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Graphical Abstract

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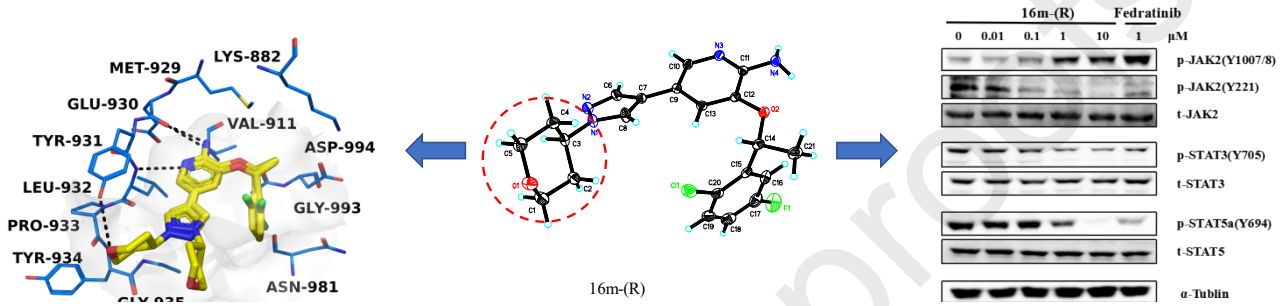
Discovery and optimization of 2-aminopyridine derivatives as novel and selective JAK2 inhibitors

A novel 2-aminopyridine derivative **16m-(R)** was designed as a potent and selective JAK2

Xiangyu Ma[#,a] , Yanyan Diao[#,a], Huan Ge[#,a], Fangling Xu[a], Lili Zhu[a,*], Zhenjiang Zhao[a,*], Honglin Li[a,*]



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Discovery and optimization of 2-aminopyridine derivatives as novel and selective JAK2 inhibitors

Xiangyu Ma[#,a] , Yanyan Diao[#,a], Huan Ge[#,a], Fangling Xu[a], Lili Zhu[a,*], Zhenjiang Zhao[a,*], Honglin Li[a,*]

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ABSTRACT

Janus kinases (JAKs) including JAK1, JAK2, JAK3, and TYK2 are members of a family of intracellular nonreceptor tyrosine kinases, which have been demonstrated to be critical in the cell signaling pathway and involved in inflammatory diseases and cancer. V617F mutation in JAK2 has been implicated in polycythaemia vera (PV), essential thrombocythaemia (ET) and myelofibroisis (MF). Here, we described the design, synthesis, and biological evaluation of a series of 2-aminopyridine derivatives. The results of enzymatic activity assays supported compound **16m-(R)** as a potential and selective JAK2 inhibitor, which exhibited high inhibitory activity with an IC_{50} of 3 nM against JAK2, and 85- and 76-fold selectivity over JAK1 and JAK3, respectively. Structure-activity relationships (SAR) and mechanistic analysis demonstrated that **16m-(R)** might be a promising selective JAK2 inhibitor for further study.

Many cytokines exert their effects by employing the JAK-STAT cell signaling pathway, which play critical role in the regulation of cell growth, metabolism, differentiation, as well as cell migration and survival.[1] Dysregulation of the JAK-STAT pathway can result in inflammation, immunodeficiency and cancer.[2]

Janus kinases (JAKs) including JAK1, JAK2, JAK3, and TYK2 are members of a family of intracellular nonreceptor tyrosine kinases. JAK2 is known to be associated with myeloproliferative neoplasms (MPNs) including polycythaemia vera (PV), essential thrombocythaemia (ET) and myelofibroisis (MF), owing to the strong evidence provided by the identification of the activating V617F mutation in the pseudo kinase domain (JH2) of JAK2. [3] Almost all patients with PV (97%), and in half with ET (57%), MF (50%) have been found carrying V617F mutation in JAK2.[4] V617F is proximal to the SH2-JH2 linker and the catalytically active conformation of αC in JH1 is more stable in V617F than in wild-type JAK2, which can help promote the process of phosphorylation.[5] The discovery ignite interests in the exploitation of JAK2 inhibitors for targeted therapy since 2005, which culminated in the discovery of ruxolitinib, a selective JAK inhibitor, that inhibit JAK2 and JAK1, with IC_{50}s of 5.7 nM and 5.9 nM respectively.[6] In 2013, ruxolitinib was allowed by FDA for the treatment of patients with intermediate- and high-risk myelofibrosis. The success of ruxolitinib provided a strong impetus for the exploitation of other JAK2 inhibitors. For example, pacritinib[7], targeting both JAK2 and FLT3, has been proven to become more effective than ruxolitinib for reducing splenomegaly and symptoms in patients with myelofibrosis and

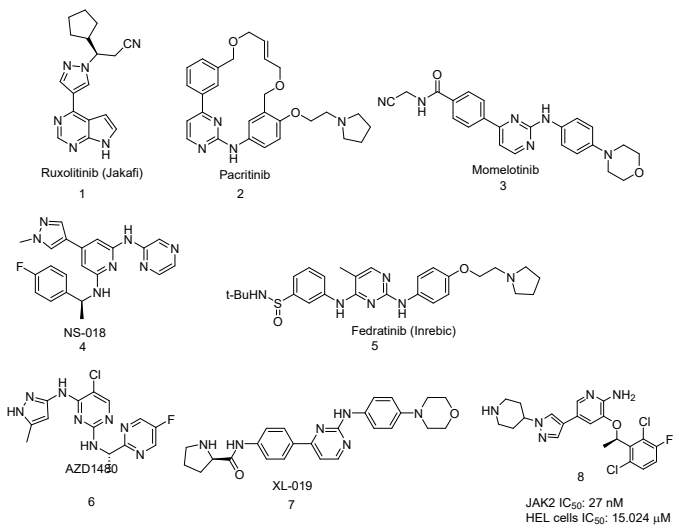

Figure 1. Structures of (1) Ruxolitinib, (2) Pacritinib, (3) Momelotinib, (4) NS-018, (5) Fedratinib, (6) AZD1480, (7) XL-019, (8) Crizotinib.

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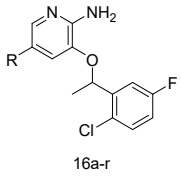
thro[illegible] phase.[8] Another JAK1 and JAK2 inhibitor, momelotinib, was shown to be effective and well tolerated in patients with myelofibrosis at an oral administration of 300 mg per day.[9]

Unfortunately, some of the discovered JAK2 inhibitors had to be cancelled, owing to their severe side effects. For example, the unexpected occurrence of neurological toxicity, especially central nervous system (CNS) neurotoxicity, in AZD1480[10] and XL019[11], lead to the suspension in the early stage. The study of fedratinib was ever suspended in phase 3 phase trial due to 3 cases of Wernicke encephalopathy.[12] The unexpected is that fedratinib has been approved by FDA for the treatment of (intermediate-2/high-risk) primary or secondary myelofibrosis. The redevelopment of fedratinib gives us the courage to develop novel selective JAK2 inhibitors.

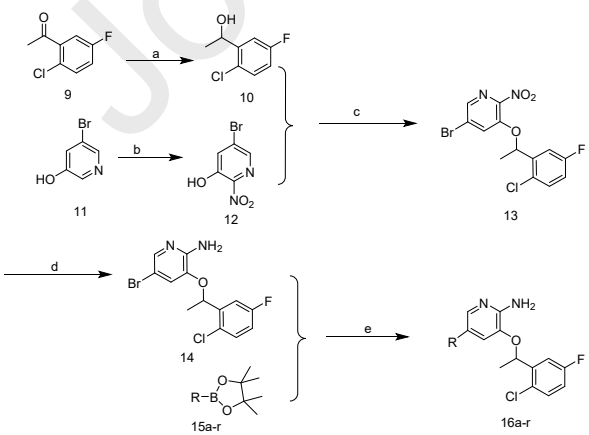
However, the process of discovering specific inhibitor against JAK2 over other JAK family is proved to be a great challenge because of the high homology in catalytic domain among the JAK family, although amino acid differences in family members do exist.[13] In our previous work, a c-MET and ALK inhibitor crizotinib (compound **8** in Figure 1) was found to have good activity against JAK2 with an IC_{50} value of 27 nM. Then, structural optimization from crizotinib had been carried out and some potent compounds were obtained.[14] In this study, we paid our attention to modify the solvent exposed area (R group in Figure 2), where the non-conserved residues are thought to be the main reason to provide high selectivity.[15] Therefore, we described the design, synthesis and biological evaluation of a series of 2-aminopyridine derivatives from crizotinib, finally leading to the discovery of some potential JAK2 inhibitors (Figure2).

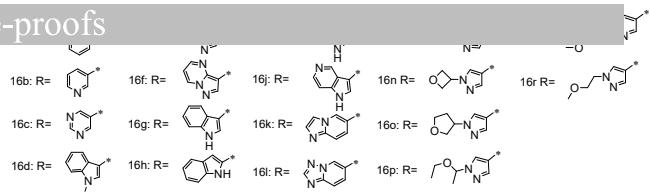


16a-r

Figure 2. General molecular formula of **16a-r.**

The general synthetic procedures for 2-aminopyridine derivatives **16a-r** were described in Scheme 1. 1-(2-chloro-5-fluorophenyl)ethanone **9** was reduced by sodium borohydride to give **10**. 3-Hydroxy-5-bromopyridine **11** was subjected to nitration to obtain **12**. Mitsunobu reaction was then conducted to gain compound **13**. The key intermediate **14** was then obtained via reduction by Fe powder. In the end, Suzuki-coupling reaction occurred between **14** and **15a-r** to obtain the final products **16a-r**.





Scheme 1. Reagents and Conditions: (a) $NaBH_4$, MeOH, 0℃; (b) concentrated H_2SO_4, HNO_3, 0 ℃; (c) PPh_3, DIAD, THF, 0 ℃; (d) Fe powder in HOAc/C_2H_5OH 1:1 78℃; (e) Cs_2CO3, $Pd(dppf)Cl_2$, toluene, H_2O, 80℃.

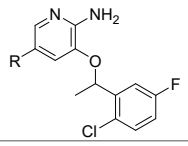
At the initial optimization stage, we designed several compounds with simple aromatic rings at R position with the purpose of providing special π-π interactions with Tyr931 at the JAK2 hinge region. Compounds **16a-c** were synthesized. However, these compounds showed weaker activities against JAK2 than crizotinib. The loss of potencies might indicate that the sizes of the aromatic six-membered ring substituents were a little small and couldn't fit the pocket well, or in another case, the single aromatic rings were probably too far away from Tyr931 to form π-π stack interactions.

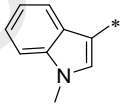
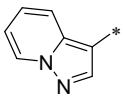
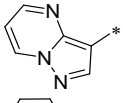
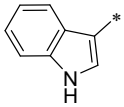
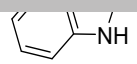
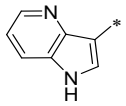
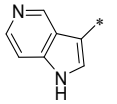
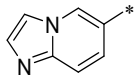
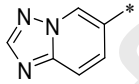
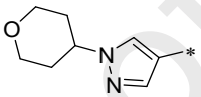
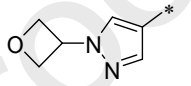
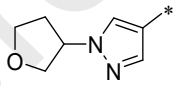
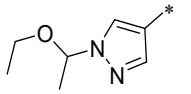
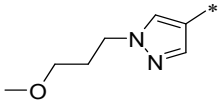
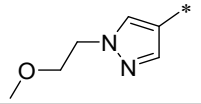
In order to verify our hypothesis, compounds **16d-j** containing five-fused-six membered heterocyclic rings were therefore synthesized. As shown in table 1, compounds **16e** and **16f** (IC_{50} = 0.061 μM and 0.145 μM, respectively) displayed better activities against JAK2 than other compounds in this series (except for compound **16j**). The improved potency may be ascribed to the advantageous π-π stack effects between the pyrazolo[1,5-a]pyridine or pyrazolo[1,5-a]pyrimidine ring and Tyr931. Interestingly, compound **16i**, with the introduction of a nitrogen atom to the ortho-position of the benzene ring, suffered 10-fold potency loss compared to compound **16g**. The same tendency was also observed between compounds **16e** and **16f.** Surprisingly, when the nitrogen atom was moved from the ortho- to the meta-position of the benzene ring, compound **16j** showed 452-fold increase in potency compared to compound **16i** (IC_{50} = 0.022 μM vs 9.963 μM). In the predicted binding mode of compound **16j** to JAK2 (Figure 3A), the 2-aminopyrimidine core participates in hydrogen bond interactions with residues Glu930 and Leu 932 in the hinge region. The added nitrogen atom possibly forms an additional hydrogen bond to hinge residue Tyr931, offering a possible explanation for the significant improvement in potency of compound **16j**.

After the second rounds of structural optimization mentioned above, obvious improvement in terms of activity against JAK2, comparing with crizotinib, still had not been made. The reason may attribute to the steric clash which was caused by the terminal six-membered ring of these substituents with adjacent residues such as Gly935 and Tyr931. Therefore, an alternative optimization strategy was adopted with different oriented di-heterocyclic R substituents. Compounds **16k** and **16l** were synthesized and then we evaluated their inhibitory activities against JAK2. The results were consistent with our expectation. Compounds **16k** and **16l** exhibited superior activity against JAK2 with the IC_{50} values of 0.051 μM and 0.071 μM, respectively. The increased activity of **16k** and **16l** compared to **16e** could be explained in Figure 3B. The predicted binding modes of compounds **16e** and **16k** docked in JAK2 were superimposed to dissect the SAR. The distal five-membered ring lies in a position farther away from the hinge region, putatively leaving sufficient space to avoid the possible steric conflicts with the surrounding amino acid residues.

A inhibitory activity, for example **16j** showed a high activity with the IC_{50} of 22nM at the JAK2 kinase level. Results were still not very encouraging when these compounds were compared with JAK2 inhibitor fedratinib (IC_{50}=3 nM). Therefore, we designed and synthesized another series of compounds (**16m-r**) based on crizotinib and our previous work. Encouragingly, this series showed remarkable potency against JAK2, especially for compound **16m** with an IC_{50} value of 8 nM. Compound **16m** was docked to the ATP-binding pocket of JAK2. As expect, **16m** shares a similar binding profile with **16j**, with the 2-aminopyrimidine core forming critical hydrogen bond interactions with the hinge region (Figure 3C). In addition, the R substituent of **16m** could swing either to the left side of the solvent channel or the right side at the bottom of the pocket. When the tail extends toward the right side, there is no direct interaction between JAK2 and the inhibitor. However, the oxygen atom of the tetrahydropyran moiety could form the third hydrogen bond with Tyr931 when it pointed to the left side. Compounds **16n** and **16o**, two analogues of **16m**, also showed good potencies against JAK2, with the IC_{50} values of 0.025 and 0.027μM, respectively. Based on the above SAR, we inferred that oxygen atoms in rings played an important role in the potency against JAK2. Subsequently, several open-chained ethers substituted compounds were synthesized. Compound **16r**, in which the oxygen atom in alkyl chains formed a hydrogen bond with Tyr931 (Figure 3D), displaying a comparable JAK2 inhibitory activity (IC_{50} = 0.011 μM) to compounds **16m**. However, **16p** showed a slight decrease in potency, probably due to the unsuitable position of the oxygen atom, which was not capable of forming the hydrogen bond interaction.

Table 1. Structure and activity profiles of compounds **16a-16r** against JAK2 kinase



Compd	R	JAK2 IC_{50}/μM
16a		0.461±0.002
16b		0.090±0.002
16c		0.566±0.029
16d		0.695±0.002
16e		0.061±0.007
16f		0.145±0.010
16g		0.925±0.102
16h		>10
16i		9.963±0.035
16j		0.022±0.001
16k		0.051±0.001
16l		0.071±0.001
16m		0.008±0.001
16n		0.025±0.001
16o		0.027±0.001
16p		0.157±0.031
16q		0.015±0.001
16r		0.011±0.001

Because of the satisfying JAK2 inhibitory potency of compound **16m**, its two optical isomers were obtained by chiral resolution. The structure of **16m-(R)** was determined through single crystal X-ray diffraction (Figure 4). After the evaluation of the enzymatic activity, the R-enantiomer was found to be the most potent compound in our study (IC_{50} =3 nM) , while the S-enantiomer showed poor activity (IC_{50} =1399 nM). Alignment of the proposed binding poses of **16m-(R)** and **16m-(S)** (figure S2) indicate that the methyl group of **16m-(S)** lies in a position closer to residue Asp994 than **16m-(R)**, and bad contacts were detected by the measurement toolbar of Maestro v10.1 with default criteria. Therefore, the less potent inhibitory activity of **16m-(S)** against JAK2 may be contributed to the steric conflicts between the methyl group and Asp994.

The fluorescence-based thermal shift assay is a general method for identification of inhibitors of the target protein. The inhibitor binding affinity can be assessed from the shift of the melting temperature in the presence of the inhibitor relative to that obtained in the absence of the inhibitor.[16] The ability of racemate **16m** and optical isomers **16m-(S), 16m-(R)** increasing thermal stability of the JAK2 (JH1) were evaluated by fluorescence-based thermal shift assays. The melting temperature (Tm) for JAK2 (JH1) alone was 45.1℃, and the present of **16m, 16m-(S)** and **16m-(R)** with an increase of 11.9℃, 5.5℃ and 13.1℃, respectively. These results declared that **16m-(R)** had a stronger ability to improve the thermal stability of JAK2 than **16m-(S)**,

whi
JAK2 than **16m-(S)**.

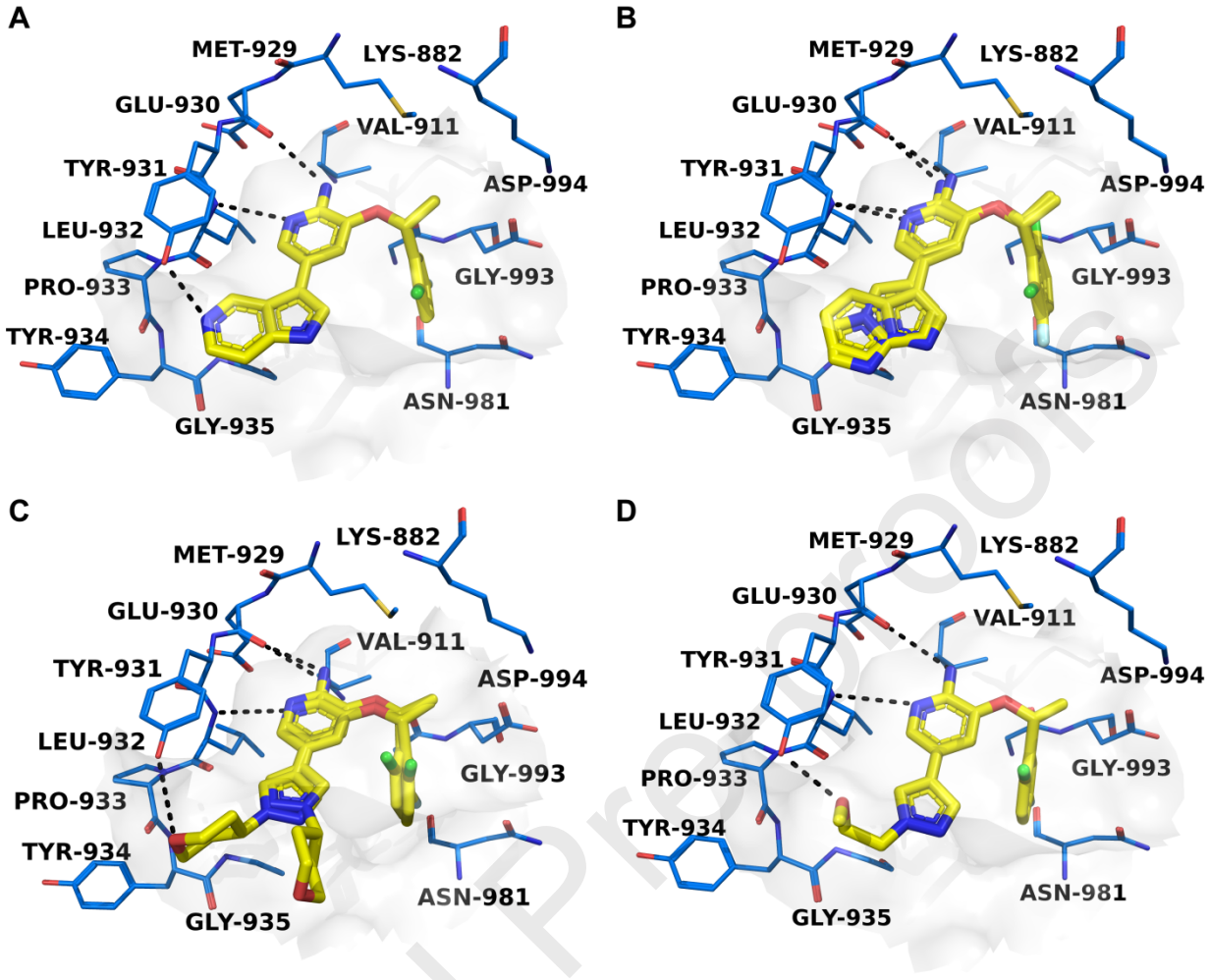


Figure 3. Predicted binding poses of compounds **16j** (A), **16e** and **16k** (B), **16m** (C), and **16r** (D) in the ATP binding pocket of JAK2 (PDB code 2XA4). The docking process was implemented using Glide SP mode of Maestro v10.1 (Schrödinger Inc.). Key residues around the binding pocket are displayed as marine lines, and the hydrogen bonds are highlighted as black dashed lines.

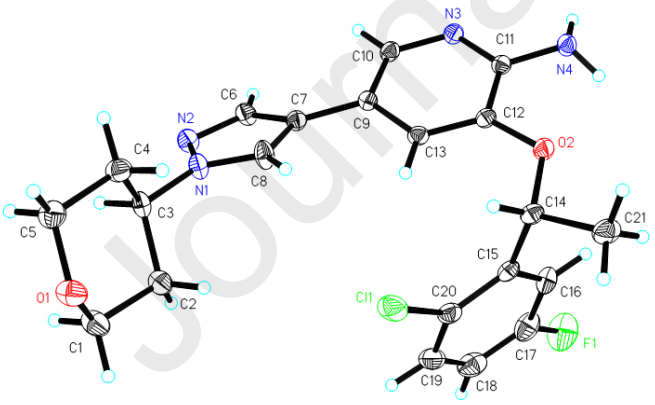


Figure 4. X-ray diffraction structure of **16m-(R)**. CCDC NO. 1922072

Subsequently, **16m- (R)** was selected to evaluate the selectivity of JAK family (JAK1 and JAK3) inhibition, and fedratinib was used as the positive control. The results were shown in table2. **16m-(R)** displayed an IC_{50} of 3 nM against JAK2 and was approximately 85-and 76-fold selectivity over JAK1 and JAK3. The tetrahydropyran ring occupied the region where amino acid difference exists (JAK2/Gln 853, JAK1/Arg 868, JAK3/Ser 826), contributing to the observed selectivity against JAK2 over JAK1 and JAK3. Then, **16m-(R)** was evaluated its antiproliferative activity against $JAK2^{V617F}$-dependent cells, Human Erythroid Leukemia cells (HEL cells). The result showed that compound **16m-(R)** exhibited potent antiproliferative effects with an IC_{50} of 5.75 μM.

In order to verify the mechanism of **16m-(R)** in the JAK2-STAT signaling pathway, the phosphorylation levels of JAK2 and its downstream substrates were evaluated. As shown in figure 6, the treatment of the HEL cells with **16m-(R)** or fedratinib led to the increase of pJAK2 (Y1007/8) level. The same phenomenon was also found in fedratinib, which are general effects of type I ATP competitive inhibitors of JAK2, such as Gö6976, SB1518 and ruxolitinib.[17, 18] Furthermore, JAK2 has been reported to have other autophosphorylation sites for the exception of Y1007/8, which is necessary for JAK2 full activated.[19] In our experiment, **16m-(R)** can effectively block the phosphorylation Y221 of JAK2, and also inhibit STAT3 and STAT5 phosphorylation. Overall, these results revealed that **16m-(R)** down-regulated the JAK2-STAT signaling pathway by inhibiting JAK2 kinase activity in HEL cell.

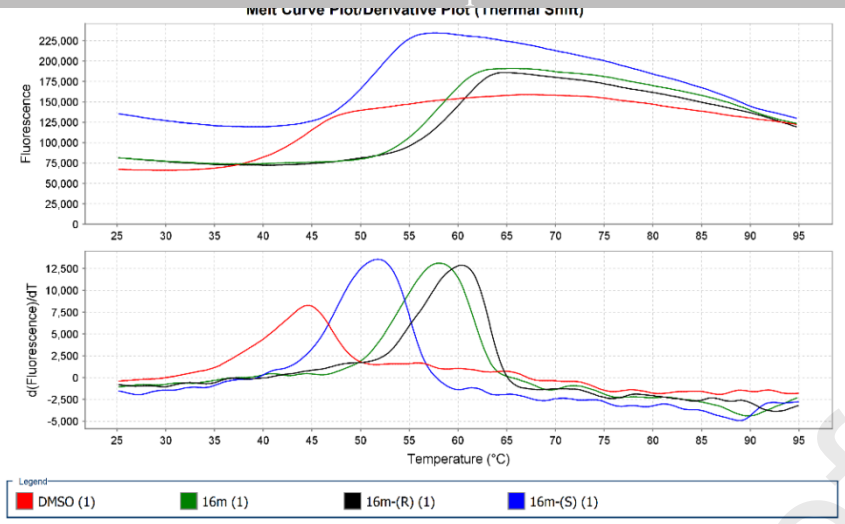


Figure 5. Thermal melting fluorescence curves (up) and derivative curves of the denaturation data (down) of JAK2(JH1) in the present of DMSO (red line)、racemate **16m** (green line),optical isomers **16m-(S)** (blue line) and **16m-(R)** (black line).

Table 2. Evaluation of the inhibitory activities of selected compounds against JAK1, JAK2 and JAK3, and antiproliferative effects in HEL cells.

Comd	Enzyme inhibitory activity (IC_{50}/μM)			Enzyme selectivity		HEL cell (IC_{50}/μM)
	JAK1	JAK2	JAK3	JAK1/JAK2 ratio	JAK3/JAK2 ratio	
16m-(R)	0.254±0.041	0.003±0.001	0.228±0.007	85	76	5.754±0.038
Fedratinib	0.096±0.001	0.003±0.001	0.263±0.002	32	88	1.519±0.023

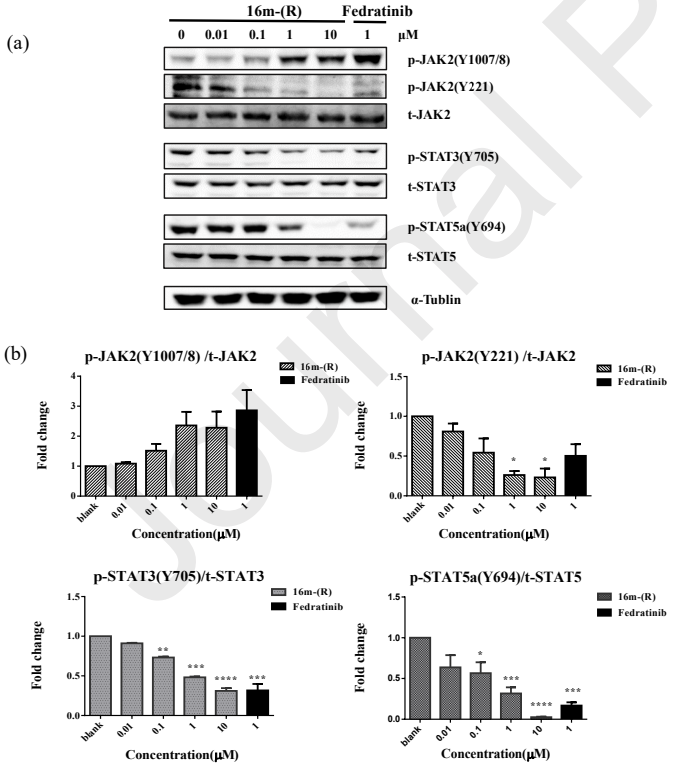


Figure 6. 16m-(R) effectively blocks the JAK2-STAT signaling pathways in HEL cells. (a) Western blot analysis of phosphorylated JAK2(Y1007/1008), JAK2 (Y221), STAT3, STAT5. (b) Quantitative analysis of the intensity of the bands of p-JAK2, p-STAT3 and p-STAT5 from western blot images (n = 3). Data are represented as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****p < 0.0001.

In conclusion, a series of 2-aminopyridine derivatives were designed, synthesized and evaluated for their biological activity against JAK2. Half of these compounds exhibited potent biological activities against JAK2 in kinase level with IC_{50} values in nanomolar range. Among them, compound **16m-(R)** [(R)-3-(1-(2-chloro-5-fluorophenyl)ethoxy)-5-(1-(tetrahydro-2H-pyran-4-yl)-1*H*-pyrazol-4-yl)pyridin-2-amine] exhibited the most potent inhibitory activity against JAK2, with IC_{50} of 3 nM and 85- and 76-fold selectivity over JAK1 and JAK3, respectively. In addition, **16m-(R)** significantly suppressed JAK-STAT signaling pathways in HEL cells. Hence, **16m-(R)** might be selected as a promising JAK2 inhibitor for further detailed investigation in the future.

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Supplementary Material

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Declaration of interests

Discovery and optimization of 2-aminopyridine derivatives as novel and selective JAK2 inhibitors

A novel 2-aminopyridine derivative **16m-(R)** was designed as a potent and selective JAK2

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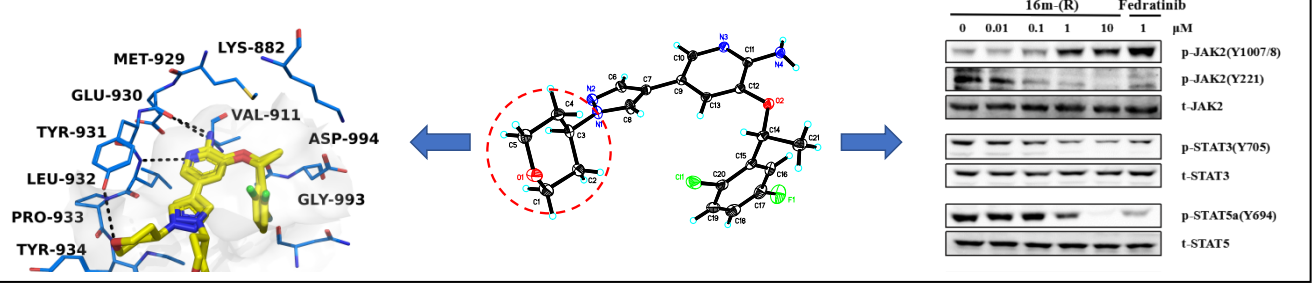

☒ competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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