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Letter

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

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**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-(1*H*)-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 $IC_{50}$ values of compound **3f** against JAK1, JAK2 and JAK3 were 3.4 nM, 2.2 nM and 3.5 nM, respectively. In cell culture experiments, compound **3f** showed potent anti-proliferative 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 ($IC_{50}$: 0.35 μM) and K562 ($IC_{50}$: 0.37 μM) cell lines. It is worth noting that both **3f** and **11b** showed more potent anti-proliferative activities than the approved JAKs inhibitor Ruxolitinib.

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

The JAK/STAT signaling pathway plays critical roles in immunity, hematopoiesis as well as 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 phosporylation of STAT3, which is a potential target for anti-cancer 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 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 Pacritinib are both developed for the treatment of myelofibrosis.[10]

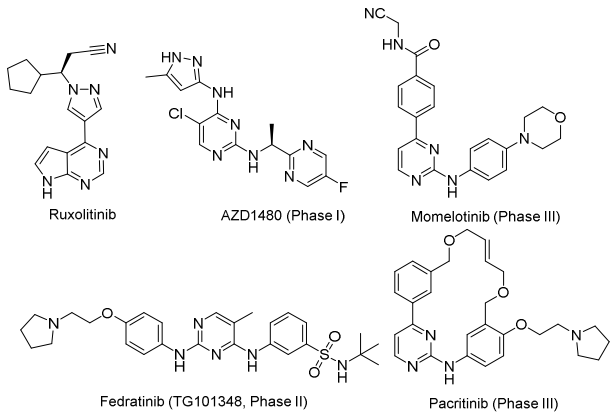



**Figure 1.** Approved or clinical JAK inhibitors for cancer treatment

In our previous work, we discovered a series of 4-aminopyrazole derivatives as novel and potent JAK inhibitors (Figure 2).[11] Structure-activity relationship (SAR) studies showed that $R_1$ group modifications in these analogs did not

influence their JAK inhibition significantly.[11] Such results indicate that the $R_1$ 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-(1*H*)-pyrazole derivatives were designed, synthesized and evaluated as potent JAKs inhibitors. Firstly, removing the $R_1$ side chain of compound **1** led to compound **3a**, which exhibited improved JAKs kinase inhibition at 10 μM. Then further structural modifications of **3a** led to pyrimidine-based 4-amino-(1*H*)-pyrazole derivatives, quinazoline-based 4-amino-(1*H*)-pyrazole derivatives and 7*H*-pyrrolo[2,3-d] pyrimidine-based 4-amino-(1*H*)-pyrazole derivatives (Figure 2)**.**

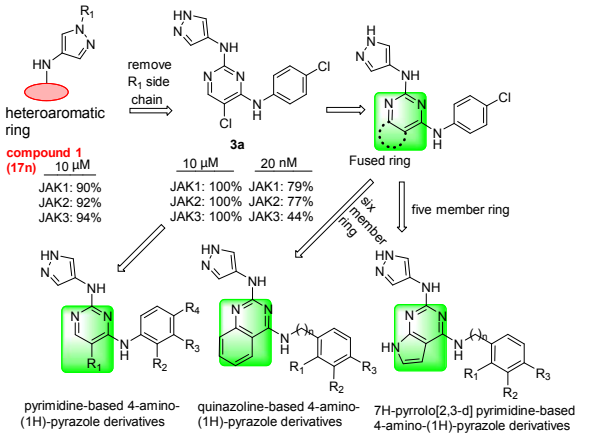
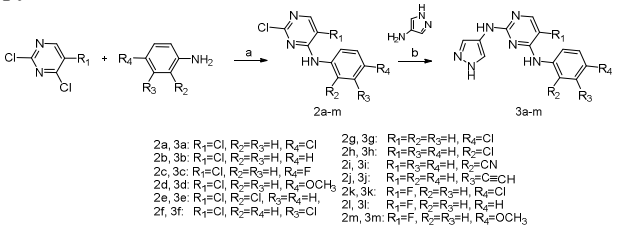



**Figure 2.** Design of 4-amino-(1*H*)-pyrazole derivatives as JAKs inhibitor

Pyrimidine-based 4-amino-(1*H*)-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, $Et_3N$, $Na_2CO_3$) conditions led to the intermediates **2a-m**, which was then reacted with 1*H*-pyrazol-4-amine using TFA as a catalyst at high temperature to give target molecules **3a-m.**[12]

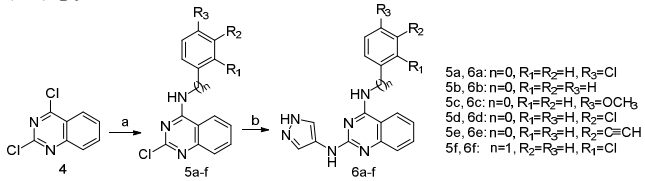
**Scheme 1. Synthesis of Pyrimidine-based 4-Amino-(1*H*)-pyrazole Derivatives 3a-m**.




Conditions and reagents: (a). For compounds **2a**, **2b** and **2d**, DIPEA, DMF, r.t. 16 h. For compound **2c**, $Na_2CO_3$, EtOH, r.t. overnight. For compound **2e**, DIPEA, NMP, 100 °C, 12 h. For compounds **2f, 2g**, $Et_3N$, EtOH, 50-80 °C, overnight. For compound **2h**, HCl, $H_2O$, r.t. 5 days. For compounds **2i**, **2l**, HCl, $H_2O$, 50 °C, 5 h. For compound **2j**, DIPEA, i- PrOH, 60 °C, overnight. For compounds **2k**, **2m**, $H_2O/CH_3OH$=3:1, 50 °C, 5 h. (b). TFA, n-BuOH, 120 °C, 1 h, microwave (MW irradiation).

Quinazoline-based 4-amino-(1*H*)-pyrazole derivatives **6a-f** were synthesized as shown in Scheme 2. The synthetic routes of 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 $CH_3COONa$.[13] Then, **5a-f** reacted with 1*H*-pyrazol-4-amine at high temperature to give target molecules **6a-f.**

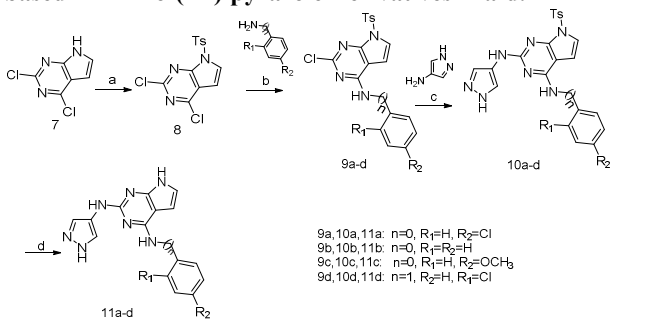
**Scheme 2. Synthesis of Quinazoline-based 4-Amino-(1*H*)-pyrazole Derivatives 6a-f.**




Conditions and reagents: (a), $CH_3COONa$, THF/$H_2O$=3:1, r.t-60 °C, overnight. (b) n-BuOH, 120 °C, 1 h, microwave (MW irradiation).

7*H*-pyrrolo[2,3-d] pyrimidine-based 4-amino-(1*H*)-pyrazole derivatives **11a-d** were synthesized as shown in Scheme 3. The starting material 2,4-dichloro-7*H*-pyrrolo[2,3-d] pyrimidine was protected by 4-methylbenzenesulfonyl chloride[14] 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 catalyst at high temperature to produce intermediates **10a-d.** Ts-Deprotection of **10a-d** yielded target molecules **11a-d.**[15]

**Scheme 3. Synthesis of 7*H*-pyrrolo[2,3-d] pyrimidine-based 4-Amino-(1*H*)-pyrazole Derivatives 11a-d.**




Conditions and reagents: (a), TsCl, $Et_3N$, DMAP, $CH_2Cl_2$. r.t. 5 h. (b) DIPEA, n-BuOH, 100 °C, overnight. (c)TFA, n-BuOH, 120 °C. 1 h. microwave. (MW irradiation). (d) $Cs_2CO_3$, $H_2O$:$CH_3OH$: dioxane=1:3:3（V/V/V）, 80 °C, 6 h.

The 4-amino-(1*H*)-pyrazole derivatives were screened for their *in vitro* kinase inhibitory activities towards JAK1, JAK2, and JAK3 at 10 μM, 1 μM, 0.1 μM, 40 nM, and 20 nM. Because we are only interested in compounds with nM inhibition activities, the final screening was done at 20 nM. Staurosporine (a prototypical ATP-competitive kinase inhibitor, $IC_{50}$: 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.

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 by 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 nM, 2.2 nM and 3.5 nM, respectively. Generally, different substituents on the phenyl ring were well tolerated.

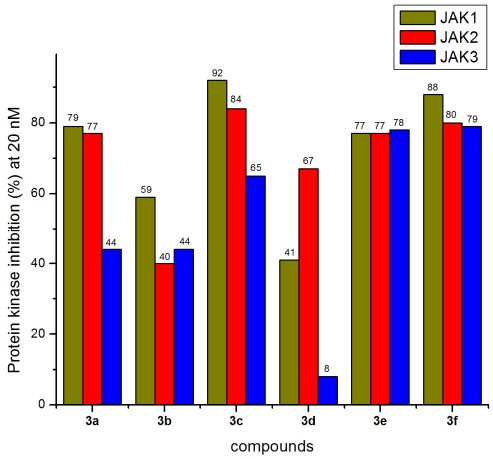



**Figure 3.** *In vitro* inhibitory activity against JAK1, JAK2 and JAK3.

Results in Figure 4 showed that replacing the Cl group on pyrimidine ring with other groups, such as H or F could lead to reduced JAKs inhibition. For example, compound **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.

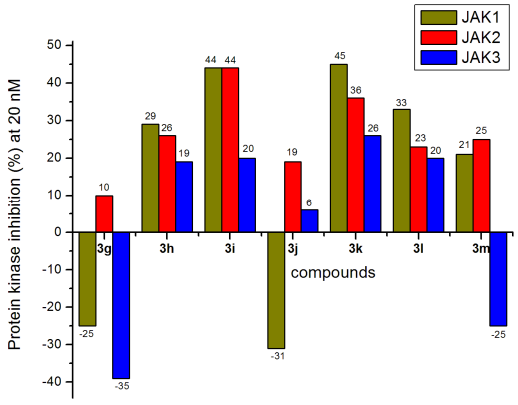



**Figure 4**. *In vitro* inhibitory activity against JAK1, JAK2 and JAK3.

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 towards JAKs at 20 nM. This indicated that a large fused ring, such as quinazoline, was not beneficial to binding with JAKs.

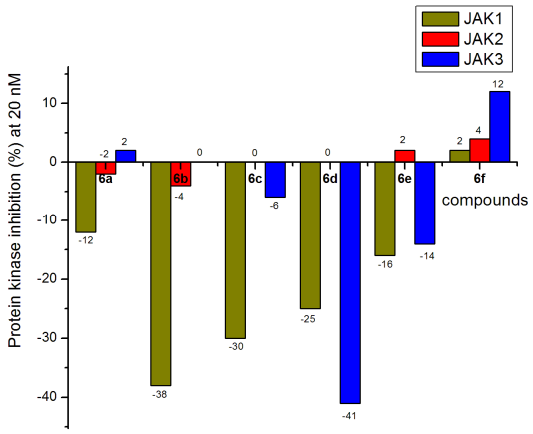



**Figure 5.** *In vitro* inhibitory activity against JAK1, JAK2 and JAK3.

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

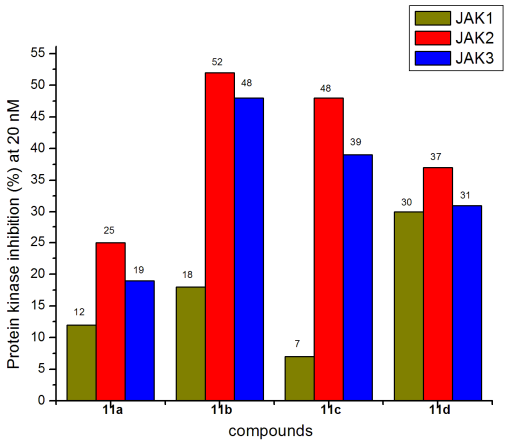



**Figure 6.** *In vitro* inhibitory activity against JAK1, JAK2 and JAK3.

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 μM to evaluate their *in vitro* anticancer activities. Results in Figure 7 showed that among these analogs, compounds **3a-f** and **11a-d** exhibited superior anti-proliferative activities against HEL cell line (indicated by the red column) than the other compounds we synthesized. These data were generally consistent with their JAKs inhibitory potency.

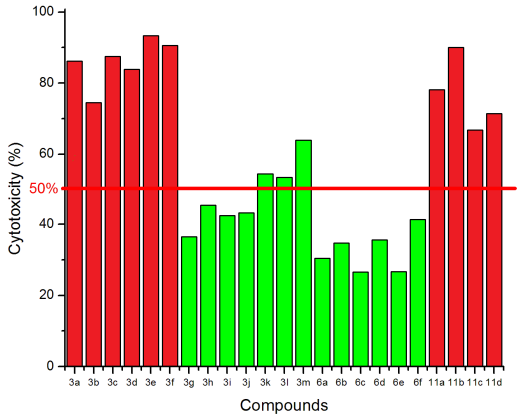



**Figure 7.** Activity screening against HEL cell line at the concentration of 5 μM. The plates containing compounds and cells were incubated for 48 h in MTT assay.

Considering their potent JAKs inhibitory activities and anti-proliferative potency against the HEL cell line, ten compounds (**3a-f, 3k, 11b, 11d,** and **6d**) were chosen for further anti-proliferative 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 anti-cancer 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 ($IC_{50}$: 0.35 μM) and K562 (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 fourteen other cancer related kinases. The results in Figure 8 showed that at 20 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.[20,21,22] 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.

**Table 1. The Inhibitory Activities of Compounds Against Tumor Cell Lines.**

| Compound | $IC_{50}$ [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 |

[a] $IC_{50}$ are mean of two or three experiments and standard deviation is given.

[b] > 8.3 or > 5 in this figure means the $IC_{50}$ value of this compound is larger than 8.3 or 5.

[c] ND, not determined

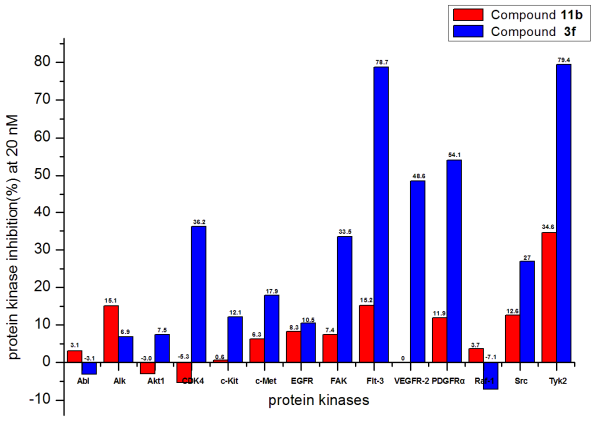



**Figure 8**. Selectivity profile of compounds **3f** and **11b** on 14 protein kinases at 20 nM.

To investigate the binding mode of these 4-amino-(1*H*)-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 showed in Figure 8 suggested that the –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-(1*H*)-pyrazole derivatives were much 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 have a fused phenyl ring (compound **6d**) was also studied. Docking result 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 sidechain of ASP939 gave a 1.50kcal/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 reasons may explain why adding an additional large hydrophobic ring, such as a fused phenyl ring, does not favor binding.

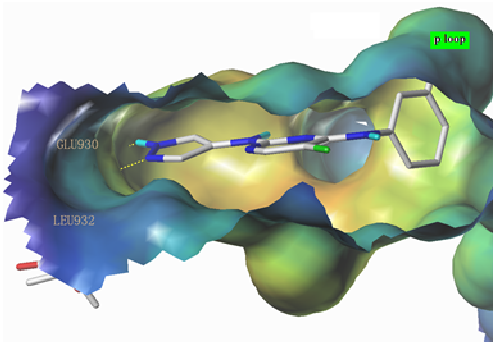



**Figure 9.** The docking of compound **3f** with JAK2 (PDB code 3FUP)

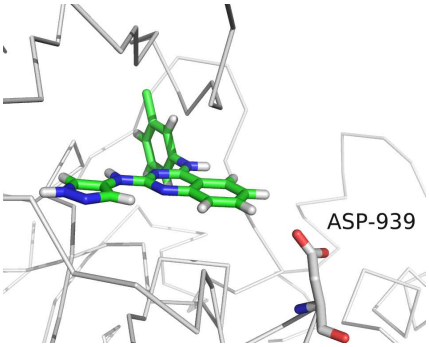



**Figure 10.** The docking of compound **6d** with JAK2 (PDB code 3FUP).

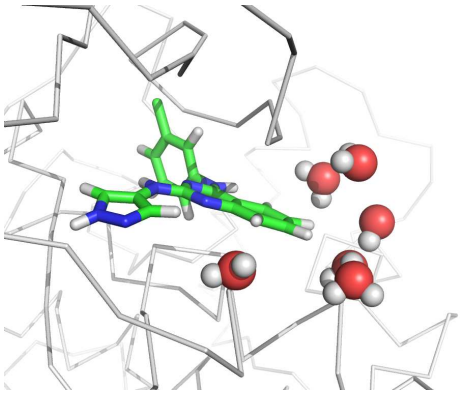


**Figure 11.** Fused phenyl ring is exposed to water in MD simulation. Water molecules within 4Å of the compound **6d** were selected for visualization (spheres).

In summary, a series of 4-amino-(1*H*)-pyrazole derivatives as potent JAKs inhibitors was 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 sub-micromolar range, which were over ten-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

Experimental section and characterization data (HRMS, $^1$H-NMR) for new compounds.

## AUTHOR INFORMATION

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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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## Graphical Abstract: