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Design, synthesis and evaluation of pyrrolo[2,3-*d*]pyrimidine-phenylamide hybrids as potent Janus kinase 2 inhibitors

Tingfang Wang, Xiaofei Liu, Meixi Hao, Jianan Qiao, Caoyun Ju, Lingjing Xue *, Can Zhang *

State Key Laboratory of Natural Medicines and Jiangsu Key Laboratory of Drug Discovery for Metabolic Diseases, Center of Drug Discovery, China Pharmaceutical University, 24 Tong Jia Xiang, Nanjing 210009, China

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ABSTRACT

Janus kinase 2 (JAK2) plays an essential role in the signaling of hormone-like cytokines and growth factors, which has been convinced as an important target of myeloproliferative neoplasms (MPNs) therapy. In this study, a series of novel pyrrolo[2,3-*d*]pyrimidine-phenylamide hybrids were designed and synthesized as potential JAK2 inhibitors through hybridization strategy. In vitro biological studies showed that most of these compounds exhibited potent activity against JAK2. Especially, compound **16c** was identified as a suitable lead compound, which showed favorable pharmacokinetic profiles in rats (F = 73.57%), excellent in vitro efficacy against erythroleukemic cells (TF-1, IC_{50} = 0.14 μM), and high selectivity for JAK2 (IC_{50} = 6 nM with >97-fold selectivity vs JAK3).

The Janus kinases (including JAK1, JAK2, JAK3, and TYK2) are a pivotal family of intracellular non-receptor protein tyrosine kinases that play prominent roles in cytokine and growth factor-mediated receptor signaling.[1] Once cytokines binding to the receptors, the receptor-associated JAKs can be activated and subsequently phosphorylate signal transducers and activators of transcription proteins (STATs). The phosphorylated STATs dimerize and migrate to the nucleus where their respective target genes expression are modulated, consequently leading to the influence of cell proliferation, differentiation and survival.[2] Among the JAKs, JAK2 is essential for signaling through hormone-like cytokines and growth factors such as interleukin-3 (IL-3), IL-5, granulocyte-macrophage-colony stimulating factor (GM-CSF), erythropoietin (EPO), and thrombopoietin (TPO).[3] Herein, the JAK2 has been implicated in the pathogenesis of myeloid malignancies.[4] Furthermore, an activating mutation of JAK2 (V617F) was reported in a majority of patients with myeloproliferative neoplasms (MPNs) (>95% in PV (polycythemia vera), >50% in essential ET (thrombocythemia) and >50% in primary MF (myelofibrosis)).[5] Since this mutation located in the JH2 pseudokinase (Janus Homology 2) domain of JAK2, which is required for the inhibition of basal kinase activity, thus leading to increased tyrosine kinase activity and cytokine hypersensitivity.[6] As a consequence, JAK2 is closely associated with the MPNs, which means that inhibition of JAK2 can lead to the inhibition of the aberrant JAK2/STATs signaling, therefore represents an attractive therapeutic approach for MPNs.

So far, many JAK2 inhibitors have been reported and tested in preclinical and clinical trials. Notably, Ruxolitinib (Fig. 1) was approved by FDA in 2011 for the treatment of patients with intermediate and high-risk MF.[7] In addition, Fedratinib (SAR302503),[8] Lestaurtinib (CEP-701),[9] Momelotinib (CYT-387),[10] Pacritinib (SB1518),[11] Gandotinib (LY2784544),[12] BMS-911543,[13] XL019[14] and CEP-33779[15] are also being investigated in clinical trials. However, Ruxolitinib, a JAK1/JAK2 dual inhibitor, has been observed to gain the immunosuppressive side effects due to inhibition of JAK1 or JAK3, which suggest to pursue the identification of a JAK2 selective inhibitor in order to increase safety.[16] The discovery of selective JAK2 inhibitors has been disclosed in numerous publications,[17] however, they are far from being a solution to all clinical issues in patients with MPNs.[18] Herein, we wish to report a series of novel pyrrolo[2,3-*d*]pyrimidine-phenylamide hybrids designed using the hybridization strategy as potential selective JAK2 inhibitors.

The pyrrolo[2,3-*d*]pyrimidines are important chemical scaffolds and have been extensively used in the design of JAKs inhibitors such as Ruxolitinib and Tofacitinib.[19] As important pharmacophores, pyrrolo[2,3-*d*]pyrimidines provided hydrogen bond acceptor and donor to interact with the amino acid residues in the kinase hinge region. Recently, Amgen developed a series of thienopyridine-phenylamide compounds as selective and potent JAK2 inhibitors using the structure-based design. Thereinto, compound **1** showed the highest selectivity (100- to >500-fold) over the other JAK family kinases in enzyme assays.[20]

* Corresponding authors.
E-mail addresses: xuelingjing65@163.com (L. Xue), zhangcan@cpu.edu.cn (C. Zhang).

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0960-894X/

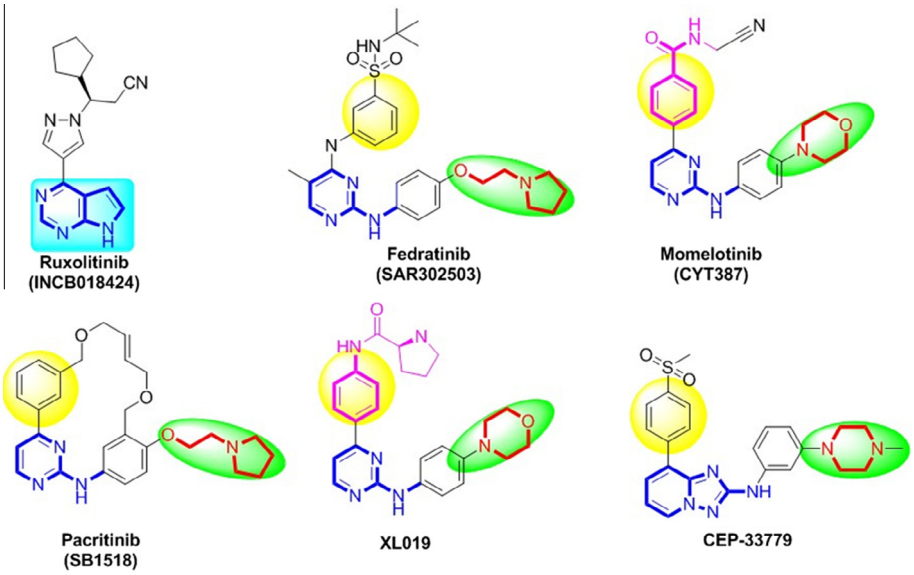


Figure 1. Selected published preclinical and clinical JAK2 inhibitors.

Structural–activity relationship analysis reveals that almost all the JAK2 inhibitors contain three crucial pharmacophores (Fig. 1): the core aromatic structure with H-bond donor/acceptor, binding with the kinase hinge region; the sulfonamide or amide moiety makes close contacts with the glycine rich P-loop; and the hydrophobic side-chain branching out of the enzyme into the solvent front. Based on these studies and also as a part of our ongoing research on the synthesis of novel anticancer agents,[21] we envisaged that hybrid of the pyrrolo[2,3-*d*]pyrimidine with an identified pharmacophore of selective JAK2 inhibitor **1** by applying scaffold hopping could be the way to provide the novel scaffold for a potent and selective JAK2 inhibitor (Fig. 2). Therefore, we designed, synthesized and evaluated the biological activity of pyrrolo[2,3-*d*]pyrimidine-phenylamide hybrids (**2**) as novel potential JAK2-selective inhibitors.

The general synthetic procedure for pyrrolo[2,3-*d*]pyrimidine-phenylamide hybrids **12a**–**g** was described in Scheme 1. Compound **4** was prepared by reacting 4,6-dihydroxylpyrimidne **3** with freshly prepared Vilsmeier–Haack reagent.[22] 4,6-Dichloropyrimidine-5-carbaldehyde **4** underwent S_NAr reaction with sarcosine benzyl ester **5** in the presence of a base and afforded **6** in moderate yield. Intermediate **6** was then cyclized to form pyrrolidine ring **7** by treatment with triethylamine in acetonitrile at reflux for 8 h. Dehydration of **7** with thionyl chloride ($SOCl_2$) and pyridine in dichloromethane at room temperature provided **8** in good yield. Suzuki coupling of compound **8** with 4-nitrophenylboronic acid pinacol ester **9**, under the catalysis of $Pd(PPh_3)_4$ with Cs_2CO_3 as the alkali at 90 °C generated the key intermediate **10**.[23] The nitro group of **10** was subjected to chemoselective catalytic hydrogenation to give pyrrolo[2,3-*d*]pyrimidine-aniline **11**,[24] which was

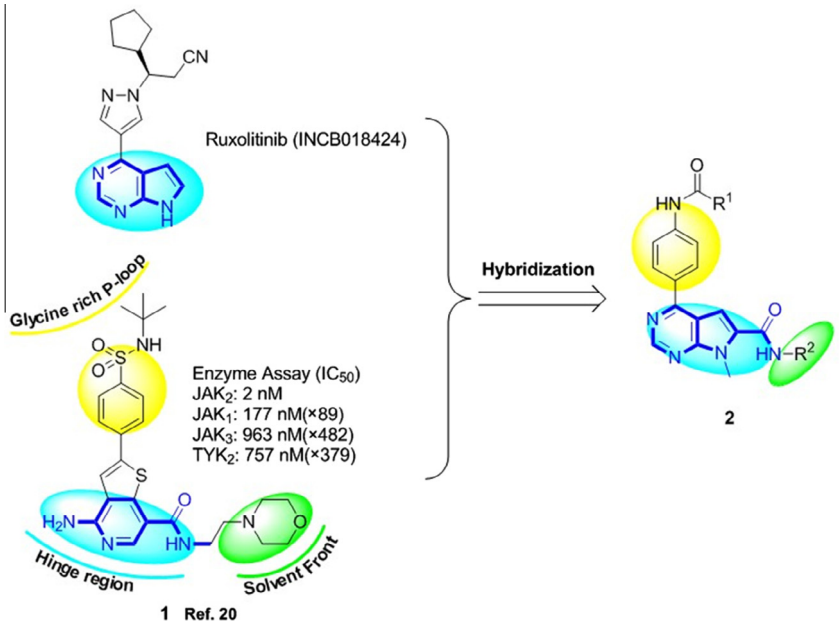
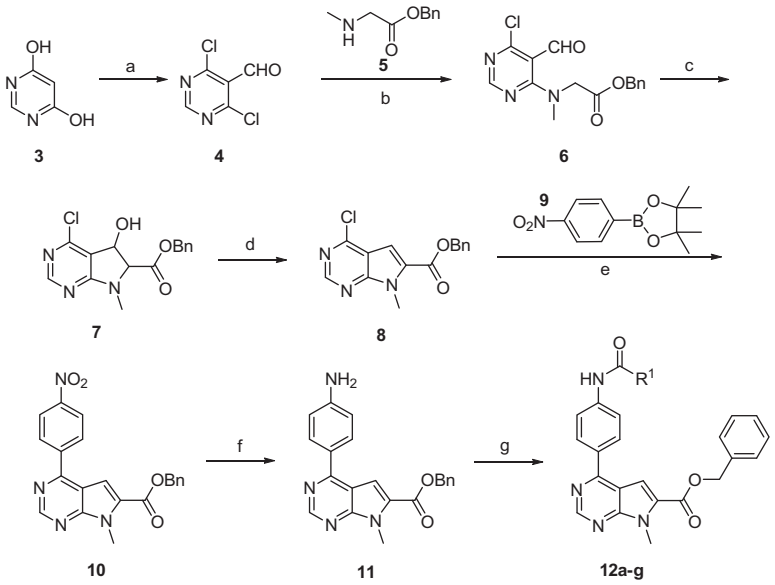


Figure 2. Design strategy for pyrrolo[2,3-*d*]pyrimidine-phenylamide hybrids.



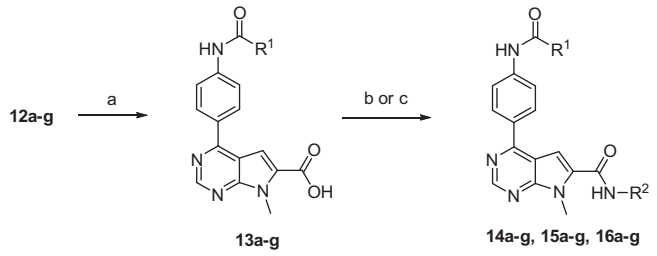
Scheme 1. Synthesis of pyrrolo[2,3-*d*]pyrimidine-phenylamide hybrids **12a–g**. Reagents and conditions: (a) $POCl_3$, DMF, 80 °C, 94.0%; (b) triethylamine, CH_3CN, 84.3%; (c) triethylamine, CH_3CN, reflux, 91.9%; (d) pyridine, $SOCl_2$, CH_2Cl_2, 92.7%; (e) $Pd(PPh_3)_4$, K_2CO_3, 90 °C, DME, 90.2%; (f) H_2, 10% Pd/C, EA, 1 h, 99.7%; (g) EDCI, HOBt, DIPEA, CH_2Cl_2, 65.1–94.5%.

subsequently acylated with various substituted carboxylic acids using *N*-ethylcarbodiimide (EDC), 1-hydroxybenzotriazole (HOBt), and *N*,*N*-diisopropylethylamine (DIPEA) to afford the benzyl ester series **12a–g**.

In Scheme 2, benzyl ester series **12a–g** were treated with catalytic hydrogenation to remove the benzyl group thereby releasing the carboxylic acid group which were reacted with primary amines in the presence of coupling reagent EDCI/NHS to afford the target pyrrolo[2,3-*d*]pyrimidine-phenylamide hybrids **15a–g** and **16a–g**. Compounds **13a–g** were converted to carboxamide series **14a–g** by coupling with ammonia in dry tetrahydrofuran (THF) at room temperature in the presence of carbonyl diimidazole (CDI),[25] which is a common activating agent for the synthesis of amide from carboxylic acid and ammonia through the acylimidazole intermediate.

The structures of pyrrolo[2,3-*d*]pyrimidine-phenylamide hybrids **12a–g**, **14a–g**, **15a–g** and **16a–g** were characterized by ^1H NMR, ^{13}C NMR and HRMS analysis. The data of the representative compounds **12c**, **16c** and **16e** are presented as examples.[26]

The kinase inhibitory activities of pyrrolo[2,3-*d*]pyrimidine-phenylamide hybrids **12a–g**, **14a–g**, **15a–g** and **16a–g** against JAK2 were evaluated by using the Z′-LYTE™ kinase assay kit (Life Technologies).[27] Tofacitinib, The FDA-approved drug, was used as positive control to validate the screening conditions. The IC_{50} values against JAK2 kinase displayed by Tofacitinib was 5 nM, which was highly consistent with the data reported previously.[21] Besides, in vitro JAK2 mediated cellular inhibitory activities of targeted hybrids were further measured in TF-1 cell line (an erythroleukemic cell line).[28] In this assay, TF-1 cells stimulated with GM-CSF was used to measure the intracellular concentration of pSTAT5, a downstream target of JAK2. The results of the biological assays were summarized in Table 1.

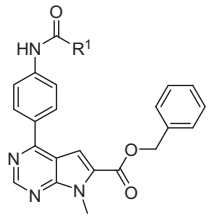


Scheme 2. Synthesis of pyrrolo[2,3-*d*]pyrimidine-phenylamide hybrids **14a–g**, **15a–g** and **16a–g**. Reagents and conditions: (a) H_2, 10% Pd/C, CH_3OH, 24 h, 99.2%; (b) R_2NH_2, EDCI, NHS, THF, 73.5–91.2%; (c) CDI, NH_3 (7 M) in MeOH, THF, 67.3–89.4%.

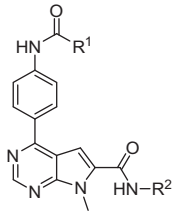
As shown in Table 1, most of the designed hybrids (**12a–g**, **14a–g**, **15a–g** and **16a–g**) presented moderate JAK2 kinase inhibitory and cellular activity, especially compounds **15c**, **15e**, **16c** and **16e**. To our delight, compound **16c** exhibited the most potent JAK2 kinase and cellular activity with IC_{50} of 6 nM and 0.14 μM, which was comparable to the reference Tofacitinib (4 nM and 0.095 μM). From the structure–activity relationship study, the data manifests that the properties of the substituents at R2-position are crucial for the potency and that follows this trend: 2-(pyrrolidin-1-yl)ethyl series **15a–g**, 3-morpholinopropyl series **16a–g** > carboxamide series **14a–g** > benzyl ester series **12a–g**. The benzyl ester series **12a–g** displayed no or very low JAK2 inhibitory activity, indicating that the benzyl ester group did not bring any advantage in comparison with the amide group. Replacement of the solvent tail R2-position with morpholine or pyrrolidine provided compounds **15a–g** and **16a–g**, both of which exhibited excellent potency toward JAK2 and showed exquisite selectivity over JAK3 relative to **14a–g** (Table 2).

We assumed that the size variation of the R1 substituent would have an influence on the inhibitory activity against JAK2 due to the fact that R1 group occupied the pocket beneath the conserved glycine-rich loop. To prove this hypothesis, several R1 substituents were examined (Table 1). Compounds with small hydrophobic substituents (Me, Et; entries **15a**, **15b**, **16a** and **16b**) showed similar JAK2 potency, with IC_{50} values in the range of 23–155 nM. However, bulky substituents ((*S*)-prolinyl moiety: entries **15e** and **16e**) showed an additional potency boost (JAK2 IC_{50} = 9 nM and 10 nM), promoting the enzymatic potency to a 10 nM. Also, compound **16c**, where R1 is a bulky substituents (*tert*-butyl), showed the highest affinity for JAK2 and selectivity over JAK3 (IC_{50} = 6 nM with >97-fold selectivity vs JAK3, Table 2), which also showed excellent cellular JAK2 potency (IC_{50} = 0.14 μM) in a TF-1 cell assay. The most disappointing result was the lack of activity showed by compounds carrying the benzamide moiety (series **g** and **h**).

Table 1
Chemical structures and activity profiles of target compounds[a]



12a-g



14a-g, 15a-g, 16a-g

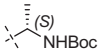
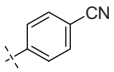
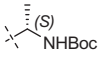
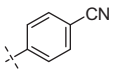
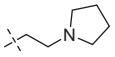
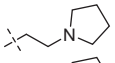
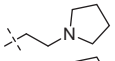
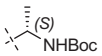
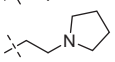
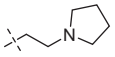
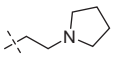
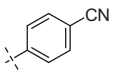
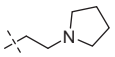
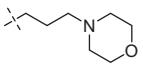
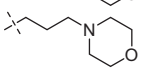
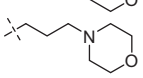
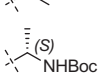
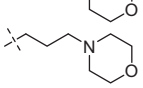
Compound	R^1	R^2	JAK2 IC_{50}[b] (nM)	TF1-GMCSF IC_{50} (μM)
12a	Me	–	732	ND[c]
12b	Et	–	531	ND[c]
12c		–	786	ND[c]
12d		–	475	>20
12e		–	>1000	>20
12f		–	>1000	ND[c]
12g		–	>1000	ND[c]
14a	Me	H	184	>20
14b	Et	H	97	15.8
14c		H	20	0.91
14d		H	51	4.8
14e		H	33	1.2
14f		H	908	>20
14g		H	>1000	ND[c]
15a	Me		155	4.3
15b	Et		67	2.9
15c			8	0.3
15d			21	1.4
15e			9	0.52
15f			78	ND[c]
15g			41	17.5
16a	Me		36	9.6
16b	Et		23	>20
16c			6	0.14
16d			17	9.3

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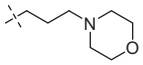
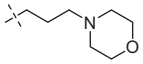
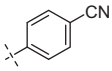
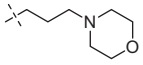
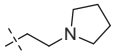
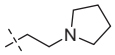
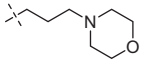
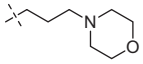
Table 1 (*continued*)

Compound	R^1	R^2	JAK2 IC_{50}[b] (nM)	TF1-GMCSF IC_{50} (μM)
16e			10	0.27
16f			94	ND[c]
16g			81	>20
Tofacitinib	—	—	5	0.095

[a] Assays done in replicates ($n \geqslant 3$). Mean values are shown and the standard deviations are <30% of the mean.
[B] Enzymatic IC_{50}s obtained with Z′-Lyte activity assays.
[c] ND: not determined.

Table 2
JAK selectivity profiles for Tofacitinib, **15c**, **15e**, **16c** and **16e**[a]

Compound	R^1	R^2	JAK2 IC_{50}[b] (nM)	JAK3 IC_{50}[b] (nM)	JAK3/JAK2
15c			8	953	119
15e			9	716	80
16c			6	584	97
16e			10	507	50.7
Tofacitinib	—	—	5	4	0.8

[a] Assays done in replicates ($n \geqslant 3$). Mean values are shown and the standard deviations are <30% of the mean.
[b] Enzymatic IC_{50}s obtained with Z′-Lyte activity assays.

Compounds **15c**, **15e**, **16c** and **16e** exhibited significant biochemical, cellular potencies and high selectivity (50- to >119-fold, Table 2), whereas other series **15** and **16** showed moderate activity. It is worth mentioning that all these active molecules carry a bulkier hydrophobic substituent at R1.

To further explain the potent activities of compound **16c**, we performed a docking analysis utilizing the C-DOCKER program within Discovery Studio 3.0 software package (Fig. 3). Docking simulations were performed on the kinase domain of JAK2 (PDB ID: 3TJC). The docking results showed that: the pyrrolo[2,3-*d*]pyrimidine nitrogen interacted with the hinge region by hydrogen bonding with the Leu932; the *tert*-butyl made close contacts with the glycine rich P-loop where the amide moiety was form hydrogen bond with Asp994; the 3-morpholinopropyl hydrophobic side-chain at R2 was exposed to solvent region.

Based on the significant biochemical and cellular potencies as well as high selectivity, the in vivo pharmacokinetics parameters of compound **16c** were calculated following intravenous bolus administration (iv) and oral gavage administration (po) in Sprague–Dawley rat.[29] As shown in Table 3, compound **16c** displayed low plasma clearance (CL = 3.30 mL/min/kg). The volume of distribution (V_{ss}) was 612 mL/kg leading to a moderate half-life of approximately 2 h. Oral dosing of compound **16c** yielded a C_{max} value of 6.14 μg/mL with a moderate T_{max} at 4 h. The drug exposure ($AUC_{0-24\,h}$) of compound **16c** was 19.72 or 36.27 μg h/mL when delivered by iv at 4 mg/kg or by po at 10 mg/kg, resulting in a good bioavailability (F = 73.57%). The pharmacokinetic parameters results suggested that compound **16c** might be an excellent candidate for the treatment of MPNs.

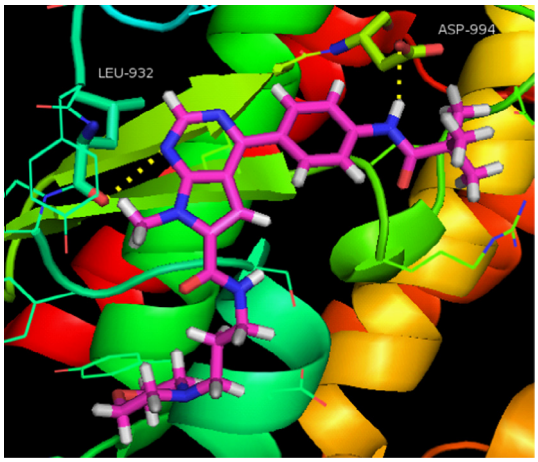


Figure 3. Compound **16c** docked into the ATP-binding site of the kinase domain of JAK2 (PDB code: 3TJC). **16c** is shown as sticks. Hydrogen bonds are shown as yellow dashed lines.

Table 3
Pharmacokinetic profiles of compound **16c**[a] in mice[b]

Route	iv	po
Dose (mg/kg)	4	10
$AUC_{0-24\,h}$ (μg h/mL)	19.72	36.27
CL (mL/min/kg)	3.30	
V_{ss} (mL/kg)	612	
C_{max} (μg/mL)	9.73	6.14
T_{max} (h)		4.00
$t_{1/2}$ (h)	2.09	2.32
F (%)		73.57%

[a] Formulated in a solution of 5% ethanol, 5% emulsifier EL, and 90% normal saline.
[b] n = 4.

In conclusion, a series of novel pyrrolo[2,3-*d*]pyrimidine-phenylamide hybrids were designed, synthesized and evaluated for their biological activities in vitro. Most of these compounds exhibited potent activities against JAK2 kinase with IC_{50} values in nanomolar range. Among them, compound **16c** exhibited the most potent inhibitory activity against JAK2 with IC_{50} value of 6 nM and high selectivity (>97-fold selectivity vs JAK3). Additionally, compound **16c** also demonstrated an excellent cellular activity (IC_{50} = 0.14 μM) in TF-1 cell assay and had a good rat pharmacokinetic profiles (F = 73.57%). Molecular docking study indicated that hydrogen bond and hydrophobic interaction are essential for **16c** to interact within the putative binding site of JAK2. The promising results indicated compound **16c** is a potent lead compound for the

discovery of inhibitors of JAK2 tyrosine kinase. We believe that it has potential for further detailed investigation and optimization to afford novel anti-MPNs drugs.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.04.027.

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26. *Selected spectroscopic data*. Compound **12c**: ^{1}H NMR (300 MHz, $CDCl_3$-*d*): δ (ppm) 9.04 (s, 1H), 8.13 (d, J = 8.8 Hz, 2H), 7.77 (d, J = 8.8 Hz, 2H), 7.59 (s, 1H), 7.53 (br s, 1H), 7.43 (m, 5H), 5.41 (s, 2H), 4.19 (s, 3H), 1.36 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 176.3, 160.5, 159.0, 153.3, 152.8, 140.0, 134.9, 132.4, 130.4, 129.4, 129.0, 128.8, 128.4, 128.3, 128.1, 127.9, 119.5, 113.3, 108.3, 66.5, 30.3, 29.2, 27.1; HRMS (ESI) calcd for $C_{26}H_{26}N_4O_3$ $[M+H]^+$: 443.2038; found, 443.2072. Compound **16c**: ^{1}H NMR (300 MHz, $CDCl_3$-*d*): δ (ppm) 8.97 (s, 1H), 8.28 (m, 1H), 8.05 (d, J = 8.7 Hz, 2H), 7.73 (d, J = 8.8 Hz, 2H), 7.61 (br s, 1H), 7.09 (s, 1H), 4.14 (s, 3H), 3.70–3.65 (m, 4H), 3.60–3.54 (m, 2H), 2.56 (t, J = 5.9 Hz, 2H), 2.51–2.53 (m, 4H), 1.86–1.78 (m, 2H), 1.33 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 169.1, 161.4, 158.4, 153.0, 152.9, 140.1, 133.9, 133.3, 129.7, 120.0, 113.9, 101.6, 66.8, 58.3, 54.0, 40.0, 30.5, 27.7, 24.2. HRMS (ESI) calcd for $C_{26}H_{35}N_6O_3$ $[M+H]^+$: 479.2765; found, 479.2726. Compound **16e**: ^{1}H NMR (300 MHz, $CDCl_3$-*d*): δ (ppm) 9.87 (br s, 1H), 8.93 (s, 1H), 8.13 (br s, 1H), 7.98 (d, J = 8.8 Hz, 2H), 7.71 (d, J = 8.8 Hz, 2H), 7.02 (s, 1H), 4.53–4.50 (m, 1H), 4.13 (s, 3H), 3.72–3.69 (m, 4H), 3.61–3.55 (m, 2H), 3.56–3.41 (m, 2H), 2.62–2.57 (t, 2H), 2.54–2.53 (m, 4H), 2.22–2.17 (m, 2H), 1.97–1.95 (s, 2H), 1.89–1.85 (m, 2H), 1.50 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 170.6, 161.4, 158.3, 152.9, 152.8, 140.5, 133.7, 129.4, 122.0, 120.9, 119.6, 113.7, 101.7, 80.9, 66.7, 60.6, 58.1, 53.7, 47.2, 39.8, 30.5, 29.6, 28.3, 25.6, 24.3; HRMS (ESI) calcd for $C_{31}H_{41}N_7O_5$ $[M+H]^+$: 592.3242; found, 592.3237.
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