Design, Synthesis, and Antitumor Evaluation of 4-Amino-(1H)-pyrazole Derivatives as JAKs Inhibitors

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Supporting Information

ABSTRACT: Abnormalities in the JAK/STAT signaling pathway lead to many diseases such as immunodeficiency, inflammation, and cancer. Herein, we designed and synthesized a series of 4-amino-(1H)-pyrazole derivatives as potent JAKs inhibitors for cancer treatment. Results from *in vitro* protein kinase inhibition experiments indicated that compounds **3a–f** and **11b** are potent JAKs inhibitors. For example, the IC₅₀ values of compound **3f** against JAK1, JAK2, and JAK3 were 3.4, 2.2, and 3.5 nM, respectively. In cell culture experiments, compound **3f** showed potent antiproliferative activity against various cell lines (PC-3, HEL, K562, MCF-7, and MOLT4) at low micromolar levels, while compound **11b** showed selective cytotoxicity at submicromolar levels against HEL (IC₅₀: 0.35 μM) and K562 (IC₅₀: 0.37 μM) cell lines. It is worth noting that both **3f** and **11b** showed more potent antiproliferative activities than the approved JAKs inhibitor Ruxolitinib.

KEYWORDS: JAKs, Inhibitors, 4-Amino-(1H)-pyrazole, Anticancer

The JAK/STAT signaling pathway plays critical roles in immunity, hematopoiesis, and cell growth.¹ Abnormalities in the JAK/STAT signaling pathway lead to many diseases such as immunodeficiency, inflammation, and cancers.² Constitutive activations of JAKs are correlated to oncogenesis. Dysregulation of JAK2 is discovered in patients with myeloproliferative neoplasms and childhood T cell acute lymphoblastic leukemia.³ Several hematologic malignancies including malignant lymphoma and myeloproliferative disorders are associated with mutations of JAK2.⁴ Some cytokines and growth factors bind to their receptors and then stimulate JAKs for the phosphorylation of STAT3, which is a potential target for anticancer therapy.⁵ Persistent activation of the JAK/STAT3 signaling promotes proliferation and survival of tumor cells.⁶ Thus, the JAK/STAT signaling pathway is a promising antitumor target.

Inhibitors of JAKs are widely explored for treatment of immunodeficiency, inflammation, and cancer. Among the synthetic JAK inhibitors for the treatment of cancer identified to date (Figure 1), Ruxolitinib (Incyte's Jakafi) was approved by the US Food and Drug Administration (FDA) in 2013 for the treatment of myelofibrosis, a rare bone marrow cancer. Besides, it is in phase III clinical trials for the treatment of metastatic pancreatic cancer and phase II clinical trials for the treatment of multiple myeloma, leukemia, and colon cancer.⁷ There are several other JAKs inhibitors in clinical trials for cancer treatment (Figure 1). JAK2 inhibitor AZD1480 can inhibit STAT5

Figure 1. Approved or clinical JAK inhibitors for cancer treatment.

signaling in prostate cancer cells and then effectively inhibit castration-resistant growth of prostate cancer.⁸ JAK2 inhibitor TG101348 blocks JAK/STAT signaling leading to suppression of proliferation and induction of apoptosis and is used for the treatment of myelofibrosis.⁹ JAKs inhibitors Momelotinib and

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Pacritinib are both developed for the treatment of myelofibrosis.¹⁰

In our previous work, we discovered a series of 4-amino-pyrazole derivatives as novel and potent JAK inhibitors (Figure 2).¹¹ Structure–activity relationship (SAR) studies showed that

Figure 2. Design of 4-amino-(1H)-pyrazole derivatives as JAKs inhibitor.

R₁ group modifications in these analogues did not influence their JAK inhibition significantly.¹¹ Such results indicate that the R₁ side chain is not crucial to the interaction between these 4-aminopyrazole derivatives and JAKs proteins. Herein, using 1 as a lead, a series of 4-amino-(1H)-pyrazole derivatives were designed, synthesized, and evaluated as potent JAKs inhibitors. First, removing the R₁ side chain of compound 1 led to compound 3a, which exhibited improved JAKs kinase inhibition at 10 μ M. Then further structural modifications of 3a led to pyrimidine-based 4-amino-(1H)-pyrazole derivatives, quinazoline-based 4-amino-(1H)-pyrazole derivatives, and 7H-pyrrolo[2,3-d] pyrimidine-based 4-amino-(1H)-pyrazole derivatives (Figure 2).

Pyrimidine-based 4-amino-(1H)-pyrazole derivatives 3a–m were synthesized as described in Scheme 1. Generally, the reaction of 5-substituted-2,4-dichloropyrimidine with various aromatic amines under acidic (HCl) or basic (DIPEA, Et₃N, Na₂CO₃) conditions led to the intermediates 2a–m, which was

Scheme 1. Synthesis of Pyrimidine-Based 4-Amino-(1H)-pyrazole Derivatives 3a–m^a

^aConditions and reagents: (a) For compounds 2a, 2b, and 2d, DIPEA, DMF, r.t., 16 h. For compound 2c, Na₂CO₃, EtOH, r.t. overnight. For compound 2e, DIPEA, NMP, 100 °C, 12 h. For compounds 2f and 2g, Et₃N, EtOH, 50–80 °C, overnight. For compound 2h, HCl, H₂O, r.t., 5 days. For compounds 2i and 2l, HCl, H₂O, 50 °C, 5 h. For compound 2j, DIPEA, i-PrOH, 60 °C, overnight. For compounds 2k and 2m, H₂O/CH₃OH = 3:1, 50 °C, 5 h. (b) TFA, *n*-BuOH, 120 °C, 1 h, microwave (MW irradiation).

then reacted with 1H-pyrazol-4-amine using TFA as a catalyst at high temperature to give target molecules 3a–m.¹²

Quinazoline-based 4-amino-(1H)-pyrazole derivatives 6a–f were synthesized as shown in Scheme 2. The synthetic routes of

Scheme 2. Synthesis of Quinazoline-Based 4-Amino-(1H)-pyrazole Derivatives 6a–f^a

^aConditions and reagents: (a) CH₃COONa, THF/H₂O = 3:1, r.t. to 60 °C, overnight. (b) *n*-BuOH, 120 °C, 1 h, microwave (MW irradiation).

derivatives 6a–f were similar to that of Scheme 1. Intermediates 5a–f were obtained from reaction of 2,4-dichloroquinazoline with various amines under basic condition of CH₃COONa.¹³ Then, 5a–f reacted with 1H-pyrazol-4-amine at high temperature to give target molecules 6a–f.

7H-Pyrrolo[2,3-d] pyrimidine-based 4-amino-(1H)-pyrazole derivatives 11a–d were synthesized as shown in Scheme 3. The

Scheme 3. Synthesis of 7H-Pyrrolo[2,3-d]pyrimidine-Based 4-Amino-(1H)-pyrazole Derivatives 11a–d^a

^aConditions and reagents: (a), TsCl, Et₃N, DMAP, CH₂Cl₂, r.t., 5 h. (b) DIPEA, *n*-BuOH, 100 °C, overnight. (c) TFA, *n*-BuOH, 120 °C, 1 h, microwave (MW irradiation). (d) Cs₂CO₃, H₂O/CH₃OH/dioxane = 1:3:3 (v/v/v), 80 °C, 6 h.

starting material 2,4-dichloro-7H-pyrrolo[2,3-d] pyrimidine was protected by 4-methylbenzenesulfonyl chloride¹⁴ to give compound 8, which was reacted with various amines, leading to intermediates 9a–d, under basic conditions at high temperature. Intermediates 9a–d reacted with 1H-pyrazol-4-amine using TFA as a catalyst at high temperature to produce intermediates 10a–d. Ts-deprotection of 10a–d yielded target molecules 11a–d.¹⁵

The 4-amino-(1H)-pyrazole derivatives were screened for their *in vitro* kinase inhibitory activities toward JAK1, JAK2, and JAK3 at 10, 1, and 0.1 μ M and 40 and 20 nM. Because we are only interested in compounds with nanomolar inhibition activities, the final screening was done at 20 nM. Staurosporine (a prototypical ATP-competitive kinase inhibitor; IC₅₀: JAK1 3 nM, JAK2 2 nM, JAK3 1 nM) and Ruxolitinib (an approved JAK inhibitor; inhibition at 20 nM: JAK1 97%, JAK2 99%, JAK3 95%) were used as positive controls.¹⁶ All the inhibition results were shown in Figures 3–6.

Figure 3. *In vitro* inhibitory activity against JAK1, JAK2, and JAK3.

Results in Figure 3 showed that compounds 3a–3f exhibited remarkable inhibitory activities against JAK1, JAK2, and JAK3 at 20 nM with the exception of compound 3d, which was not active against JAK3 at 20 nM. For example, at 20 nM, compound 3f inhibited protein kinase activities of 88%, 80%, and 79% against JAK1, JAK2, and JAK3 respectively. Further evaluation revealed that the IC_{50} values of 3f against JAK1, JAK2, and JAK3 were 3.4, 2.2, and 3.5 nM, respectively. Generally, different substituents on the phenyl ring were well tolerated.

Results in Figure 4 showed that replacing the Cl group on pyrimidine ring with other groups, such as H or F could lead to

Figure 4. *In vitro* inhibitory activity against JAK1, JAK2, and JAK3.

reduced JAKs inhibition. For example, compounds 3g and 3k were much less potent than 3a (Figure 3). Taking the results in Figure 3 and Figure 4 together, we conclude that R_1 group on pyrimidine ring contributed much more to JAKs inhibition than R_2 , R_3 , and R_4 groups on the phenyl ring.

From the data shown in Figure 5, we could see that quinazoline-based 4-amino-(1*H*)-pyrazole derivatives 6a–f almost completely lost their inhibitory activities toward JAKs at 20 nM. This indicated that a large fused ring, such as quinazoline, was not beneficial to binding with JAKs.

Comparing the compounds in Figure 6 with compounds in Figure 3, we could see that 7*H*-pyrrolo[2,3-*d*] pyrimidine-based 4-amino-(1*H*)-pyrazole derivatives (Figure 6) showed moderate JAKs inhibition at 20 nM, but less potent than pyrimidine-based 4-amino-(1*H*)-pyrazole derivatives (Figure 3). For example,

Figure 5. *In vitro* inhibitory activity against JAK1, JAK2, and JAK3.

Figure 6. *In vitro* inhibitory activity against JAK1, JAK2, and JAK3.

compounds 11a, 11b, and 11c in Figure 6 were less potent than 3a, 3b, and 3d in Figure 3, respectively.

It was reported that mutation in the JH2 pseudokinase domain of the Janus kinase 2 gene (JAK2 V617F) existed in HEL (human erythroleukemia) cell line.¹⁷ Therefore, all target compounds were screened against HEL cell line at the concentration of 5 μ M to evaluate their *in vitro* anticancer activities. Results in Figure 7 showed that among these analogues, compounds 3a–f and 11a–d exhibited superior antiproliferative activities against HEL cell line (indicated by the red column) than the other compounds we

Figure 7. Activity screening against HEL cell line at the concentration of 5 μ M. The plates containing compounds and cells were incubated for 48 h in MTT assay.

Table 1. Inhibitory Activities of Compounds Against Tumor Cell Lines

compd	IC ₅₀ ^a (μM)				
	PC-3	MCF-7	HEL	K562	MOLT4
3a	2.57 ± 0.22	1.93 ± 0.02	1.53 ± 0.15	1.70 ± 0.27	1.37 ± 0.23
3b	5.38 ± 0.62	3.66 ± 1.29	5.93 ± 0.01	>8.3 ^b	>5
3c	1.03 ± 0.25	1.87 ± 0.01	1.18 ± 0.15	1.86 ± 0.29	3.28 ± 0.45
3d	2.30 ± 0.98	ND ^c	1.76 ± 0.24	2.08 ± 0.33	ND
3e	1.13 ± 0.08	1.10 ± 0.01	1.24 ± 0.19	1.29 ± 0.21	1.26 ± 0.15
3f	1.08 ± 0.05	1.33 ± 0.42	1.08 ± 0.06	0.77 ± 0.05	1.61 ± 0.35
3k	10.38 ± 0.97	ND	3.96 ± 1.05	3.79 ± 0.86	ND
11b	4.47 ± 1.29	>5	0.35 ± 0.07	0.37 ± 0.11	>5
11d	13.52 ± 1.98	>5	ND	3.72 ± 0.71	>5
6d	ND	>5	9.71 ± 0.99	>8.3	ND
Ruxolitinib	>5	>5	2.62 ± 0.19	10.3 ± 0.3	15.8 ± 1.4

^aIC₅₀ are mean of two or three experiments, and standard deviation is given. ^bIC₅₀ value of this compound is larger than 8.3 or 5. ^cND, not determined.

synthesized. These data were generally consistent with their JAKs inhibitory potency.

Considering their potent JAKs inhibitory activities and antiproliferative potency against the HEL cell line, ten compounds (3a–f, 3k, 11b, 11d, and 6d) were chosen for further antiproliferative evaluation against human prostate cancer PC-3, human breast cancer MCF-7, human erythroleukemia HEL, human myelogenous leukemia K562, and human lymphoid leukemia MOLT4 cell lines. Ruxolitinib was used as a positive control. The results in Table 1 showed that most of the ten compounds possessed potent anticancer activity *in vitro*. Among these compounds, 3a, 3c, 3e, and 3f were cytotoxic to all five tested cell lines, while 11b exhibited remarkably selective cytotoxicity to HEL (IC₅₀: 0.35 μM) and K562 (IC₅₀: 0.37 μM). It is worth emphasizing that, though less potent than Ruxolitinib in JAK inhibition, most of our compounds exhibited more potent cytotoxicity than Ruxolitinib (Table 1), indicating that our compounds might have off-target effects. Therefore, representative compounds 3f and 11b were evaluated against 14 other cancer related kinases. The results in Figure 8 showed that at 20

Figure 8. Selectivity profile of compounds 3f and 11b on 14 protein kinases at 20 nM.

nM compound 3f was active against a number of kinases including Flt-3, VEGFR-2, PDGFRα, and TYK2, while compound 11b exhibited very good selectivity against JAK2 and JAK3 over the other tested kinases. These results could explain why 3f were cytotoxic to all five cell lines, while 11b was more selective against JAK/STAT pathway promoted cell lines,

such as HEL^{18,19} and K562.^{20–22} However, our kinase panel screening results still could not explain why 11b were more cytotoxic than Ruxolitinib. Further anticancer mechanism research of 11b is warranted.

To investigate the binding mode of these 4-amino-(1H)-pyrazole derivatives in JAK2, the most potent compound 3f was docked into the ATP binding pocket of JAK2 using SYBYL-X 2.0 (Figure 9). The results shown in Figure 9 suggested that the

Figure 9. Docking of compound 3f with JAK2 (PDB code 3FUP).

–NH and =N moieties of pyrazole in compound 3f could form hydrogen bonds with GLU930 and LEU932. These hydrogen interactions were the crucial part for the protein kinase inhibition. The –NH moiety of pyrazole as hydrogen donor was necessary for the improvement of binding to JAKs. This maybe the reason why the kinase inhibitory activities of 4-amino-(1H)-pyrazole derivatives were much more potent than their parent 4-amino-pyrazole derivatives. For example, compound 3a reported here is much more potent than its parent 17n (compound 1) reported in our previous research.¹¹ Additional docking with the compound that has a fused phenyl ring (compound 6d) was also studied. Docking results showed compounds 3f and 6d share a similar binding mode. To understand why compound 6d showed weak activity, a molecular dynamics (MD) simulation and a molecular mechanics Poisson–Boltzmann surface area (MM/PBSA) energy decomposition calculation experiment were conducted. Our result showed that the fused phenyl ring and the side chain of ASP939 gave a 1.50 kcal/mol negative energy contribution in binding (Figure 10). Besides, MD simulation indicated that the fused ring position is solvent accessible: it is exposed to water (Figure 11). These two

Figure 10. Docking of compound **6d** with JAK2 (PDB code 3FUP).

reasons may explain why adding an additional large hydrophobic ring, such as a fused phenyl ring, does not favor binding.

Figure 11. Fused phenyl ring is exposed to water in MD simulation. Water molecules within 4 Å of compound **6d** were selected for visualization (spheres).

In summary, a series of 4-amino-(1*H*)-pyrazole derivatives as potent JAKs inhibitors were designed, synthesized, and evaluated. In enzyme inhibition screenings, many of the compounds reached IC_{50} values below 20 nM. In cell-based assays, compound **3f** showed potent cytotoxicity against a wide variety of cell lines at micromolar levels. Compound **11b** possessed very potent cytotoxicity against HEL and K562 cell lines with IC_{50} values in the submicromolar range, which were over 10-fold lower than in PC-3, MCF-7, and MOLT4 cell lines. Further kinase panel screening results revealed that compound **3f** is a pan-kinase inhibitor, while **11b** is a highly selective JAK2 and JAK3 inhibitor, which could be used as lead compound for further structural optimizations to find more potent and selective JAKs inhibitors. Moreover, considering the discrepancy between JAKs inhibition and cytotoxicity when compared with Ruxolitinib, more detailed mechanistic studies are warranted for **11b**.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00247.

Experimental section and characterization data (HRMS, 1H NMR) for new compounds (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

JAK, Janus kinase; STAT, signal transducers and activators of transcription; DIPEA, *N,N*-diisopropylethylamine; DMAP, 4-dimethylaminopyridine; TFA, trifluoroacetic acid; DMF, dimethylformamide; NMP, *N*-methyl-2-pyrrolidone

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