SLAS Discovery 28 (2023) 180–187

Contents lists available at ScienceDirect

SLAS Discovery

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

Original Research

Development of an enzyme-coupled activity assay for Janus kinase 2 inhibitor screening

Angelika Pölläniemi[a], Anniina Virtanen[a,b], Olli Silvennoinen[a,b,c], Teemu Haikarainen[a,c,*]

[a] Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
[b] Institute of Biotechnology, HiLIFE, University of Helsinki, Helsinki, Finland
[c] Fimlab Laboratories, Tampere, Finland

ARTICLE INFO

Keywords:
Janus kinase
JAK2
Screening assay
Inhibitor

ABSTRACT

JAK2 transmits signals of several important cytokines, such as growth hormone and erythropoietin. The interest toward the therapeutic targeting of JAK2 was boosted in 2005, when the somatic JAK2 V617F mutation, responsible for the majority of myeloproliferative neoplasms (MPNs) was discovered. JAK2 inhibitors have been approved for MPN therapy and they are effective in alleviating symptoms and improving the quality of life of the patients, but they do not lead to molecular remission. This calls for the discovery of new compounds for JAK2-targeted therapeutic approaches. Here we describe the development of a fluorescence-based activity assay for the screening of versatile inhibitor types against JAK2. The assay was utilized to screen a diverse set of small molecule weight natural products and the assay performance was compared to that of differential scanning fluorimetry. We identified 37 hits and further analysis of the most potent hits revealed that most of them displayed non-ATP competitive binding modes. The hits were profiled against other JAK family members and showed distinctive selectivity profiles. The developed assay is consistent, simple and inexpensive to use, and can be utilized for inhibitor screening of diverse compound classes against all JAK family members.

Introduction

Protein phosphorylation is a post-translational modification that regulates a variety of cellular functions. Thus, it is not surprising that protein kinases form one of the largest protein families corresponding to approximately 2% of human genes [1]. Deregulation of kinase activity via mutations, deletions, chromosomal translocations or changes in expression levels is tightly linked to several diseases, including cancer and immunological disorders [2,3]. Since the approval of the first kinase inhibitors Fasudil (1995 in Japan and China) and Imatinib (2001 in USA) more than 70 kinase inhibitors have been approved for clinical use [4]. The success of kinase inhibitors in various therapeutic areas have made them one of the most prevalent targets for drug development.

Janus kinases (JAKs) are non-receptor tyrosine kinases controlling and regulating development, as well as immune and hematopoietic signaling [5–7]. In mammals, the JAK family includes JAK1-3 and TYK2 (Tyrosine kinase 2) that are characterized by a unique tandem kinase domain structure; a regulatory pseudokinase domain (JH2), followed by a typical tyrosine kinase domain (JH1). FERM-SH2 domains in the N-terminus link JAKs to their cognate cytokine receptors, which have no intrinsic catalytic activity. Cytokine binding to their cognate receptors induces JAK activation, which initiates a signaling cascade leading to an active receptor-JAK signaling complex [8]. Subsequent phosphorylation events of JAKs, the receptor, and STAT transcription factors lead to the translocation of active STAT dimers to the nucleus and transcription of target genes.

After the discovery of the hyperactive JAK2 JH2 domain mutation (V617F), the driver of most myeloproliferative neoplasms (MPNs), numerous mutations in JAK2 have been linked to malignant diseases, including acute myeloid, lymphoblastic, and megakaryoblastic leukemias [9–12]. This has led to several drug discovery efforts against JAK2 and to significant advances in the therapeutic field: JAK inhibitors ruxolitinib (Jakavi), fedratinib (Inrebic), and pacritinib (Vonjo) have been approved for the treatment of MPNs, and several other JAK inhibitors for autoimmune diseases [13]. Despite their clinical efficacy in relieving symptoms and improving blood counts, JAK2 inhibitors have limited disease-modifying properties or impact on the molecular pathogenesis of MPNs. MPN cells can acquire adaptive resistance against type I JAK2 inhibitors via heterodimerization and transactivation by JAK1 and TYK2 decreasing the effect of JAK2 inhibitors [14]. Type II inhibitors, which stabilize JAK2 in an inactive form attenuate JAK2 transphosphorylation in the heterodimers, and have been shown to decrease mutant allele burden in mice [15]. Compounds that not only function as ATP-competitors

* Corresponding author at: Kauppi Campus, Arvo Building, Arvo Ylpön katu 34, 33520 Tampere, Finland.
E-mail address: teemu.haikarainen@tuni.fi (T. Haikarainen).

https://doi.org/10.1016/j.slasd.2023.05.001
Received 6 February 2023; Received in revised form 5 April 2023; Accepted 2 May 2023
Available online 5 May 2023

but also have allosteric effects might therefore provide better clinical responses than canonical type I inhibitors.

The developed JAK2 screening assays [16,17] are cell-based assays and/or utilize expensive reagents and can be difficult to adapt for a high-throughput setting, especially without a high cost. We adapted and optimized an activity-based enzyme-coupled fluorescence intensity kinase inhibitor screening assay for JAK2 [18]. The optimized assay was used to screen a natural product library against the JAK2 kinase domain, and the assay performance was compared to differential scanning fluorimetry (DSF). The binding modes of the hit molecules were examined with fluorescence polarization (FP) tracer displacement assay, which detects compounds binding to the ATP pocket. Furthermore, the hits were profiled for selectivity against other JAK family members.

Materials and methods

Materials

Recombinant glucose-6-phosphate dehydrogenase (G6PDH) from *Leuconostoc mesenteroides* and recombinant diaphorase I from *Clostridium kluyveri*, poly (Glu, Tyr) substrate, ATP, ADP, glucose, and resazurin sodium salt were purchased from Merck. β-Nicotinamide adenine dinucleotide phosphate sodium salt hydrate ($NADP^+$) was purchased from Thermo Fischer Scientific.

Protein expression and purification

Codon optimized *Thermococcus litoralis* ADP-hexokinase with an N-terminal 6xHis-tag in pET-19b vector (Genscript) was expressed in *Escherichia coli* Rosetta 2 (DE3) cells. The cells were grown in Terrific broth autoinduction media (Formedium) supplemented with 30 µg/ml kanamycin and 30 µg/ml chloramphenicol at 37°C until OD600 reached 1, after which the temperature was lowered to 18°C for protein expression (16 h). The cells were collected by centrifugation and suspended in lysis buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, 0.5 mM TCEP, 20 mM imidazole). Cell lysates were supplemented with 9000 U/ml of lysozyme, 20 µg/ml of DnaseI, 5 mM $MgCl_2$ and 0.5 mM PMSF and lysed by five freeze-thaw cycles in liquid nitrogen. After centrifugation at 30 000 x g at 4°C for 50 min, the supernatant was incubated with Ni-NTA (Protino Ni-NTA agarose, Macherey-Nagel) sepharose at 4°C for 1 hour. The resin was loaded onto a gravity-flow column and washed with 50 ml of lysis buffer. The proteins were eluted with lysis buffer containing 250 mM imidazole. The proteins were further purified by gel filtration using the Superdex 75 16/600 (Cytiva) column equilibrated with 20 mM Tris-HCl pH 8.5, 300 mM NaCl, 0.5 mM TCEP and 10% glycerol. The purified protein was aliquoted and frozen in liquid nitrogen and stored at -70°C.

Human JAK2 (840-1132) was cloned in pFB-LIC-Bse vector (pFB-LIC-Bse was a gift from Opher Gileadi, Addgene plasmid # 26108) [19] with an N-terminal 6xHis-tag and protein expression was done using baculovirus system in High Five™ cells. The cells were collected by centrifugation and suspended in lysis buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 10 % glycerol, 0.5 mM TCEP, 20 mM imidazole) supplemented with 20 µg/ml of DnaseI, 5 mM $MgCl_2$ and 0.5 mM PMSF, and lysed by three freeze-thaw cycles. Protein was purified as described above using 20 mM Tris-HCl pH 8.5, 300 mM NaCl, 10% glycerol, 0.5 mM TCEP as the gel filtration buffer.

Human JAK1 (866-1154), JAK3 (811-1124), and TYK2 (886-1187) with a C-terminal 6xHis-tag were cloned in pFB1-vector and expressed in High Five™ (TYK2) or Sf9 (JAK1, JAK3) cells. Cells were collected by centrifugation, resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 10 % glycerol, 20 mM imidazole, 1 mM sodium vanadate, 1 mM PMSF, 1 µg/ml Pepstatin A, 1 µg/ml Leupeptin, 5 U/ml benzonase and 5 mM $MgCl_2$), and lysed either by two freeze-thaw cycles (JAK3) or by incubating in lysis buffer supplemented with 1 % Triton X-100, for 1 h on ice (JAK1, TYK2). Recombinant proteins were purified using Ni-NTA-affinity and size exclusion chromatography as described earlier [20] in buffer containing 20 mM Tris-HCl pH 8.0 (JAK3) or 50 mM Tris-HCl pH 8.5 (JAK1, TYK2), 300 mM NaCl (JAK3) or 500 mM NaCl (JAK1, TYK2), 10 % glycerol, and 0.5 mM TCEP.

Enzyme-coupled JAK-activity assay

Kinase reactions were carried out in black 384-well plates (Proxiplate™-384 F Plus, PerkinElmer) in a total volume of 5 µl. The reactions consisted of 20 nM JAK2 kinase, 0.2 mg/ml Poly (Glu, Tyr) substrate and 100 µM ATP in optimized assay buffer (100 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 0.01% Tween-20, 0.01% BSA). The plate was incubated at room temperature with shaking at 300 rpm using PST-60 HL plus Thermo Shaker (Biosan) for 2 hours. ADP produced in the kinase reaction was detected by the addition of 5 µl of the optimized ADP detection solution (2 mM glucose, 200 µM $NADP^+$, 100 µM resazurin, 1 µM ADP-hexokinase, 0.02 U/ml G6PDH, and 0.02 U/ml diaphorase in 100 mM Tris-HCl pH 7.5). The fluorescence was measured with excitation at 540 nm and emission at 590 nm after 1 hour of incubation (with shaking as above) with the Tecan Spark plate reader.

Inhibitor screening

The inhibitor screening was conducted with the JAK2 activity assay using NIH Natural Products Set V from the chemical repository of the National Cancer Institute (NCI). Altogether, 391 compounds were screened at two concentrations. The kinase reaction was prepared by dispensing 1 µl of compound (final concentration 100 and 10 µM, DMSO concentration 1 and 0.1%, respectively) in each well, followed by 2 µl of the ATP-substrate mix (100 µM and 0.2 mg/ml respectively) and 2 µl of JAK2 (20 nM). The samples were incubated for 2 hours, after which 5 µl of ADP detection solution was added to each well. The fluorescence intensity was measured after 1 hour. Each plate included control wells with JAK2 inhibitor pacritinib and wells without JAK2 as positive controls, and control wells without inhibitor and wells containing 100 µM ADP as negative controls.

Potency measurements

Dose–response measurements were done in triplicates from 100 µM to 10 nM using half-logarithmic dilutions of the compounds. Compounds (1 µl) were transferred to the assay plates, followed by the addition of 2 µl of ATP-substrate mix (final concentrations 100 µM and 0.2 mg/ml, respectively). The reaction was initiated by the addition of 2 µl of JAK2 (final concentration 10 nM). After 2 hours of incubation, 5 µl of ADP detection solution was added. The plates were incubated for 1 hour at room temperature before fluorescence intensity measurements. The dose-response curves were fitted with the nonlinear regression analysis, dose-response fitting with variable slope (four-parameters), using GraphPad Prism version 9.0.0.

Differential scanning fluorimetry

DSF screening was performed with a final protein concentration of 5 μM and an inhibitor concentration of 100 μM in duplicates in clear 384-well PCR plates (Hard-Shell, Bio-Rad). SYPRO Orange (Life Technologies) was used as the reporter dye with a final concentration of 6x. The samples were prepared by mixing 1 µl of the compound with 4 µl of SYPRO Orange and JAK2 solution in DSF buffer (10 mM Tris-HCl pH 8.0, 500 mM NaCl). The experiment was performed on a real-time PCR machine (Bio-Rad CFX) with the temperature increasing from 4°C up to 80°C with a 1°C increment per minute. The melting curves were analyzed using SimpleDSFviewer [21]. The melting temperature change (ΔT_m) values were obtained by subtracting the T_m value of DMSO control from the reaction T_m values.

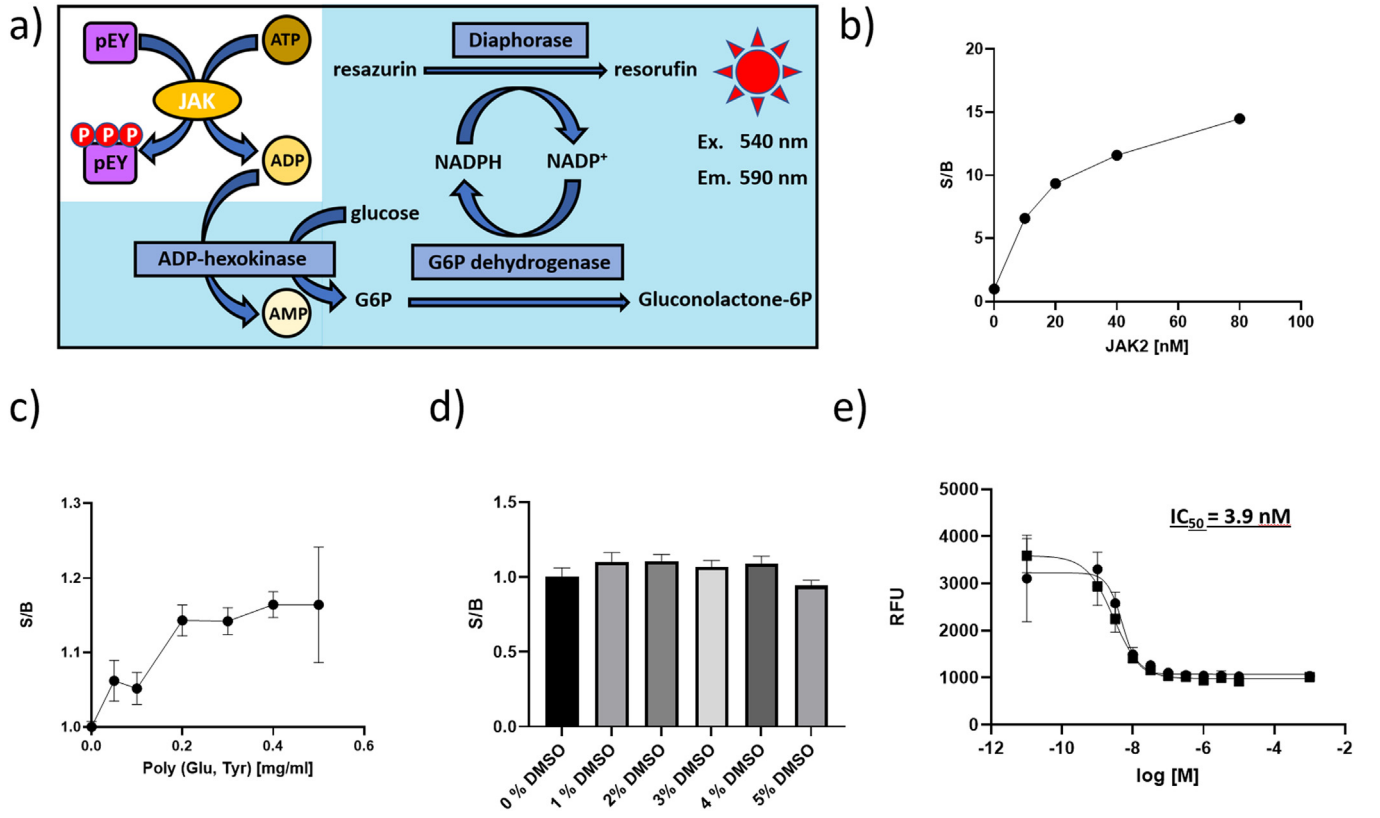

Fig. 1. JAK2 assay development. a) Principle of the enzyme-coupled assay. b) Assay signal dependency on JAK2 concentration. c) The effect of the substrate concentration to the assay signal. d) DMSO tolerance of the assay. e) JAK2 inhibition by ruxolitinib from two independent measurements. pIC_{50} ± SEM = 8.4 ± 0.06 (IC_{50} = 3.9 nM). Kinase reaction time was 2 hours and ADP detection time 1 hour prior to fluorescence signal reading with the plate reader.

Fluorescence polarization assay

Fluorescence polarization was measured in 384-well black polystyrene plates (Proxiplate^{TM}-384 F Plus, PerkinElmer) in a final volume of 5 µl. The reaction was carried out in 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 20% glycerol, 0.01% Brij-35, 2 mM DTT with 1.5 nM of JAK2 JH2 tracer (HY-102055, MedChemExpress) and 30 nM JAK2. For potency measurements, half-logarithmic dilutions of the compounds were prepared, with final concentrations ranging from 100 µM to 10 nM. The triplicate samples were done by mixing 1 µl of the compound dilution with 2 µl of tracer (1.5 nM) and 2 µl of JAK2 (30 nM). Fluorescence polarization was measured on a PerkinElmer Envision plate reader with FITC FP filters (excitation 480 nm, emission 535 nm). The data were fitted with the nonlinear regression analysis, dose-response fitting with variable slope (four-parameters), using GraphPad Prism version 9.0.0.

Results

Assay development for JAK2

To facilitate drug discovery against JAK2, we sought to develop a simple, affordable, and robust activity-based screening assay for JAK2. To this end, we adapted a general kinase screening assay [18] for JAK2. The assay works by utilizing the universal product of kinase reactions, ADP, to produce red-shifted fluorescence emission from resorufin via an enzyme-coupled reaction cascade (Fig. 1a). We optimized all assay components and reaction buffers for JAK2 activity detection. First, the ADP detection solution buffer and enzyme concentrations of the coupling reaction were optimized. Optimization of buffers and pH values revealed HEPES pH 7.0 as the optimal buffer for the ADP detection solution. As no major differences in pH were observed between the different buffers, we decided to use a buffer corresponding to the physiological pH. Based on this criterion, Tris-HCl pH 7.5 yielded a better signal than HEPES pH 7.5 (Supplementary Fig. 1a). After this step, many different additives were tested in Tris-HCl pH 7.5. The tested additives did not have a positive effect on the signal-to-background value of the detection solution, except $MgCl_2$, which is required by the ADP-hexokinase (Supplementary Fig. 1b). However, even this additive was omitted from the final buffer as $MgCl_2$ carryover from the kinase reaction buffer was sufficient for ADP-hexokinase activity.

Following the buffer optimization, the concentrations of the enzymes in the coupling reaction of the ADP detection solution were optimized. The concentration of the first enzyme of the reaction, ADP-hexokinase, was set to 1 µM as this gave a good signal-to-background ratio with low enzyme consumption (Supplementary Fig. 1c). The concentrations of glucose-6-phosphodehydrogenase and diaphorase were optimized together as they perform a codependent reaction cycle. The concentration of 0.02 U/ml was selected for both enzymes based on a good signal-to-background value and reasonable enzyme consumption (Supplementary Fig. 1d). To ensure the linearity of ADP conversion to the final fluorescent product, an ADP standard curve was measured, showing excellent linearity at three timepoints (R^2: 0.95, 0.99 and 0.99) within the assay range (Supplementary Fig. 1e).

Next, we optimized the assay buffer for the kinase reaction. Several components and their concentrations in the reaction buffer known to affect kinase activity were tested. The optimized reaction buffer consisted of 5 mM $MgCl_2$, 0.01% Tween-20 and 0.01% BSA in Tris-HCl pH 7.5. Tris-HCl pH 8.0 gave a slightly better signal than pH 7.5 but we decided to use the physiological pH in the assay (Supplementary Fig. 2a,c). After buffer optimization, the concentrations of the kinase reaction components, JAK2, ATP and the substrate, were optimized sepa-

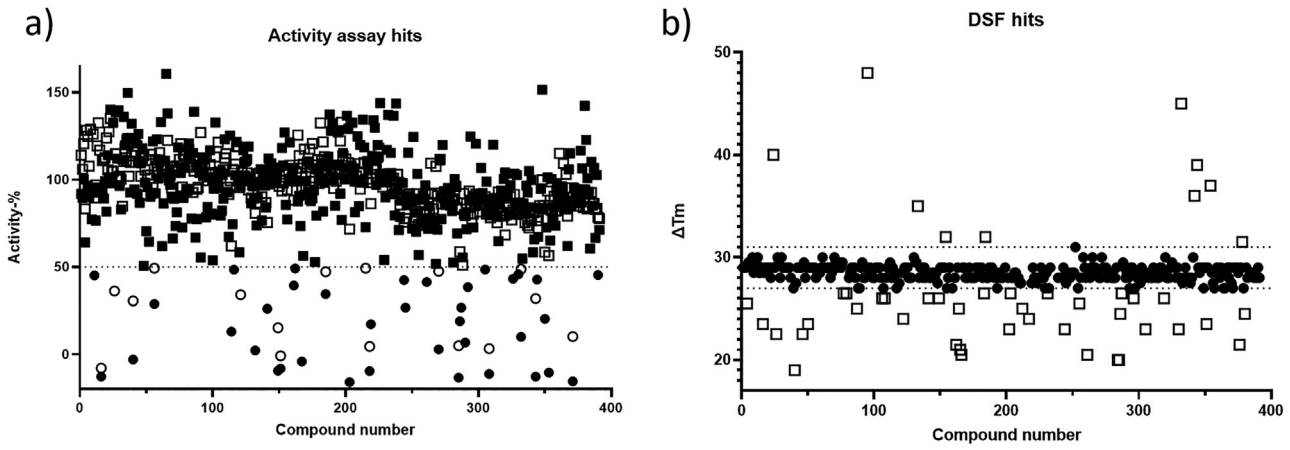

Fig. 2. Library screening. a) Screening of the natural product library with the activity assay in singlets at 100 μM and 10 μM compound concentration. Hit limit of 50% is shown as a dotted line. Hits from the 100 μM compound screening are represented by solid dots and 10 μM compound screening hits are represented by hollow dots. b) Comparative screening with DSF in duplicates at a 100 μM compound concentration. ΔTm ≥ 2 and ΔTm ≤ -2 hit limits are shown as dotted lines.

Table I
Assay performance.

CV-% max	2.89
CV-% min	3.23
Z'	0.87
S/N	26.58
S/B	7.42

rately. Sufficient signal-to-background was achieved with 20 nM JAK2, 100 μM ATP and 0.2 mg/ml Poly (Glu, Tyr) substrate with the lowest possible component consumption (Fig. 1b,c, Supplementary Fig. 2b).

Finally, we tested the DMSO tolerance of the assay and found no statistically significant deviations with DMSO concentrations up to 5% according to the one-way ANOVA analysis (Fig. 1d). Lastly, we measured the IC_{50} values of approved JAK2 inhibitors, ruxolitinib (Fig. 1e) and pacritinib (Supplementary Fig. 2d). Ruxolitinib and pacritinib displayed IC_{50} values of 3.9 nM and 25.0 nM respectively, similar to the literature values of 2.8 nM for ruxolitinib [22], and 23.0 nM for pacritinib [23].

The assay performance was tested by measuring the consistency of the maximal and minimal signals between different wells (Table I). 64 maximum signal wells (kinase reaction with 20 nM JAK2, 100 μM ATP and 0.2 mg/ml substrate) were prepared alongside 64 minimal signal wells (kinase reaction without JAK2). The assay had a Z' value of 0.87 and a S/B of 7.4 indicating that the assay is robust and of high quality.

Screening of natural product library

Natural products have played an important role in drug discovery in many diseases, including cancer. Several natural products are also potent tyrosine kinase inhibitors both *in vitro* and *in vivo* [24]. Importantly, natural products offer a vast source of diverse scaffolds and structurally complex molecules when compared to synthetic libraries [25]. We validated the assay by screening a structurally diverse set of natural products from the National Cancer Institute (NCI) at a 100 μM and 10 μM concentration in singlets (Fig. 2a). Altogether, 37 hits were identified that inhibited JAK2 activity by more than 50% at 100 μM compound concentration when compared to the no inhibitor and 10 μM pacritinib control wells. With 10 μM concentration, 16 hits were identified that inhibited JAK2 by more than 50% (Supplementary Table I). Out of these hits, 13 compounds were the same as in the 100 μM screening. Three compounds were hits identified only at a 10 μM compound concentration.

We selected four most active compounds, producing the lowest activity-% from the screening, Coprinin (**1**), Aquaymycin (**2**), NSC169517 (**3**), and NSC111041 (**4**) for dose-response measurements. All the compounds inhibited JAK2 activity with micromolar potencies (Fig. 3). The mean IC_{50} values for the triplicate measurements were 2.2 μM, 1.2 μM, 1.2 μM and 3.4 μM for **1, 2, 3**, and **4**, respectively (Table II). The compounds were also tested for the inhibition of the detection reaction. Two compounds (**1** and **3**) inhibited the reaction without the kinase with 10 μM compound concentration (43% and 55% activity with **1** and **3**). However, the inhibition was significantly lower than JAK2 inhibition (4.5% and 3.3% for **1** and **3** at 10 μM as a comparison). Two other compounds, **2** and **4** had activities of 92% and 70%, respectively in the detection reaction (at 10 μM concentration) and therefore did not significantly inhibit the reaction. Due to the inhibition of the detection reaction, we tested whether the possible redox reactivity of the compounds would explain the detection reaction inhibition. We tested the inhibition in the presence of ascorbic acid and reduced glutathione (final concentrations 10 μM), but these free radical scavengers did not have significant effect on the kinase reaction or detection reaction with or without the inhibitors (Supplementary Fig. 3). It is possible that compounds **1** and **3** inhibit one or several enzymes in the coupling reaction albeit with lower potencies than they inhibit JAK2.

DSF screening

To compare the activity-based assay to DSF, a commonly used method for kinase inhibitor screening, we used it to screen the same natural product library. The screening was performed with a 100 μM compound concentration in duplicates (Fig. 2b). Initially, 49 hits were identified, and the screening was repeated for selected compounds (hits and compounds producing noninterpretable melting curves) in triplicates. Even after the second DSF analysis, some compounds remained noninterpretable as the melting curves could not be reliably analyzed. Several melting curves also required manual inspection as automated analysis could not produce a reliable result. Both JAK2 (in assay buffer without DMSO or inhibitor) and the DMSO control had a Tm of 29°C. Interestingly, two types of compounds were identified in the screening, ones that decreased and others that increased the melting temperature of the protein. 16 common hits between the activity assay and DSF were identified, i.e. 43% of the hits were identified from both DSF using ΔTm of 2 as a limit and the activity-based screening (100 μM concentration). The best hits (**1**-**4**) decreased the melting temperature of JAK2, destabilizing the kinase (Table II). The comparison of hits with arbitrary hit limits between the two assays is somewhat artificial but gives an indi-

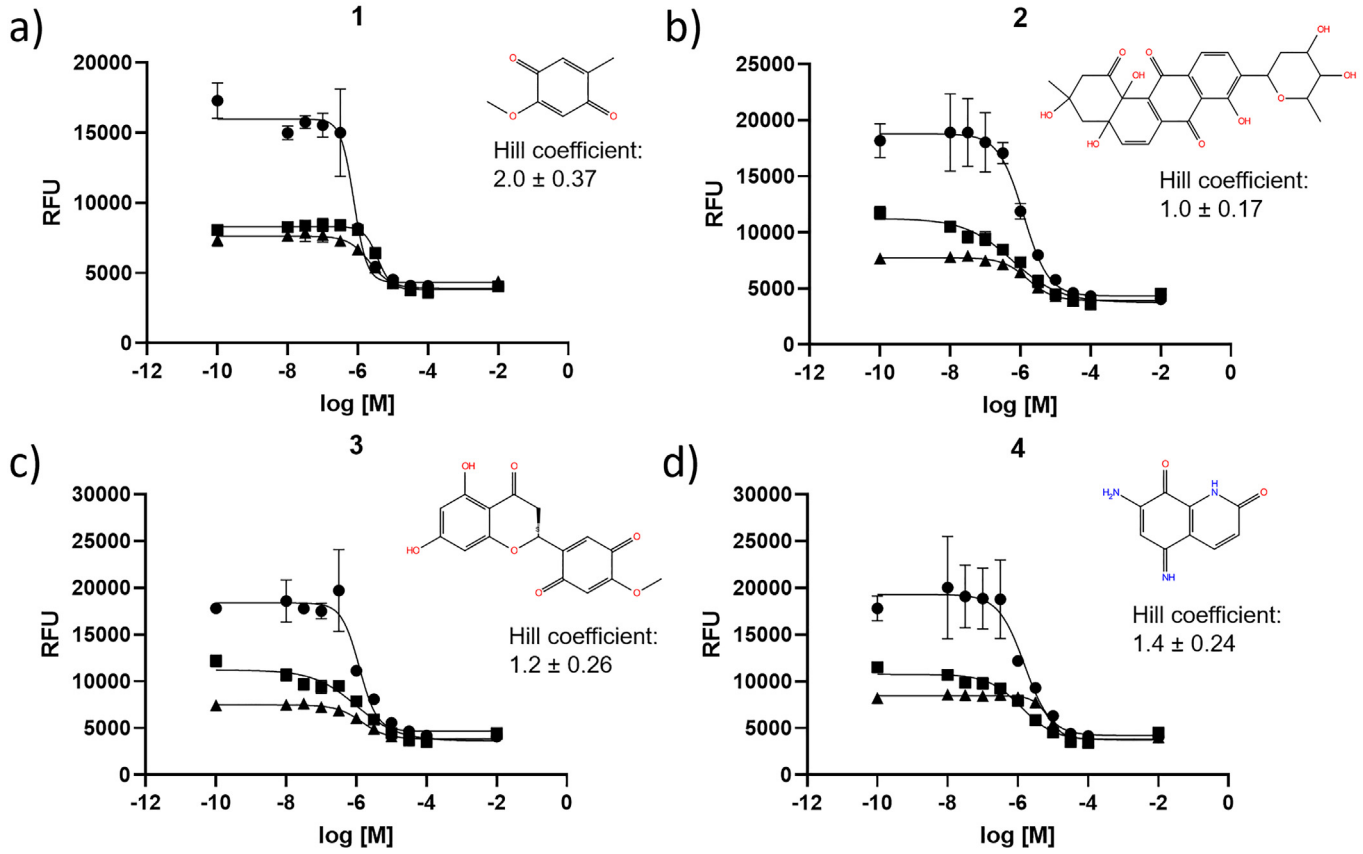

Fig. 3. Dose-response analysis of hit compounds. a) Coprinin (**1**), b) Aquaymycin (**2**), c) NSC169517 (**3**), and d) NSC111041 (**4**). All compounds were measured three times in triplicates. Error bars for single data points are presented as standard deviations. The average hill coefficient and SEM are indicated for each compound.

Table II
Summary of JAK2 hit characterization. Compound potencies from the activity assay and fluorescence polarization assay. DSF values are from the library screening (DMSO control has a Tm of 29°C).

	Activity assay: pIC_{50} ± SEM (IC_{50})	FP: pIC_{50} ± STD (IC_{50})	DSF: Tm ± SEM
1	5.7 ± 0.05 (2.2 μM)	NB	20°C*
2	6.0 ± 0.08 (1.2 μM)	NB	21 ± 1.4°C
3	5.9 ± 0.09 (1.2 μM)	NB	24 ± 0.92°C
4	5.6 ± 0.08 (3.4 μM)	4.7 ± 0.09 (21 μM)	27°C*

NB: no binding.
* Based on one melting curve from duplicate measurement due to a noninterpretable second melting curve.

cation of correlation between the assays. Both the activity assay and the DSF assay identified hits that were not detected with the other method. Compounds with similar potencies can produce very different ΔTm values leading to difficulties in detecting compounds producing low thermal shifts in DSF. Also, several activity assay hits did not produce an interpretable melting curve in DSF and thus were not detected as hits. Hits that were only detected in DSF but not in the activity assay can bind e.g. to sites that do not inhibit enzymatic activity, at least in an *in vitro* setting without the other domains of JAK2. Altogether, the activity assay was more robust for inhibitor screening even in singlets, although DSF did identify some compounds which did not show as hits in the activity assay.

Binding site characterization

To further characterize the possible binding sites of the four hit compounds, we utilized a fluorescence polarization assay for determining whether the compound binds to the ATP-binding pocket of the protein (Fig. 4a, Table II). The assay utilizes an ATP-competitive kinase inhibitor linked to a fluorescent probe, and its displacement from the protein indicates that compounds compete with it in binding to the pocket. The compounds were tested in triplicate with concentrations ranging from 100 μM to 10 nM. Interestingly, only one of the hits, compound **4**, showed binding in the FP assay (IC_{50} = 21 μM), indicating an ATP-competitive binding mode. Other hit compounds did not interfere with the binding of the tracer and based on this preliminary analysis have a non-ATP competitive binding mode. Notably, few non-ATP competitive JAK2 inhibitors have been identified to date [26], but their binding modes have not been studied in detail.

JAK2 selectivity of the hits

To profile the selectivity of the hit compounds in the JAK family and to extend the usability of the assay, we measured the inhibition of

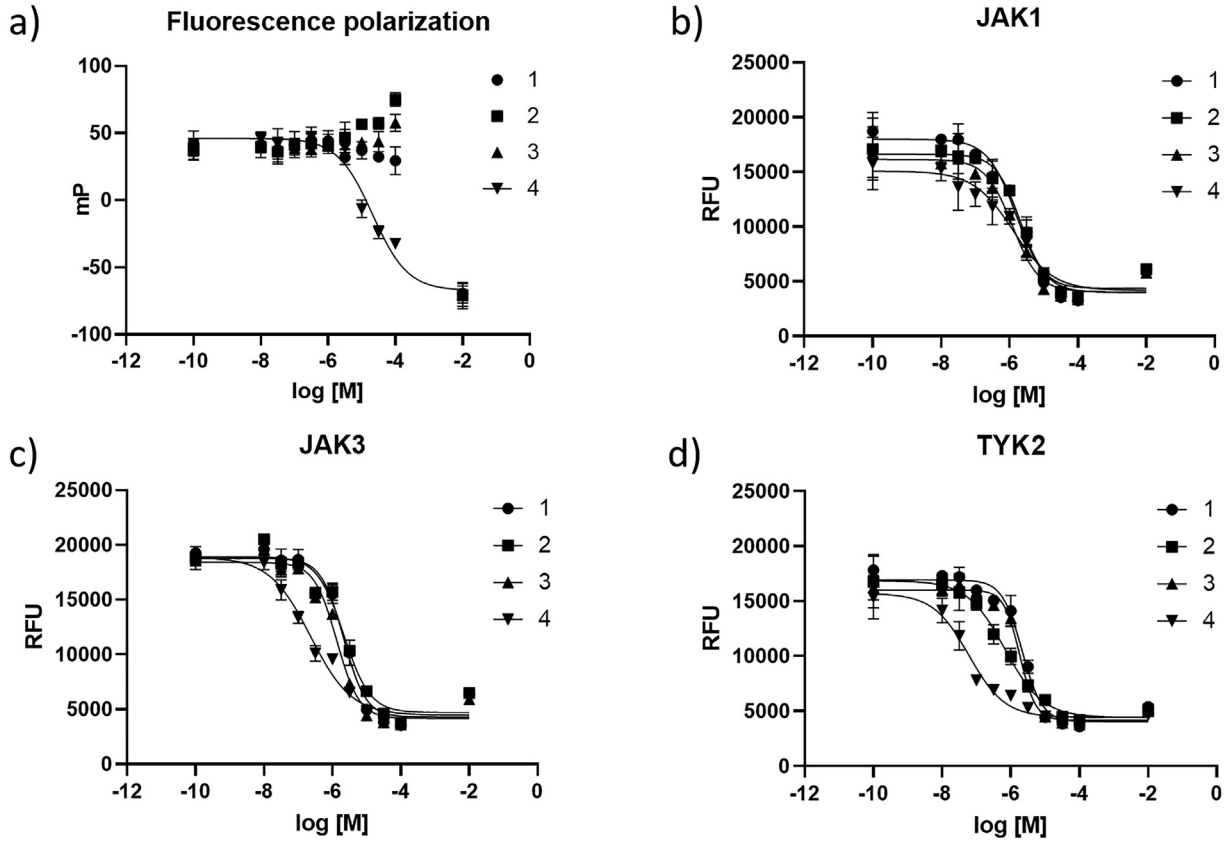

Fig. 4. Binding site and selectivity profiling. a) Fluorescence polarization assay with JAK2 and four hit compounds measured in triplicates (n=1). Potencies of the hit compounds to JAK1 (b), JAK3 (c), and TYK2 (d) with the activity assay measured in triplicates (n=3). The error bars are presented as standard deviations. Hill coefficients for compounds 1-4: JAK1 (1.0, 1.2, 1.1, 0.7), JAK3 (1.3, 1.1, 1.3, 0.7) and TYK2 (1.5, 0.8, 1.9, 0.8).

Table III
JAK selectivity analysis. Potencies are measured with the activity assay in triplicates (n=1) and shown as pIC_{50} ± SD (IC_{50}).

	JAK1 (μM)	JAK2 (μM)	JAK3 (μM)	TYK2 (μM)
1	5.8 ± 0.08 (1.6)	5.7 ± 0.05 (2.2)	5.7 ± 0.06 (2.1)	5.7 ± 0.05 (2.2)
2	5.7 ± 0.09 (2.1)	6.0 ± 0.08 (1.2)	5.7 ± 0.07 (2.1)	6.2 ± 0.09 (0.68)
3	5.9 ± 0.08 (1.2)	5.9 ± 0.09 (1.2)	5.9 ± 0.05 (1.3)	5.7 ± 0.03 (1.8)
4	5.9 ± 0.15 (1.3)	5.6 ± 0.08 (3.4)	6.6 ± 0.09 (0.23)	7.2 ± 0.09 (0.06)

the hits using JAK1, JAK3, and TYK2 (Fig. 4b-d, Table III). JAKs share the same domain structure, and the sequence identities of their kinase domains to JAK2 are 54%, 58%, and 50%, for JAK1, JAK3, and TYK2 respectively. In addition, residues around their ATP-pockets are highly conserved. The assay performed well with other JAKs without any further optimization of reaction buffers or substrate concentrations. Only JAK concentrations were optimized to give a sufficient signal in the assay (JAK1: 100 nM, JAK3: 20 nM, and TYK2: 50 nM). All identified hits inhibited the other JAKs with varying affinities. JAK1 was inhibited by all hits at micromolar concentrations, which were in line with the JAK2 inhibitory potencies. Also, JAK3 was inhibited to the same extent by the compounds, except for **4**, which inhibited JAK3 with a submicromolar potency. TYK2 was clearly the most potently inhibited family member by the hits: **1** and **3** had micromolar potencies, while **2** and **4** displayed submicromolar IC_{50} values. Compound **4** was clearly the most potent TYK2 inhibitor displaying 60 nM potency with 4 to 57-fold selectivity to TYK2 over other JAK family members. Due to its small molecular weight (189.17 Da) the compound has a high ligand efficiency towards TYK2 and could be further used as a TYK2 inhibitor scaffold.

Discussion

After the discovery of the somatic pathogenic V617F mutation, JAK2 has emerged as a prime therapeutic target against Philadelphia negative (Ph-) myeloproliferative neoplasms. JAK2 V617F mutation is causative of over 95% of polycythemia vera, 50–70% of essential thrombocythemia and 50% of idiopathic myelofibrosis cases [27]. Also, in MPNs caused by MPL and CALR mutations hyperactivation of JAK2 is the key pathogenic factor. Consequently, several JAK2 inhibitors have been developed against MPNs, and few have already been approved for clinical use. However, currently approved drugs do not significantly affect the molecular pathogenesis of the disease or decrease the V617F mutant burden of the patients. This highlights the need to develop new JAK2 inhibitors with disease-modifying properties.

Only a few JAK2 inhibitor screening assays have been described before. To facilitate inhibitor development against JAK2, we developed an activity-based screening assay based on a previously described general kinase inhibitor screening assay [18]. The assay performs well with a Z' value of 0.87 and a signal-to-background value of 7.4. Due to the high DMSO tolerance, the assay is also suitable for the screening of libraries requiring high compound concentrations, e.g. small molecule fragment libraries. We utilized this assay to screen a diverse natural product library and identified several hits inhibiting JAK2 activity by more than 50% with a 100 μM compound concentration. The screening was compared to a commonly used kinase inhibitor screening method, DSF. Many DSF hits were not identified as activity assay hits, as these compounds did not lead to the modulation of kinase activity despite binding to the protein. Similar to DSF, the activity assay can also be susceptible to false positives in the form of autofluorescent or quenching compounds. The activity assay was overall more robust, consumed

less protein, and produced results requiring no manual interpretation. Another clear advantage of the activity assay compared to DSF is that it directly measures a functional outcome. We selected four hits for further characterization, and surprisingly, only one of them displayed an ATP-competitive binding mode in an FP-based ligand displacement assay. Few allosteric JAK inhibitors have been described to date, implying the usefulness of activity-based assays in identifying allosteric JAK modulators. Non-ATP competitive compounds benefit from having binding sites outside the highly conserved ATP-pocket of protein kinases thus potentially resulting in higher selectivity. The hits were also profiled against other JAK family members. All the identified hits inhibited the other JAKs with micromolar to nanomolar potencies. TYK2 emerged as the most potently inhibited family member by the hit compounds. Two compounds, **2** and **4**, had nanomolar potencies with the ATP-competitive compound **4** displaying 60 nM potency together with high ligand efficiency.

While compounds **1** and **2** do not have any reported activities (https://pubchem.ncbi.nlm.nih.gov/), **3** and **4** have been identified as hits in reported screens. Compound **3** has few reported activities, such as micromolar activity against polo-like kinase 1 polo-box domain. Compound **4**, in turn, has previously been identified having activity against tyrosyl-DNA phosphodiesterase 2 [28]**.** All the identified hits contain quinone or quinone-like ring system, which can function as a Michael's acceptor and is often associated with poor solubility [29]. As such they are not suitable for direct medicinal chemistry optimization but the allosteric binding modes of the three compounds invite further characterization to determine their exact inhibitory mechanisms. This could lead to the identification of novel druggable pockets within the JAK family. Altogether, the screening of the natural products library resulted in a very high hit rate (9.5% and 4.1% for 100 μM and 10 μM concentrations, respectively) indicating a promiscuous nature of the compounds in the library. Due to this promiscuity and the presence of redox active compounds in the library, care should be taken when utilizing this assay for screening of natural products.

In conclusion, we have developed an activity-based assay that facilitates the screening of ATP-competitive and allosteric JAK2 inhibitors in a simple, homogenous format. The assay utilizes inexpensive commercially available reagents and materials and can be utilized to screen even large inhibitor libraries with a modest cost. In addition, the same assay can be utilized for other family members, JAK1, JAK3, and TYK2, allowing convenient and fast inhibitor selectivity profiling.

Declaration of Competing Interest

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

Teemu Haikarainen reports financial support was provided by Academy of Finland and Tampere Tuberculosis Foundation. Olli Silvennoinen reports financial support was provided by Academy of Finland, Sigrid Jusélius Foundation, Finnish Cancer Foundation, Jane and Aatos Erkko Foundation, Tampere Tuberculosis Foundation, and Tampere University Hospital. Olli Silvennoinen reports a relationship with Pfizer, Abbvie, and Novartis that includes: speaking and lecture fees and with Ajax Therapeutics and Finnish hematology registry and biobank that includes: board membership. Olli Silvennoinen reports a relationship with St. Jude Children's Research Hospital and Ajax Therapeutics that includes: equity or stocks. Olli Silvennoinen has patent #US Patent no. 5,728,536 issued to St Jude Childrens Research Hospital and the following patents issued to GENON BIOTECHNOLOGIES Oy: #US patent no. 8,841,078, #AU 2011214254, #CAN 2789186, and #EPO 11741946.5.

Acknowledgements

Compounds set for screening was obtained from the open chemical repository of the National Cancer Institute (NCI) Developmental Therapeutics Program (DTP) (http://dtp.cancer.gov). This work was funded by Academy of Finland (grant numbers 340572 and 346303) and Tampere Tuberculosis Foundation (grant number 2022) for TH, and supported by Academy of Finland, Sigrid Jusélius Foundation, Finnish Cancer Foundation, Jane and Aatos Erkko Foundation, Tampere Tuberculosis Foundation, and Competitive State Research Financing of the Expert Responsibility area of Tampere University Hospital for OS.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.slasd.2023.05.001.

References

[1] Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science 2002;298:1912–34. doi:10.1126/science.1075762.
[2] Lahiry P, Torkamani A, Schork NJ, Hegele RA. Kinase mutations in human disease: interpreting genotype–phenotype relationships. Nat Rev Genet 2010;11:60–74. doi:10.1038/nrg2707.
[3] Cicenas J, Zalyte E, Bairoch A, Gaudet P. Kinases and Cancer. Cancers 2018;10:63. doi:10.3390/cancers10030063.
[4] Cohen P, Cross D, Jänne PA. Kinase drug discovery 20 years after imatinib: progress and future directions. Nat Rev Drug Discov 2021;20:551–69. doi:10.1038/s41573-021-00195-4.
[5] Leonard WJ, O'Shea JJ. Jaks and STATs: biological implications. Annu Rev Immunol 1998;16:293–322. doi:10.1146/annurev.immunol.16.1.293.
[6] Ihle JN, Witthuhn BA, Quelle FW, Yamamoto K, Silvennoinen O. Signaling through the hematopoietic cytokine receptors. Annu Rev Immunol 1995;13:369–98. doi:10.1146/annurev.iy.13.040195.002101.
[7] Hammarén HM, Virtanen AT, Raivola J, Silvennoinen O. The regulation of JAKs in cytokine signaling and its breakdown in disease. Cytokine 2019;118:48–63. doi:10.1016/j.cyto.2018.03.041.
[8] Haan C, Kreis S, Margue C, Behrmann I. Jaks and cytokine receptors–an intimate relationship. Biochem Pharmacol 2006;72:1538–46. doi:10.1016/j.bcp.2006.04.013.
[9] Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet Lond Engl 2005;365:1054–61. doi:10.1016/S0140-6736(05)71142-9.
[10] James C, Delhommeau F, Marzac C, Teyssandier I, Couédic J-PL, Giraudier S, et al. Detection of JAK2 V617F as a first intention diagnostic test for erythrocytosis. Leukemia 2006;20:350–3. doi:10.1038/sj.leu.2404069.
[11] Kralovics R, Passamonti F, Buser AS, Teo S-S, Tiedt R, Passweg JR, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med 2005;352:1779–90. doi:10.1056/NEJMoa051113.
[12] Dunbar AJ, Rampal RK, Levine R. Leukemia secondary to myeloproliferative neoplasms. Blood 2020;136:61–70. doi:10.1182/blood.2019000943.
[13] Spinelli FR, Meylan F, O'Shea JJ, inhibitors Gadina MJAK. Ten years after. Eur J Immunol 2021;51:1615–27. doi:10.1002/eji.202048922.
[14] Koppikar P, Bhagwat N, Kilpivaara O, Manshouri T, Adli M, Hricik T, et al. Heterodimeric JAK-STAT activation as a mechanism of persistence to JAK2 inhibitor therapy. Nature 2012;489:155–9. doi:10.1038/nature11303.
[15] Meyer SC, Keller MD, Chiu S, Koppikar P, Guryanova OA, Rapaport F, et al. CHZ868, a type II JAK2 inhibitor, reverses type I JAK inhibitor persistence and demonstrates efficacy in myeloproliferative neoplasms. Cancer Cell 2015;28:15–28. doi:10.1016/j.ccell.2015.06.006.
[16] Robers MB, Machleidt T, Carlson CB, Bi K. Cellular LanthaScreen and beta-lactamase reporter assays for high-throughput screening of JAK2 inhibitors. Assay Drug Dev Technol 2008;6:519–29. doi:10.1089/adt.2008.144.
[17] Yu V, Pistillo J, Archibeque I, Han Lee J, Sun B-C, Schenkel LB, et al. Differential selectivity of JAK2 inhibitors in enzymatic and cellular settings. Exp Hematol 2013;41:491–500. doi:10.1016/j.exphem.2013.01.005.
[18] Imamura RM, Kumagai K, Nakano H, Okabe T, Nagano T, Kojima H. Inexpensive high-throughput screening of kinase inhibitors using one-step enzyme-coupled fluorescence assay for ADP detection. SLAS Discov 2019;24:284–94. doi:10.1177/2472555218810139.
[19] Savitsky P, Bray J, Cooper CDO, Marsden BD, Mahajan P, Burgess-Brown NA, et al. High-throughput production of human proteins for crystallization: the SGC experience. J Struct Biol 2010;172:3–13. doi:10.1016/j.jsb.2010.06.008.
[20] Hammarén HM, Virtanen AT, Abraham BG, Peussa H, Hubbard SR, Silvennoinen O. Janus kinase 2 activation mechanisms revealed by analysis of suppressing mutations. J Allergy Clin Immunol 2019;143:1549–59 e6. doi:10.1016/j.jaci.2018.07.022.
[21] Sun C, Li Y, Yates EA, Fernig DG. SimpleDSFviewer: a tool to analyze and view differential scanning fluorimetry data for characterizing protein thermal stability and interactions. Protein Sci Publ Protein Soc 2020;29:19–27. doi:10.1002/pro.3703.
[22] Quintás-Cardama A, Vaddi K, Liu P, Manshouri T, Li J, Scherle PA, et al. Preclinical characterization of the selective JAK1/2 inhibitor INCB018424: therapeutic implications for the treatment of myeloproliferative neoplasms. Blood 2010;115:3109–17. doi:10.1182/blood-2009-04-214957.
[23] Hart S, Goh KC, Novotny-Diermayr V, Hu CY, Hentze H, Tan YC, et al. SB1518, a novel macrocyclic pyrimidine-based JAK2 inhibitor for the treatment of myeloid and lymphoid malignancies. Leukemia 2011;25:1751–9. doi:10.1038/leu.2011.148.

[24] Siveen KS, Prabhu KS, Achkar IW, Kuttikrishnan S, Shyam S, Khan AQ, et al. Role of non receptor tyrosine kinases in hematological malignances and its targeting by natural products. Mol Cancer 2018;17:31. doi:10.1186/s12943-018-0788-y.
[25] Atanasov AG, Zotchev SB, Dirsch VM. International natural product sciences taskforce, supuran CT. Natural products in drug discovery: advances and opportunities. Nat Rev Drug Discov 2021;20:200–16. doi:10.1038/s41573-020-00114-z.
[26] Raivola J, Haikarainen T, Abraham BG, Silvennoinen O. Janus kinases in leukemia. Cancers 2021;13:800. doi:10.3390/cancers13040800.
[27] Girodon F, Bonicelli G, Schaeffer C, Mounier M, Carillo S, Lafon I, et al. Significant increase in the apparent incidence of essential thrombocythemia related to new WHO diagnostic criteria: a population-based study. Haematologica 2009;94:865–9. doi:10.3324/haematol.2008.004234.
[28] Kont YS, Dutta A, Mallisetty A, Mathew J, Minas T, Kraus C, et al. Depletion of tyrosyl DNA phosphodiesterase 2 activity enhances etoposide-mediated double-strand break formation and cell killing. DNA Repair 2016;43:38–47. doi:10.1016/j.dnarep.2016.04.009.
[29] Klopčič I, Dolenc MS. Chemicals and drugs forming reactive quinone and quinone imine metabolites. Chem Res Toxicol 2019;32:1–34. doi:10.1021/acs.chemrestox.8b00213.