/z* 346 $[M+H]^+$.

*5.1.28. N-(3-(((6-((4-fluorophenyl)amino)pyrimidin-4-yl)amino)methyl) phenyl)acrylamide (**11f**)*

To a solution of **10f** (40 mg, 0.13 mmol) in DCM (5 mL) were added DIEA (16.7 mg, 0.13 mmol) and acryloyl chloride (11.7 mg, 0.13 mmol) at −5 °C. The resulting mixture was stirred for 30 min. Then it was quenched by MeOH (2 mL), concentrated, and purified by silica gel column chromatography (DCM: MeOH = 40:1) to afford the title compound **5** (27.8 mg, 59%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.28 (s, 1H), 8.78 (s, 1H), 8.23 (s, 1H), 8.07 (s, 1H), 7.60 (d, J = 6 Hz, 2H), 7.44 (t, J = 9 Hz, 1H), 7.26 (d, J = 9 Hz, 1H), 7.06 (d, J = 9 Hz, 1H), 6.99(d, J = 9 Hz, 2H), 6.46(dd, J = 15 Hz, 12 Hz, 1H), 6.17(d, J = 12 Hz, 1H), 5.64 (s, 1H), 5.56(d, J = 12 Hz, 1H), 4.41(d, J = 6 Hz, 2H); ^{13}C NMR (300 M DMSO-d_6) δ 163.58, 163.14, 158.04, 139.62, 139.56, 132.35, 129.16, 127.18, 124.61, 124.37, 122.59, 118.27, 116.23, 115.66, 115.37, 78.83, 49.04; MS (ESI) *m/z* 364 $[M+H]^+$.

*5.1.29. N-(3-(((6-((1-methyl-1H-pyrazol-4-yl)amino)pyrimidin-4-yl) amino)methyl)phenyl)acrylamide (**11a**)*

To a solution of **10a** (40 mg, 0.13 mmol) in DCM (5 mL) were added DIEA (16.7 mg, 0.13 mmol) and acryloyl chloride (11.7 mg, 0.13 mmol) at −5 °C. The resulting mixture was stirred for 30 min. Then it was quenched by MeOH (2 mL), concentrated, and purified by silica gel column chromatography (DCM: MeOH = 20:1) to afford the title compound **11a** (29.9 mg, 63.6%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.17 (s, 1H), 8.66 (s, 1H), 8.05 (s, 1H), 7.75 (s, 1H),7.62 (d, J = 6 Hz, 1H), 7.56 (s, 1H),7.30 (s, 1H),7.24(t, 1H), 7.00 (d, J = 9 Hz, 1H),6.95(t, 1H), 6.25 (dd, J = 15, 6 Hz, 1H), 5.76 (s, 1H), 5.72 (m, 2H), 4.41 (s, 1H), 4.27 (s, 1H), 3.77 (s, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 163.18, 162.59, 160.13, 157.82, 140.90, 139.18, 131.94, 131.02, 128.83, 128.78, 126.91, 122.20, 117.79, 117.70, 114.51, 82.44, 43.89, 38.73; MS (ESI) *m/z* 350 $[M+H]^+$.

*5.1.30. N-(3-(((6-((1-methyl-1H-pyrazol-4-yl)amino)pyrimidin-4-yl) amino)methyl)phenyl)but-2-enamide (**12a**)*

Compound **12a** was prepared from **10a** in 73% yield by a method similar to that described for **11a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.11 (s, 1H), 8.94 (br, 1H), 8.34 (s, 1H), 7.83 (s, 1H),7.60 (s, 1H), 7.54 (d, J = 6 Hz,1H),7.45 (s, 1H),7.24(m, 1H), 6.97 (d, J = 6 Hz, 1H),6.77(dd, J = 15, 6 Hz, 1H) 6.20 (d, J = 15 Hz, 1H), 5.84 (s, 1H), 4.48 (s, 2H), 3.81 (s, 3H),1.83(d, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 164.00, 159.27, 157.85, 141.17, 139.99, 139.21, 130.75, 128.24, 126.59, 122.17, 121.48, 119.80, 119.08, 116.47, 83.62, 70.87, 44.76, 17.94; MS (ESI) *m/z* 364 $[M+H]^+$.

*5.1.31. N-(4-(((6-((1-methyl-1H-pyrazol-4-yl)amino)pyrimidin-4-yl) amino)methyl)phenyl)acrylamide (**11a'**)*

Compound **11a'** was prepared from **10a'** in 70% yield by a method similar to that described for **11a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.51 (s, 1H), 10.06 (br, 1H), 8.78 (s, 1H), 8.31 (s, 1H),7.81(s, 1H), 7.70(d, J = 9 Hz, 1H), 7.44 (s, 1H), 7.28 (d, J = 6 Hz, 2H), 7.09 (d, J = 9 Hz, 1H), 6.55 (dd, J = 12,9 Hz, 1H), 6.23 (d, J = 18 Hz, 1H), 5.82 (s, 1H), 5.71 (d, J = 9 Hz, 1H), 3.81 (s, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 163.65, 159.54, 158.82, 150.94, 138.84, 133.32, 132.43, 128.89, 128.23, 127.06, 120.50, 119.90, 119.68, 81.31, 49.01, 44.63; MS (ESI) *m/z* 350 $[M+H]^+$.

*5.1.32. N-(4-(((6-((1-methyl-1H-pyrazol-4-yl)amino)pyrimidin-4-yl) amino)methyl)phenyl)but-2-enamide (**12a'**)*

Compound **12a'** was prepared from **10a'** in 75% yield by a method similar to that described for **11a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.27 (s,1H), 10.15(br, 1H),8.87 (s, 1H), 8.33 (s, 1H), 7.82(s, 1H),7.67 (d, J = 9 Hz, 2H), 7.45 (s, 1H), 7.26 (d, J = 9 Hz, 2H), 6.76(q, 1H),6.22(d, J = 15 Hz, 1H),5.85 (s, 1H), 4.46 (s, 2H), 3.81 (s, 3H),1.83(d, J = 6 Hz, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 163.98, 158.16, 157.72, 141.15, 139.99, 139.16, 128.90, 128.21, 126.59, 121.86, 121.13, 119.81, 84.69, 44.66, 17.93; MS (ESI) *m/z* 364 $[M+H]^+$.

*5.1.33. N-(3-(((6-((1-(2-hydroxyethyl)-1H-pyrazol-4-yl)amino) pyrimidin-4-yl)amino)methyl)phenyl)acrylamide (**11b**)*

Compound **11b** was prepared from **10b** in 85% yield by a method similar to that described for **11a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.36 (s, 1H), 10.23 (br, 1H), 9.08 (br, 1H), 8.34 (s, 1H), 7.87(s, 1H), 7.56 (s, 1H), 7.44 (s, 1H), 7.37 (t, 1H), 7.25(d, J = 12 Hz, 1H), 7.00 (d, J = 6 Hz, 1H), 6.70 (dd, J = 15, 9 Hz, 1H), 6.33 (d, J = 15 Hz, 1H), 5.89 (s, 1H), 5.77 (d, J = 9 Hz, 1H), 4.59 (s, 2H),4.11(t, 2H), 3.71(t, 2H); ^{13}C NMR (300 M DMSO-d_6) δ 163.96, 162.06, 160.05, 155.99, 139.81, 138.13, 132.45, 130.36, 129.36, 128.93, 127.47, 122.88, 122.44, 121.99, 120.56, 81.12, 60.38, 54.95, 49.00; MS (ESI) *m/z* 380 $[M+H]^+$.

*5.1.34. N-(4-(((6-((1-(2-hydroxyethyl)-1H-pyrazol-4-yl)amino) pyrimidin-4-yl)amino)methyl)phenyl)acrylamide (**11b'**)*

Compound **11b'** was prepared from **10b'** in 80% yield by a method similar to that described for **11a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.51 (s, 1H), 8.91 (br, 1H), 8.34 (s, 1H), 7.85 (s, 1H), 7.70(d, J = 6 Hz, 2H), 7.47 (s, 1H), 7.29 (d, J = 6 Hz, 2H), 6.54 (dd, J = 15,9 Hz, 1H), 6.23 (d, J = 15 Hz, 1H), 5.89 (s, 1H), 5.71 (d, J = 9 Hz, 1H), 4.54 (s, 2H),4.11(t, 2H), 3.71(t, 2H); ^{13}C NMR (300 M DMSO-d_6) δ 163.67, 162.77, 161.08, 157.84, 138.87, 135.47, 132.40, 128.28, 127.07, 123.72, 119.92, 117.58, 81.72, 60.41, 54.94, 48.99; MS (ESI) *m/z* 380 $[M+H]^+$.

*5.1.35. N-(3-(((6-((1-(2-methoxyethyl)-1H-pyrazol-4-yl)amino) pyrimidin-4-yl)amino)methyl)phenyl)acrylamide (**11c**)*

Compound **11c** was prepared from **10c** in 85% yield by a method similar to that described for **11a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ10.57 (s, 1H), 10.35 (s, 1H), 9.07 (s, 1H), 8.18 (s, 1H), 7.97 (d, 2H), 7.85 (d, 2H), 7.67 (d, 2H), 7.30 (t, 1H), 6.98 (d, 1H), 6.55 (dd, J = 18, 9 Hz, 1H), 6.22 (d, J = 15 Hz, 1H), 5.70 (d, J = 15 Hz, 1H), 4.41 (s, 4H), 3.57 (s, 5H); ^{13}C NMR (300 M DMSO-d_6) δ 163.63, 157.32, 154.14, 153.58, 140.90, 139.82, 137.81, 136.57, 134.55, 132.29, 129.37, 127.11, 122.96, 122.07, 119.88, 118.98, 118.63 109.29, 81.12, 63.89, 54.49, 50.41; MS (ESI) m/z 394 $[M+H]^+$.

*5.1.36. N-(4-(((6-((1-(2-methoxyethyl)-1H-pyrazol-4-yl)amino) pyrimidin-4-yl)amino)methyl)phenyl)acrylamide (**11c'**)*

Compound **11c'** was prepared from **10c'** in 82% yield by a method similar to that described for **11a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.50 (s, 1H), 10.14 (br, 1H), 8.85 (s, 1H), 8.34 (s, 1H), 7.84 (s, 1H),7.70 (d, J = 9 Hz, 2H), 7.49 (s, 1H),7.29 (d, J = 9 Hz, 2H), 6.54 (dd, J = 15, 9 Hz, 1H), 6.24 (d, J = 18 Hz, 1H), 5.85 (s, 1H), 5.73 (d, J = 9 Hz, 1H), 4.46 (s, 2H), 4.23 (s, 2H), 3.67 (s, 2H), 3.22 (s, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 163.64, 159.13, 157.96, 138.87, 138.40, 132.45, 128.25, 127.06, 126.26, 124.50, 122.88, 119.92, 83.17, 70.89, 58.44, 51.95, 44.67; MS (ESI) m/z 394 $[M+H]^+$.

*5.1.37. N-(3-(((6-((4-morpholinophenyl)amino)pyrimidin-4-yl)amino) methyl)phenyl)acrylamide (**11d**)*

Compound **11d** was prepared from **10d** in 80% yield by a method similar to that described for **11a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.50 (s,1H), 9.82(br, 1H),8.49 (s, 1H), 8.27 (s, 1H), 7.67 (s, 1H),7.30(t, J = 6 Hz, 1H), 7.18 (d, J = 6 Hz, 2H), 7.07 (d, J = 6 Hz, 1H), 7.00(d, J = 6 Hz, 1H),6.94(d, J = 6 Hz, 2H),6.56(dd, J = 18,12 Hz,1H),6.25(d, J = 15 Hz,1H),5.73 (d, 2H), 4.41 (d, 2H), 3.73 (s, 4H),3.07(s, 4H); ^{13}C NMR (300 M DMSO-d_6) δ 163.68, 162.17, 158.90, 158.13, 148.71, 139.85, 132.44, 129.81, 129.62, 129.33, 127.14, 124.07, 122.53, 118.65, 118.34, 116.13, 115.32, 114.98, 81.47, 66.52, 49.10, 44.85; MS (ESI) m/z 431 $[M+H]^+$.

*5.1.38. N-(3-(((6-((4-morpholinophenyl)amino)pyrimidin-4-yl)amino) methyl)phenyl)but-2-enamide (**12d**)*

Compound **12d** was prepared from **10d** in 83% yield by a method similar to that described for **11a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.17 (s,1H), 8.90(br, 1H),8.36 (s, 1H), 7.65 (d, J = 6 Hz, 2H),7.45(d, J = 6 Hz, 2H), 7.34 (d, J = 6 Hz, 1H), 6.93 (d, J = 6 Hz, 2H), 6.78(dd, J = 15,9 Hz,1H),6.18(d, J = 15 Hz,1H),5.81 (s, 1H), 4.48 (s, 2H), 3.78 (s, 4H),3.16(s, 4H),1.85(d, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 163.99, 161.39, 158.88, 158.03, 146.11, 140.26, 136.36, 136.10, 129.43, 126.50, 122.26, 118.74, 118.23, 116.95, 116.59, 78.80, 66.17, 49.59, 17.97; MS (ESI) m/z 445 $[M+H]^+$.

*5.1.39. N-(3-(((6-((4-morpholinophenyl)amino)pyrimidin-4-yl)amino) methyl)phenyl)propionamide (**13d**)*

Compound **13d** was prepared from **10d** in 89% yield by a method similar to that described for **11a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.44 (br, 1H), 10.12(s, 1H), 8.99(br, 1H),8.38 (s, 1H), 7.61 (s, 1H),7.49(d, J = 9 Hz, 1H), 7.35 (m, 3H), 7.19 (d, J = 6 Hz, 1H), 6.96(d, J = 6 Hz,2H), 5.92 (s, 1H), 4.49 (s, 2H), 3.86 (s, 4H),3.26(s, 4H),2.32(d, 2H)1.05(t, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 172.56, 165.49, 157.69, 157.18, 150.42, 147.06, 140.23, 132.76, 130.36, 129.33, 124.27, 122.12, 118.45, 118.09, 80.77, 65.63, 50.78, 45.09, 29.95; MS (ESI) m/z 433 $[M+H]^+$.

*5.1.40. N-(4-(((6-((4-morpholinophenyl)amino)pyrimidin-4-yl)amino) methyl)phenyl)acrylamide (**11d'**)*

Compound **11d'** was prepared from **10d'** in 88% yield by a method similar to that described for **11a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.61 (s, 1H), 10.37 (s, 1H), 8.98 (s, 1H), 8.36 (s, 1H),7.72 (s, 2H), 7.25 (m, 4H), 7.15 (s, 2H), 6.59 (dd, J = 15, 9 Hz, 1H), 6.24 (d, J = 15 Hz, 1H), 5.88 (s, 1H), 5.72 (d, J = 9 Hz, 1H), 4.45 (s, 2H), 3.80 (s, 4H), 3.19 (s, 4H); ^{13}C NMR (300 M DMSO-d_6) δ 163.67, 162.17, 159.90, 157.43, 150.31, 139.24, 138.92, 132.49, 128.25, 127.00, 124.06, 119.60, 117.50, 82.07, 65.95, 49.01, 44.76; MS (ESI) m/z 431 $[M+H]^+$.

*5.1.41. N-(3-(((6-((4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)amino)methyl)phenyl)acrylamide (**11e**)*

Compound **11e** was prepared from **10e** in 86% yield by a method similar to that described for **11a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.45 (s, 1H), 10.58 (s, 1H), 8.99 (s, 1H), 8.36 (s, 1H), 7.67 (d, 2H), 7.32 (m, 2H), 7.20 (m, 2H), 7.01 (s, 2H), 6.57 (dd, J = 18, 9 Hz ,1H), 6.24 (d, J = 18 Hz, 1H), 5.87 (s, 1H), 5.74 (d, J = 9 Hz, 1H), 4.49 (s, 2H), 3.09 (s, 4H), 2.57 (s, 4H),2.30 (s, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 163.71, 161.86, 158.13, 156.11, 150.32, 139.92, 138.42, 132.43, 129.44, 127.20, 125.03, 122.65, 118.87, 118.41, 117.04, 116.67, 80.12, 67.47, 52.37, 45.91, 42.31; MS (ESI) m/z 444 $[M+H]^+$.

*5.1.42. N-(3-(((6-((4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)amino)methyl)phenyl)but-2-enamide (**12e**)*

Compound **12e** was prepared from **10e** in 80% yield by a method similar to that described for **11a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.41 (br, 1H), 10.29 (s, 1H), 8.69 (s, 1H), 8.30 (s, 1H), 7.65 (m, 2H), 7.21 (m, 2H), 6.99 (s, 2H), 6.79 (m, 2H), 6.23 (d, J = 15 Hz ,1H), 5.81 (s, 1H), 4.46 (s, 2H), 3.74 (s, 4H), 3.40 (s, 4H), 2.78 (s, 3H), 1.85 (s, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 147.43, 142.79, 140.14, 138.81, 130.20, 129.30, 126.57, 124.26, 122.29, 118.68, 118.30, 117.03, 81.27, 67.47, 52.41, 46.01, 42.32, 17.98; MS (ESI) m/z 458 $[M+H]^+$.

*5.1.43. N-(3-(((6-((4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)amino)methyl)phenyl)propionamide (**13e**)*

Compound **13e** was prepared from **10e** in 81% yield by a method similar to that described for **11a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.37 (s,1H),10.30(s, 1H), 9.05 (br, 1H), 8.35 (s, 1H), 7.47 (d, 1H), 7.34 (s, 3H), 7.20 (s, 2H), 7.01 (d, J = 6 Hz, 2H), 5.83 (s, 1H), 4.57 (s, 2H), 3.79 (s, 2H), 3.46 (d, J = 6 Hz, 2H), 3.15 (d, J = 6 Hz, 2H),2.79(s, 3H),2.32(q, 2H),1.06(t, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 172.02, 162.99, 161.16, 159.86, 150.43, 140.29, 139.61, 138.06, 132.99, 130.35, 127.09, 122.62, 122.18, 118.65, 117.08, 81.34, 52.43, 45.92, 42.36, 10.15; MS (ESI) m/z 446 $[M+H]^+$.

*5.1.44. N-(4-(((6-((4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)amino)methyl)phenyl)acrylamide (**11e'**)*

Compound **11e'** was prepared from **10e** in 83% yield by a method similar to that described for **11a**. ^{1}H NMR (300 MHz, HCl salt, DMSO-d_6) δ 10.34 (s,1H), 8.59 (s, 1H), 8.03 (s, 1H), 7.50 (d, J = 9 Hz, 2H), 7.24 (m, 4H), 6.86 (d, J = 9 Hz, 2H), 6.49(dd, J = 9 Hz, 9 Hz, 1H), 6.16(d, J = 15 Hz, 1H), 5.65 (s, 1H), 5.59(d, J = 12 Hz, 1H), 4.37 (s, 2H), 3.09 (s, 4H), 2.57 (s, 4H), 2.30 (s, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 163.67, 162.64, 158.20, 157.92, 147.26, 138.76, 132.49, 130.49, 128.81, 128.14, 124.10, 119.88, 118.56, 117.03, 81.66, 52.43, 46.06, 44.56, 42.34; MS (ESI) m/z 444 $[M+H]^+$.

*5.1.45. N-(4-(((6-((4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)amino)methyl)phenyl)but-2-enamide (**12e'**)*

Compound **12e'** was prepared from **10e** in 83% yield by a method similar to that described for **11a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.34 (s, 1H), 8.59 (s, 1H), 8.03 (s, 1H), 7.50 (d, J = 9 Hz, 2H), 7.24 (m, 5H), 6.85 (d, J = 9 Hz, 2H), 6.64 (q, 1H), 6.17 (d, J = 15 Hz, 1H), 5.65 (s, 1H), 4.37 (s, 2H), 3.09 (t, 4H), 2.57 (t, 4H), 2.30 (s, 3H), 1.88 (d, J = 6 Hz, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 163.96, 162.35, 160.36, 159.64, 146.25, 139.89, 138.69, 132.39, 128.66, 127.92, 123.01, 119.72, 117.11, 115.48, 82.37, 52.53, 46.37, 44.38, 42.39, 17.96; MS (ESI) m/z 458 $[M+H]^+$.

*5.1.46. N-(4-(((6-((4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)amino)methyl)phenyl)propionamide **(13e')***

Compound **13e'** was prepared from **10e** in 83% yield by a method similar to that described for **11a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.34 (s, 1H), 8.59 (s, 1H), 8.03 (s, 1H), 7.50 (d, J = 9 Hz, 2H), 7.24 (m, 4H), 6.85 (d, J = 9 Hz, 2H), 5.65 (s, 1H), 4.37 (s, 2H), 3.09 (t, 4H), 2.57 (t, 4H), 2.30 (s, 3H), 2.13 (q, 2H), 1.09 (t, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 172.58, 166.89, 158.52, 157.33, 147.73, 139.18, 134.62, 128.86, 128.14, 122.23, 119.59, 116.99, 80.87, 52.42, 45.94, 44.67, 42.35, 29.91, 10.16; MS (ESI) m/z 446 $[M+H]^+$.

*5.1.47. 2-cyano-N-(3-(((6-((1-(2-methoxyethyl)-1H-pyrazol-4-yl)amino) pyrimidin-4-yl)amino)methyl)phenyl)acetamide **(14c)***

To a stirred solution of **10c** (80 mg, 0.24 mmol) in THF (15 mL) was added DIEA (76 mg, 0.74 mmol), HATU (220 mg, 0.59 mmol), and 2-cyanoacetic acid (22 mg, 0.26 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 16 h. It was quenched with water and extracted with ethyl acetate. The organic layer was washed with water followed by brine, dried over anhydrous sodium sulfate, and was evaporated to dryness. The crude mass was purified by silica gel column chromatography (DCM: MeOH = 15:1) to afford compound **14c** (50 mg, 53%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.25 (s, 1H), 8.60 (s, 1H), 8.04 (s, 1H), 7.90 (s, 1H), 7.74(s, 1H), 7.52 (d, J = 9 Hz, 1H), 7.40 (t, J = 6 Hz, 1H), 7.36(s, 1H), 7.25 (d, J = 6 Hz, 1H), 5.57 (s, 1H), 4.37 (d, J = 6 Hz, 2H), 4.18 (t, J = 6 Hz, 2H), 3.86 (s, 2H), 3.65 (t, J = 6 Hz, 2H), 3.22 (s, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 163.16, 162.51, 150.18, 141.26, 139.18, 131.28, 128.96, 128.20, 120.30, 119.62, 117.07, 116.60, 115.28, 80.52, 60.39, 54.95, 49.01, 44.72, 29.91; MS (ESI) m/z 407 $[M+H]^+$.

*5.1.48. 2-cyano-N-(3-(((6-((4-morpholinophenyl)amino)pyrimidin-4-yl) amino)methyl)phenyl)acetamide **(14d)***

Compound **14d** was prepared from **10d** in 65% yield by a method similar to that described for **14c**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.27 (s,1H), 8.78 (s, 1H), 8.06 (s, 1H), 7.54 (s, 1H), 7.46 (d, J = 9 Hz, 1H), 7.42 (s, 1H), 7.28 (t, J = 9 Hz, 1H), 7.23 (d, J = 9 Hz, 2H), 7.01 (d, J = 9 Hz, 1H), 6.87 (d, J = 9 Hz, 2H), 5.64 (s, 1H),4.41 (d, 2H), 3.86 (s, 2H), 3.73 (m, 4H),3.03(m, 4H). MS (ESI) m/z 444 $[M+H]^+$.

*5.1.49. 2-cyano-N-(3-(((6-((4-(4-methylpiperazin-1-yl)phenyl)amino) pyrimidin-4-yl)amino)methyl)phenyl)acetamide **(14e)***

Compound **14e** was prepared from **10e** in 68% yield by a method similar to that described for **14c**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.26 (s, 1H), 8.57 (s, 1H), 8.03 (s, 1H), 7.48 (d, J = 9 Hz, 1H), 7.41 (s, 1H), 7.28 (d, 1H), 7.25(d, 3H),7.02(d, J = 6 Hz, 1H),6.86(d, J = 9 Hz, 2H),5.64 (s, 1H), 4.40 (d, 2H), 3.86 (s, 2H), 3.09 (s, 4H), 2.57 (s, 4H), 2.31 (s, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 163.07, 161.36, 161.03, 158.11, 146.79, 141.46, 138.88, 132.93, 129.25, 122.99, 118.08, 116.58, 116.34, 83.24, 54.82, 48.90, 45.68, 44.09, 27.14; MS (ESI) m/z 457 $[M+H]^+$.

*5.1.50. 2-cyano-N-(4-(((6-((4-(4-methylpiperazin-1-yl)phenyl)amino) pyrimidin-4-yl)amino)methyl)phenyl)acetamide **(14e')***

Compound **14e'** was prepared from **10e'** in 68% yield by a method similar to that described for **14c**. ^{1}H NMR (300 MHz, HCl salt, DMSO-d_6) δ 10.34 (s, 1H), 8.59 (s, 1H), 8.03 (s, 1H), 7.50 (d, J = 9 Hz, 2H), 7.25 (m, 4H), 6.85 (d, J = 9 Hz,2H), 5.65 (s, 1H), 4.37 (s, 2H), 3.89 (s, 2H), 3.09 (s, 4H), 2.57 (s, 4H), 2.30 (s, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 163.06, 161.29, 161.02, 158.09, 146.78, 137.40, 135.99, 132.93, 127.98, 122.23, 119.75, 116.57, 116.38, 83.29, 54.78, 48.86, 45.64, 43.84, 27.08; MS (ESI) m/z 457 $[M+H]^+$.

*5.1.51. N-(3-(((6-((1-(2-methoxyethyl)-1H-pyrazol-4-yl)amino) pyrimidin-4-yl)amino)methyl)phenyl)cyanamide **(15c)***

To a stirred solution of **10c** (80 mg, 0.29 mmol) in THF (5 mL) was added KOAc (58 mg, 0.59 mmol) and BrCN (37 mg, 0.35 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 16 h. It was quenched with water, and was evaporated to dryness. The crude mass was purified by silica gel column chromatography (DCM: MeOH = 15:1) to afford compound **15c** (52 mg, 61%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.25 (s, 1H), 8.60 (s, 1H), 8.04 (s, 1H), 7.90 (s, 1H), 7.74(s, 1H), 7.52 (d, J = 9 Hz, 1H), 7.40 (t, J = 6 Hz, 1H), 7.36(s, 1H), 7.25 (d, J = 6 Hz, 1H), 5.57 (s, 1H), 4.37 (d, J = 6 Hz, 2H), 4.18 (t, J = 6 Hz, 2H), 3.65 (t, J = 6 Hz, 2H), 3.22 (s, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 164.35, 157.72, 157.59, 140.18, 139.08, 133.16, 129.87, 127.32, 118.49, 118.10, 117.66, 114.99, 80.77, 70.33, 58.22, 50.25, 45.02; MS (ESI) m/z 407 $[M+H]^+$.

*5.1.52. N-(3-(((6-((4-morpholinophenyl)amino)pyrimidin-4-yl)amino) methyl)phenyl)cyanamide **(15d)***

Compound **15d** was prepared from **10d** in 68% yield by a method similar to that described for **15c**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.28 (s, 1H), 8.78 (s, 1H), 8.07 (s, 1H), 7.54 (t, 1H), 7.47 (d, J = 9 Hz, 1H), 7.43 (s, 1H), 7.29 (t, J = 9 Hz, 1H), 7.23 (d, J = 6 Hz, 2H), 7.02 (d, J = 9 Hz, 1H), 6.88 (d, J = 9 Hz, 2H), 5.64 (s, 1H),4.41 (d, J = 6 Hz, 2H), 3.73 (t, J = 6 Hz, 4H),3.03(t, J = 6 Hz, 4H); ^{13}C NMR (300 M DMSO-d_6) δ 162.56, 160.52, 157.61, 146.55, 141.85, 138.74, 135.87, 132.56, 129.64, 121.72, 121.14, 115.73, 113.22, 112.06, 82.75, 66.12, 49.15, 43.47; MS (ESI) m/z 402 $[M+H]^+$.

*5.1.53. N-(3-(((6-((4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)amino)methyl)phenyl)cyanamide **(15e)***

Compound **15e** was prepared from **10e** in 69% yield by a method similar to that described for **15c**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.61 (s, 1H), 8.59 (s, 1H), 8.01 (s, 1H), 7.38(s, 1H), 7.33 (d, J = 9 Hz, 1H), 7.28 (s, 1H), 7.22 (d, J = 6 Hz, 2H), 6.93(d, J = 9 Hz, 1H), 6.84(d, J = 9 Hz, 2H), 5.63(s, 1H), 4.37(s, 2H), 3.64 (s, 4H), 3.06(s, 4H),2.24(s, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 163.04, 158.09, 154.43, 146.95, 139.65, 139.31, 132.79, 130.12, 122.20, 117.14, 116.48, 114.99, 113.71, 79.58, 55.04, 52.00, 49.12, 46.05; MS (ESI) m/z 415 $[M+H]^+$.

*5.1.54. N-(4-(((6-((4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)amino)methyl)phenyl)cyanamide **(15e')***

Compound **15e'** was prepared from **10e'** in 65% yield by a method similar to that described for **15c**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.34 (s, 1H), 8.59 (s, 1H), 8.03 (s, 1H), 7.76 (d, J = 9 Hz, 2H), 7.50 (d, 4H), 7.11 (d, J = 9 Hz, 2H), 5.65 (s, 1H), 4.37 (s, 2H), 3.09 (t, 4H), 2.57 (t, 4H), 2.30 (s, 3H); ^{13}C NMR (300 M DMSO-d_6) δ 162.32, 160.08, 157.46, 149.39, 137.16, 136.73, 134.16, 128.47, 122.70, 118.96, 117.58, 114.85, 82.48, 58.45, 51.65, 46.59, 43.31; MS (ESI) m/z 415 $[M+H]^+$.

*5.1.55. 2,2,2-trifluoro-N-(3-((((6-((4-morpholinophenyl)amino)pyrimidin-4-yl)amino)methyl)phenyl)acetamide **(16d)***

To a stirred solution of **10d** (120 mg, 0.32 mmol) in THF (15 mL) was added triethylamine (48 mg, 0.47 mmol) and trifluoroacetic anhydride (70 mg, 0.35 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 16 h. It was quenched with water, and was evaporated to dryness. The crude mass was purified by silica gel column chromatography (DCM: MeOH = 15:1) to afford compound **15c** (80 mg, 53%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.25 (s, 1H), 8.63 (s, 1H), 8.03 (s, 1H), 7.58(d, 2H), 7.36 (m, 2H), 7.24 (d, J = 9 Hz, 2H), 7.14 (d, J = 6 Hz, 1H), 6.86 (d, J = 9 Hz, 2H), 5.65 (s, 1H), 4.43 (s, 2H), 3.73 (s, 4H),3.02(s, 4H); ^{13}C NMR (300 M DMSO-d_6) δ 163.02, 160.96, 158.06, 155.19, 154.67, 147.45, 141.68, 140.00, 136.83, 129.33, 124.70, 122.21, 120.57, 119.86, 116.52, 116.23, 114.13, 83.26, 66.61, 49.64, 45.43; MS (ESI) m/z 473 $[M+H]^+$.

*5.1.56. 2,2,2-trifluoro-N-(4-((((6-((4-morpholinophenyl)amino)pyrimidin-4-yl)amino)methyl)phenyl)acetamide **(16d')***

Compound **16d'** was prepared from **10d'** in 65% yield by a method similar to that described for **16d**. ^{1}H NMR (300 MHz, HCl salt, DMSO-d_6) δ 11.23 (s, 1H), 8.63 (s, 1H), 8.03 (s, 1H), 7.60(d, J = 9 Hz, 2H),

7.32 (d, 3H), 7.26 (d, J = 9 Hz, 2H), 6.86 (d, J = 6 Hz, 2H), 5.65(s, 1H), 4.41 (s, 2H), 3.73 (s, 4H), 3.02(s, 4H); ^{13}C NMR (300 M DMSO-d_6) δ 163.04, 161.00, 158.11, 155.10, 154.61, 147.02, 137.94, 135.23, 133.08, 127.97, 122.20, 121.57, 118.20, 116.23, 114.98, 114.37, 83.30, 66.61, 49.64, 43.77; MS (ESI) m/z 473 $[M+H]^+$.

*5.1.57. 1-methyl-4-nitro-1H-pyrazole (**17a**)*

To a stirred solution of 4-nitro-1*H*-pyrazole (2 g, 17.7 mmol) in acetonitrile (15 mL) was added potassium carbonate (2.7 g, 19.5 mmol) and iodomethane (2.76 g, 19.5 mmol). The reaction mixture was stirred at reflux temperature for 6 h. And it was evaporated to dryness. The crude mass was purified by silica gel column chromatography (PE: EA = 10:1) to afford compound **15c** (2.2 g, 98%) as a white solid. MS (ESI) m/z 128 $[M+H]^+$.

*5.1.58. 2-(4-nitro-1H-pyrazol-1-yl)ethan-1-ol (**17b**)*

Compound **17b** was prepared from 4-nitro-1*H*-pyrazole in 99% yield by a method similar to that described for **17a**. MS (ESI) m/z 158 $[M+H]^+$.

*5.1.59. 1-(2-methoxyethyl)-4-nitro-1H-pyrazole (**17c**)*

Compound **17c** was prepared from 4-nitro-1*H*-pyrazole in 98% yield by a method similar to that described for **17a**. MS (ESI) m/z 172 $[M+H]^+$.

*5.1.60. 4-(4-Nitrophenyl)morpholine (**19d**)*

In a 25 mL round-bottom flask equipped with a stirring bar, a mixture of 2.62 g (30 mmol) of morpholine, 4.24 g (30 mmol) of 4-fluoronitrobenzene, and 4.14 g (30 mmol) of K_2CO_3 in 15 mL of DMSO was heated with stirring at 120 °C for 20 h. After cooling, the mixture was poured into 80 mL mixed solution of ethanol/water (1:1), and the formed yellow crystals were collected by filtration to give **19d** with an yield of 6.08 g (97%). MS (ESI) m/z 209 $[M+H]^+$.

*5.1.61. 1-Methyl-4-(4-nitrophenyl)piperazine (**19e**)*

A solution of 4-fluoronitrobenzene (5 g, 35.5 mmol), and K_2CO_3 (4.9 g, 35.5 mmol) in DMSO (10 mL) was stirred at room temperature for 0.5 h. Subsequently, 1-methylpiperazine (3.55 g, 35.5 mmol) was added dropwise, and the resulting reaction mixture was stirred at room temperature for 6 h. The mixture was then poured into ice-water. A yellow precipitate formed and was collected by filtration to give **19e** with a yield of 6.66 g (85%). MS (ESI) m/z 222 $[M+H]^+$.

*5.1.62. 1-methyl-1H-pyrazol-4-amine (**18a**)*

To **17a** (1 g, 7.8 mmol) in EtOH (20 mL) was added 10% Pd/C 50 mg. The reaction mixture was stirred for 3 h under hydrogen at room temperature. The mixture was then filtered with Celite, and the filtrate was concentrated and dried under vacuum to yield 0.74 g (97%) of **18a** as a white solid. MS (ESI) m/z 98 $[M+H]^+$.

*5.1.63. 2-(4-amino-1H-pyrazol-1-yl)ethan-1-ol (**18b**)*

Compound **18b** was prepared from **17b** in 98% yield by a method similar to that described for **18a**. MS (ESI) m/z 128 $[M+H]^+$.

*5.1.64. 1-(2-methoxyethyl)-1H-pyrazol-4-amine (**18c**)*

Compound **18c** was prepared from **17c** in 97% yield by a method similar to that described for **18a**. MS (ESI) m/z 142 $[M+H]^+$.

*5.1.65. 4-morpholinoaniline (**20d**)*

Compound **20d** was prepared from **19d** in 99% yield by a method similar to that described for **18a**. MS (ESI) m/z 179 $[M+H]^+$.

*5.1.66. 4-(4-methylpiperazin-1-yl)aniline (**20e**)*

Compound **20e** was prepared from **19e** in 99% yield by a method similar to that described for **18a**. MS (ESI) m/z 192 $[M+H]^+$.

5.2. Computational analysis

5.2.1. Docking calculation

We used the X-ray cocrystal structure of JAK3 in the Protein Data Bank (PDB ID: 3PJC). The protein structure for the docking study was prepared with Protein Preparation Workflow. All crystallographic waters were removed and chain A was kept. Hybridization states, charges, and angles were assigned in the protein structure with missing bond orders, and hydrogen atoms were added. The energy of the protein structure was minimized in 100 steps of the smart minimize method. Docking grids were generated and defined based on the centroid of compound **11e** in the ATP binding site incorporating hydrogen-bond constraints to the hinge and hydrophobic regions. Ligands were prepared using Discovery Studio 3.0 (DS 3.0). energy-minimized conformation of each ligands were used to docking calculation input molecules. Ligand-Fit and CDOCKER docking programs implemented in DS 3.0 were used in this study. Other docking parameters were kept to the default values. The top-scoring pose was employed for discussions.

5.3. Bioactivity

5.3.1. JAK1-3 and TYK2 enzyme assay

Human JAK1-3 and TYK2 kinases were obtained from Invitrogen and assays were performed using HTRF (Homogenous Time-Resolved Fluorescence) detection technology. Briefly, the enzyme reaction was run in reaction buffer, which consisted of 50 mM HEPES (pH7.0), 5 mM $MgCl_2$, 1 mM DTT, 0.1% NaN_3 and 0.1% orthovanadate. The assay was done by a 384-well plate (10 µL) assay format. A typical enzyme reaction contains 1 ng/µL JAK1, 1 µM TK-substrate-biotin, 4 µM ATP; 0.004 ng/µL JAK2, 1 µM TK-substrate-biotin, 4 µM ATP; 0.012 ng/µL JAK3, 1 µM TK-substrate-biotin, 1.43 µM ATP; 0.012 ng/µL TYK2, 1 µM TK-substrate-biotin, 1.43 µM ATP. Compounds were screened at serial diluted concentration in the presence of 2% DMSO with a 5 min pre-incubation of kinase and compounds. All reactions were started by the addition of ATP and TK-substrate-biotin, incubated at 30 °C for 30 min and quenched with the stop buffer containing 25 nM Strep-XL665 and TK Ab-Cryptate. The plates were incubated for 1 h. Time-resolved fluorescence was monitored with a Synergy H1 (Biotek) by excitation at 330 nm and emission donor at 620 nm or emission acceptor at 665 nm, respectively. The files recorded by the Synergy H1 were read with Excel and contained the acceptor and donor counts for each sample. And IC_{50} values were determined using the Graphpad Prism 5.0 Software.

5.3.2. Rat T cell proliferation

Spleen cells were suspended in RPMI1640 medium, supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 µM 2-mercaptoethanol at a density of 1.5×10^6 cells/mL. Splenocytes were cultured with Concanavalin A for 24 h at 37 °C in 5% CO_2 to induce IL-2 receptor expression and then incubated with IL-2 and test compounds at designated concentrations in 96-well tissue culture plates. After incubation for 72 h, cell viability was monitored using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI) according to the manufacturer's recommended protocols. Colorimetric assay of each well was read using microplate reader synergy H1 (Biotek). Values were transformed to percent inhibition relative to vehicle control, and IC_{50} curves were fitted according to nonlinear regression analysis of the data using PRISM GraphPad 5.0.

Acknowledgments

The authors are grateful to financial supports from National Natural science Foundation of China (81573445, 81172934) and the Beijing Municipal Natural Science Foundation (7182115).

References

1. Musumeci Francesca, Greco Chiara, Giacchello Ilaria, Fallacara Anna Lucia, Ibrahim Munjed M, Grossi Giancarlo, Brullo Chiara, Schenone Silvia. *Curr Med Chem.* 2018;25:1.
2. Wrobleski Stephen T, Pitts William J. *Ann Rep Med Chem.* 2009;44:247.
3. O'Sullivan LA, Liongue C, Lewis RS, et al. *Mol Immunol.* 2007;44:2497–2506.
4. Ivashkiv Lionel B, Xiaoyu Hu. *Arthritis Res Therapy.* 2004;6:159.
5. Smirnova OV, Ostroukhova TY, Bogorad RL. *World J Gastroenterol.* 2007;13:6478–6491.
6. O'Shea JJ, Gadina M, Schreiber RD. *Cell.* 2002;109:S121.
7. Ihle JN, Kerr IM. *Trends Genet.* 1995;11:69.
8. Menet Christel J, Van Rompaey Luc, Geney Raphaël. *Progress Med Chem.* 2013;52:153.
9. Papageorgiou Anastassios C, Wikman Linnea EK. *TRENDS Pharm Sci.* 2004;25:558.
10. Dymock Brian W, Shang See Cheng. *Expert Opin Ther Patents.* 2013;23:449–501.
11. Yamaoka K, Saharinen P, Pesu M, et al. *Genome Biol.* 2004;5:253.
12. Yamaoka K, Min B, Zhou YJ, et al. *Blood.* 2005;106:3227–3233.
13. Kisseleva T, Bhattacharya S, Braunstein J, et al. *Gene.* 2002;285:1–24.
14. Changelian Paul S, et al. *Science.* 2003;302:875.
15. Flanagan ME, Blumenkopf TA, Brissette WH, et al. *J Med Chem.* 2010;53:8468.
16. Quintas-Cardama A, et al. *Blood.* 2010;115:3109–3117.
17. Telliez JB, et al. *ACS Chem Biol.* 2016;11:3442–3451.
18. Yin Yuan, Chen Cheng-Juan, Ru-Nan Yu, et al. *Bioorg Med Chem.* 2018;26:4774.
19. Forster Michael, Gehringer Matthias, Laufer Stefan A. *Bioorg Med Chem.* 2017;27:4229.
20. Kempson James, Ovalle Damaso, Guo Junqing, et al. *Bioorg Med Chem.* 2017;27:4622.
21. Yamagishi Hiroaki, Inoue Takayuki, Nakajima Yutaka, et al. *Bioorg Med Chem.* 2017;25:5311.
22. Yamagishi Hiroaki, Inoue Takayuki, Nakajima Yutaka, et al. *Bioorg Med Chem.* 2015;23:4846.