

Electronic Supporting Information

Virtual screening and optimization of Type II inhibitors of JAK2 from a natural product library

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Table of Contents

General Experimental	2
Scheme S1	6
Preparation of 1b–j and characterizations.....	7
Tables S1–2	12
Figures S1–6	15

General Experimental

Materials

HTScan[®] JAK2 Kinase Assay Kit (ELISA) was obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-JAK2 Y1007/Y1008 rabbit monoclonal antibody and anti-JAK2 goat monoclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-STAT3, anti-STAT3, anti-GAPDH antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Human erythroleukemia (HEL) cells cultured in RPMI-1640 containing 10% fetal bovine serum were incubated at 37 °C/5% CO₂ and maintained at a cell density of 0.2–1×10⁶ cells/mL. HEL cells were generously provided by Wendy Yeung (The Hong Kong University of Science and Technology, Hong Kong). Deuterated solvents for NMR purposes were obtained from Armar and used as received.

Molecular docking and virtual screening

Structure selection. We initially docked a panel of known JAK2 inhibitors against twelve X-ray crystal structures of JAK2 (PDB code: 2B7A,¹ 3LPB,² 3JY9,³ 3KCK,⁴ 3IO7,⁵ 3IOK,⁵ 3FUP,⁶ 3EYG,⁶ 3EYH,⁶ 3E62,⁷ 3E63,⁷ 3E64⁷). The X-ray co-crystal structure of JAK2 with pan-Janus kinase inhibitor CMP6 (PDB code: 2B7A)¹ was deemed to be the most predictive structure according to our molecular modelling methods as it yielded the highest average docking score. Additionally, the X-ray co-crystal structure of the Type II inhibitor NVP-BBT594 bound to the DFG-out conformation of JAK2 (PDB: 3UGC)⁸ was reported during the preparation of this manuscript. However, the docking of compound **1a** against the molecular model generated from this structure yielded a poor binding score. This could be due to the fact NVP-BBT594 binds to JAK2 in a different conformation compared to compound **1a**, thus inducing changes in protein structure that might not be efficiency replicated by compound **1a**, leading to a lower predicted binding affinity for **1a**. Therefore, the initial molecular model generated from the DOLPHIN protocol was used for further computer-aided lead optimization of the amentoflavone derivatives.

Model construction. The deletion-of-loop Asp-Phe-Gly-in (DOLHPIN) protocol, as described by Abagyan and co-workers,⁹ was used to convert a DFG-in structure of JAK2 into a Type II-compatible conformation that can be used for molecular docking. This protocol consists of two fully automated steps: (i) the removal of all atoms of the Phe residue of DFG (DFG Phe) and the next four residues in the sequence and (ii) the generation of a pharmacophore-like field from the side-chain atoms of DFG Phe and backbone atoms of the following residues with DFG Gly skipped. The pharmacophore-like density was generated using a single property (lipophilicity) and slightly rewarded the ligand docking poses occupying the hydrophobic selectivity pocket. No ligand-related information was employed by the algorithm. This protocol was applied to the X-ray co-crystal structure of JAK2 with the pan-Janus kinase inhibitor CMP6 (PDB code: 2B7A).¹

ICM full-atom ligand-receptor complex refinement and scoring. According to the ICM method,¹⁰ the molecular system was described using internal coordinates as variables. Energy calculations were based on the ECEPP/3 force field with a distance-dependent dielectric constant. The biased probability Monte Carlo (BPMC) minimization procedure was used for global energy optimization. The BPMC global-energy-optimization method consists of 1) a random conformation change of the free variables according to a predefined continuous probability distribution; 2) local-energy minimization of analytical differentiable terms; 3) calculation of the complete energy including nondifferentiable terms such as

entropy and solvation energy; 4) acceptance or rejection of the total energy based on the Metropolis criterion and return to step (1). The binding between the small molecules and JAK2 were evaluated with a full-atom ICM ligand binding score¹¹ from a multireceptor screening benchmark as a compromise between approximated Gibbs free energy of binding and numerical errors. The score was calculated by:

$$S_{\text{bind}} = E_{\text{int}} + T\Delta S_{\text{Tor}} + E_{\text{vw}} + \alpha_1 E_{\text{el}} + \alpha_2 E_{\text{hb}} + \alpha_3 E_{\text{hp}} + \alpha_4 E_{\text{sf}}$$

where E_{vw} , E_{el} , E_{hb} , E_{hp} , and E_{sf} are van der Waals, electrostatic, hydrogen bonding, and nonpolar and polar atom solvation energy differences between bound and unbound states, respectively. E_{int} is the ligand internal strain, ΔS_{Tor} is its conformational entropy loss upon binding, and $T = 300$ K, and α_i are ligand- and receptor independent constants.

High-throughput molecular docking. We performed *in silico* screening on natural product and natural product-like databases on the DOLPHIN kinase model using the internal coordinate mechanics (ICM) method [ICM-Pro 3.6-1d molecular docking software (Molsoft) to identify natural product scaffolds as Type II JAK2 inhibitors. Three natural product databases were used in our virtual screening campaign: the Analyticon Discovery NATx and MEGabolite databases (20,000 compounds, Germany), the ZINC natural product database (90,000 compounds, USA) and the Hongcam natural products database (160,000 compounds, China). Due to the overlap of the three databases, the total number of unique compounds screened was about 150,000. Molecular docking was performed using the virtual library screening (VLS) module in the ICM-Pro 3.6-1d program (Molsoft). Each compound in the library was assigned the MMFF3 force field atom types and charges and was then subjected to Cartesian minimization. During the docking analysis, the ligand was considered flexible and the binding pose and internal torsions were sampled by the BPMC minimization procedure, which involved local energy minimization after each random move. Each compound was docked to the protein binding pocket, and a score from the docking was assigned to each compound according to the weighed component of the ICM scoring function described above. Each compound was docked three times to ensure the convergence of the Monte Carlo optimization, and the minimum score of each ligand from the three independent docking experiments was retained and used for ranking.

ELISA

Recombinant JAK2 (15 μM) was incubated with the indicated concentrations of compound or DMSO for 5 min at room temperature in reaction buffer (60 mM HEPES pH 7.5, 5 mM MgCl_2 , 5 mM MnCl_2 , 3 μM Na_3VO_4 , 1.25 mM DTT). The reaction was initiated by the addition of indicated concentrations of ATP and biotinylated peptide substrate (1.5 μM), and the mixture was incubated at room temperature for 30 min. The reaction was stopped by the addition of EDTA (50 mM, pH 8), transferred to a 96-well streptavidin coated plate and incubated at room temperature for 60 min. The wells washed with three times with PBS/0.05% Tween 20 (PBST) and incubated with anti-phospho-tyrosine mouse monoclonal antibody at room temperature for 60 min. The wells were washed as before and incubated with horseradish peroxide-conjugated anti-mouse secondary antibody at room temperature for 30 min. The wells were washed five times with PBST, incubated with 100 μL TMB solution, quenched with 100 μL 2N sulfuric acid, and the absorbance was measured at $\lambda = 450$ nm. Three independent experiments were performed.

Cytotoxicity test (MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide) assay)

HEL cells were seeded in a 96-well flat-bottomed microplate at 8000 cells/well in 100 μL of Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum

(Invitrogen) and 1% antibiotic and antimycotic solution (Sigma). The compounds were dissolved in DMSO and mixed with the growth medium (final DMSO concentration $\leq 4\%$) respectively. Serial dilution of each complex were added to each well. The microplate was incubated at 37 °C, 5% CO₂, 95% air in a humidified incubator for 72 h. After incubation, 10 μ L MTT reagent (5 mg/mL) was added to each well. The microplate was re-incubated at 37 °C in 5% O₂ for 4 h. DMSO (100 μ L) was added to each well. The microplate was further incubated for 15 min. The absorbance at 570 nm was measured using a microplate reader. The IC₅₀ values of the compounds (concentration required to reduce the absorbance by 50% compared to the control) were determined by the dose-dependence of surviving cells. This assay was repeated three times independently.

Antiviral assay

The firefly (FF)-luciferase reporter activity was used to monitor the replication of HCV replicons in Huh-luc/neo-ET cells as previously described.¹² Replicon cells were seeded at a density of 5×10^3 cells per well in 96-well plates and were incubated in duplicate with dimethyl sulfoxide (DMSO) or serially diluted NVP-BBT594, or amentoflavone analogues (**1a**, **1b**, **1c** and **1g**) at 37 °C for 72 h. Cells were lysed with ice-cold passive lysis reagent and the luciferase activity was measured with a luciferase assay kit (Promega) and a Tecan FARCyte luminometer (GE Healthcare) according to the manufacturers' instructions. The numbers of relative light units (RLU) were determined as percentage readings relative to the level for the compound-free controls. The EC₅₀ was determined by nonlinear regression analysis using Origin 6.1 (OriginLab Software). Two independent experiments were conducted for this assay.

Western blot analysis

1×10^6 HEL cells were treated with the indicated concentrations of compound or DMSO for 16 h. Cells were washed three times with cold PBS, resuspended in RIPA lysis buffer, and incubated on ice for 30 min. Cell debris was removed by centrifugation at 14,000 rpm for 30 min at 4 °C, and the protein concentration of the supernatant was determined with Bio-Rad protein assay dye reagent (Bio-Rad). Equal protein amounts were electrophoresed on a 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% milk for 1 h, and probed with anti-phospho-JAK2 Y1007/Y1008 rabbit antibody or anti-phospho-STAT3 (Y705) rabbit antibody in 5% milk overnight at 4 °C. The membrane was washed with PBST and incubated with horseradish peroxide-conjugated anti-rabbit secondary antibody in 5% milk for 1 h at room temperature. Protein bands were detected using enhanced chemiluminescence as specified by the manufacturer (ECL, Thermo). Membranes were stripped and reprobed with anti-JAK2 goat antibody or anti-STAT3 antibody to confirm equal protein loading. Three independent experiments were performed.

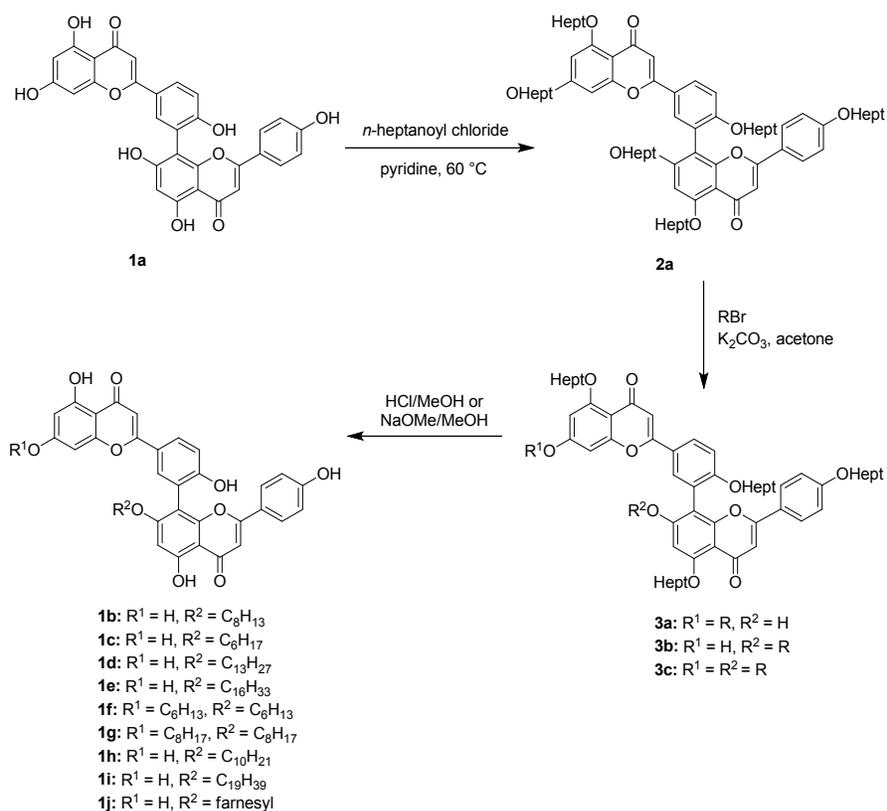
Preparative: Reactions were monitored by thin layer chromatography (TLC) using E. Merck silica gel plates, Kieselgel 60 F₂₅₄ with 0.2 mm thickness. Components were visualized by illumination with short-wavelength ultra-violet light and/or staining. Flash column chromatography was performed with E. Merck silica gel 60 (230-400 mesh ASTM).

Amentoflavone was purchased from the Shenzhen ChemStrong Scientific Co. Ltd. n-Heptanoyl chloride was purchased from Acros Organics. Solvents and commercially available reagents were used as received.

In the synthetic strategy, amentoflavone **1a** was treated with an excess of heptanoyl chloride in pyridine at 60 °C overnight to afford the peracylated derivative **2a** (Scheme S1). Warming the reaction of **2a** and the appropriate alkyl bromides in the presence of potassium carbonate promoted selective hydrolysis and alkylation at the C7 and C7'' phenolic positions, to generate mixtures of three products: the 7-monoalkylated derivatives **3a**, 7''-monoalkylated derivatives **3b** and the 7,7''-dialkylated derivatives **3c**. The reaction of **2a** with farnesyl bromide proceeded under milder conditions. Because **3a–c** were very similar in polarity, the mixtures, without further purification, were directly hydrolyzed under acidic conditions using 3M HCl/MeOH. For the synthesis of **1j**, deprotection was induced under basic conditions. The deacylated derivatives **1b–j** were isolated and purified by repeated preparative TLC or HPLC.

Analytical: ¹H and ¹³C NMR nuclear magnetic resonance spectra were recorded in deuteriochloroform (CDCl₃) or deuterioacetone (acetone-*d*₆), with tetramethylsilane (TMS) as an internal standard at ambient temperature on a Bruker Avance 400 spectrometer operating at 400 MHz for ¹H, and at 100 MHz for ¹³C. All spectra were calibrated at δ 7.26 or δ 0.00 ppm for ¹H spectra (residual CHCl₃ or TMS respectively), and 77.16 ppm for ¹³C spectra. Splitting patterns are designated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. Maldi-TOF-HRMS analysis was performed using a Bruker Autoflex II mass spectrometer (Bruker Daltonics, Germany), with accurate mass reported for the molecular ion (M⁺) or next largest fragment thereof. Preparative HPLC was carried out on a Waters HPLC with a 510 HPLC pump and 410 differential refractometer.

Scheme S1. Synthetic procedure for the preparation of top-scoring compounds.



Preparation of 1b-j and characterisation

Synthesis of 2-(3-(5,7-bis(heptanoyloxy)-2-(4-(heptanoyloxy)phenyl)-4-oxo-4H-chromen-8-yl)-4-(heptanoyloxy)phenyl)-4-oxo-4H-chromene-5,7-diyl diheptanoate (2a). Amentoflavone (100 mg, 0.186 mmol) was dissolved in 1.0 mL pyridine and *n*-heptanoyl chloride (0.35 mL, 2.3 mmol) was added dropwise. The solution was stirred at room temperature for 1 h and then heated at 60 °C overnight. DCM (20 mL) was added and the mixture was washed with H₂O and 1 M HCl. The organic phase was dried over anhydrous MgSO₄. The volatiles were removed *in vacuo*, and the residue was purified by silica gel flash column chromatography (hexane/EtOAc, 9:1) to give 193 mg of **2** as a colourless oil: 86% yield. ¹H NMR (400 MHz, CDCl₃): δ 8.00–7.96 (m, 2H), 7.48 (t, *J* = 8.0 Hz, 3H), 7.27 (s, 1H), 7.05 (d, *J* = 8.0 Hz, 2H), 6.98 (s, 1H), 6.82 (d, *J* = 4.0 Hz, 1H), 6.70 (s, 1H), 6.66 (s, 1H), 2.79–2.71 (m, 4H), 2.57–2.49 (m, 4H), 2.35–2.24 (m, 4H), 1.85–1.59 (m, 12H), 1.46–1.24 (m, 36H), 0.93–0.79 (m, 18H) ppm; ¹³C NMR (100 MHz, CDCl₃): δ 176.6, 176.4, 172.3, 172.2, 171.9, 171.2, 171.0, 170.89, 161.8, 161.0, 157.7, 155.3, 154.4, 153.6, 152.3, 151.8, 150.5, 150.3, 130.0, 128.7, 128.3, 127.5, 125.4, 124.0, 122.6, 117.3, 115.4, 115.1, 115.0, 114.2, 109.2, 108.9, 108.3, 34.5, 34.45, 34.4, 34.3, 34.2, 34.15, 34.1, 31.7, 31.68, 31.6, 31.5, 31.46, 31.4, 29.03, 29.00, 28.96, 28.9, 28.84, 28.63, 28.6, 25.0, 24.9, 24.8, 24.7, 24.6, 24.56, 24.54, 22.7, 22.6, 22.59, 22.5, 14.23, 14.2, 14.16, 14.14, 14.13 ppm; Maldi-TOF-HRMS: Calcd for C₇₂H₉₀O₁₆ [M + Na]⁺: 1233.6146 Found: 1233.6121.

General procedure for the synthesis of 3a–c. Peracylated amentoflavone (**2a**, 121 mg, 0.100 mmol) and the corresponding alkyl bromide (0.50 mmol) were dissolved in acetone (2.0 mL), and K₂CO₃ (138 mg, 1.00 mmol) was added in one portion. The mixture was stirred at 55 °C for 12 h and filtered through a pad of silica gel. The volatiles were evaporated *in vacuo* to give a mixture of three alkylation products (**3a–c**), which were used in the next step without further purification.

For the synthesis of **3a–c** using farnesyl bromide, **2a** (121 mg, 0.10 mmol), farnesyl bromide (0.40 mmol) and K₂CO₃ (138 mg, 1.0 mmol) in acetone (2.0 mL), was stirred at room temperature for 4 h, then worked up as in the General Procedure.

General procedure for the synthesis of 1b–i. The alkylation product mixture **3a–c** was dissolved in 3 M HCl/MeCN (1:2 v/v, 2.0 mL) and stirred at reflux overnight. The reaction mixture was diluted with EtOAc and washed with water then saturated NaHCO₃. The organics were dried

over anhydrous MgSO₄ and removed of volatiles *in vacuo*. Isolation and purification by either preparative TLC (DCM/MeOH, 10:1) or preparative HPLC yielded products **1b–i** as yellow solids.

Procedure for the synthesis of 1j. The alkylation product mixture **3a–c** was dissolved in MeOH, and NaOMe (12 equiv.) was added in one portion. The mixture was stirred at room temperature overnight. The reaction was neutralized with 1 M HCl to pH 2–3, and the volatiles were removed *in vacuo*. The residue was taken up in EtOAc and washed with water then saturated NaHCO₃. The organics were dried over anhydrous MgSO₄ and removed of volatiles *in vacuo*. Isolation and purification by preparative TLC (DCM/MeOH, 10:1) and preparative HPLC yielded product **1j** as a yellow solid.

8-(5-(5,7-Dihydroxy-4-oxo-4H-chromen-2-yl)-2-hydroxyphenyl)-5-hydroxy-2-(4-hydroxyphenyl)-7-(octyloxy)-4H-chromen-4-one (1b) 20% yield over 2 steps. ¹H NMR (400 MHz, acetone-*d*₆) δ 13.29 (s, 1H), 13.00 (s, 1H), 8.06 (d, *J* = 4.0 Hz, 1H), 8.00 (dd, *J* = 8.0, 4.0 Hz, 1H), 7.67 (d, *J* = 8.0 Hz, 2H), 7.26 (d, *J* = 12 Hz, 1H), 6.84 (d, *J* = 8.0 Hz, 2H), 6.71 (s, 1H), 6.66 (s, 1H), 6.56 (s, 1H), 6.52 (d, *J* = 4.0 Hz, 1H), 6.24 (d, *J* = 4.0 Hz, 1H), 4.14 (t, *J* = 8.0 Hz, 2H), 1.70–1.63 (m, 2H), 1.38–1.28 (m, 2H), 1.21–1.11 (m, 8H), 0.77 (t, *J* = 8.0 Hz, 3H) ppm; ¹³C NMR (100 MHz, acetone-*d*₆) δ 183.8, 183.3, 165.6, 165.4, 165.3, 163.8, 163.6, 163.3, 162.3, 160.4, 159.1, 155.5, 132.9, 129.5, 128.8, 123.3, 123.2, 121.2, 117.5, 117.0, 106.0, 105.7, 105.5, 104.3, 103.8, 100.0, 97.1, 95.1, 70.0, 32.7, 27.0, 23.5, 14.5 ppm; Maldi-TOF-HRMS: Calcd for C₃₈H₃₄O₁₀ [M + H]⁺: 651.2224 Found: 651.2223.

8-(5-(5,7-Dihydroxy-4-oxo-4H-chromen-2-yl)-2-hydroxyphenyl)-7-(hexyloxy)-5-hydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one (1c). 10% yield over 2 steps. ¹H NMR (400 MHz, acetone-*d*₆) δ 13.32 (s, 1H), 13.04 (s, 1H), 8.11 (d, *J* = 4.0 Hz, 1H), 8.04 (dd, *J* = 8.0, 4.0 Hz, 1H), 7.70 (d, *J* = 8.0 Hz, 2H), 7.26 (d, *J* = 12 Hz, 1H), 6.85 (d, *J* = 8.0 Hz, 2H), 6.73 (s, 1H), 6.69 (s, 1H), 6.58 (s, 1H), 6.52 (d, *J* = 4.0 Hz, 1H), 6.24 (d, *J* = 4.0 Hz, 1H), 4.16 (t, *J* = 8.0 Hz, 2H), 1.72–1.65 (m, 2H), 1.40–1.34 (m, 2H), 1.25–1.14 (m, 4H), 0.75 (t, *J* = 8.0 Hz, 3H) ppm; ¹³C NMR (100 MHz, acetone-*d*₆) δ 183.9, 183.4, 165.7, 165.5, 165.2, 165.1, 164.0, 163.5, 162.3, 160.4, 159.3, 155.8, 133.1, 129.7, 129.1, 123.7, 123.6, 121.4, 117.7, 117.1, 106.1, 105.9, 105.8, 104.8, 104.2, 100.1, 97.3, 95.3, 70.3, 32.0, 26.9, 23.7, 14.7 ppm; Maldi-TOF-HRMS: Calcd for C₃₆H₃₀O₁₀ [M + H]⁺: 623.1912 Found: 623.19.

8-(5-(5,7-Dihydroxy-4-oxo-4H-chromen-2-yl)-2-hydroxyphenyl)-5-hydroxy-2-(4-hydroxyphenyl)-7-(tridecyloxy)-4H-chromen-4-one (1d). 18% yield over 2 steps. ¹H NMR (400 MHz, acetone-*d*₆) δ 13.31 (s, 1H), 13.02 (s, 1H), 8.09 (d, *J* = 4.0 Hz, 1H), 8.03 (dd, *J* = 8.0, 4.0 Hz, 1H), 7.68 (d, *J* = 8.0 Hz, 2H), 7.25 (d, *J* = 8.0 Hz, 1H), 6.85 (d, *J* = 8.0 Hz, 2H), 6.72 (s, 1H), 6.67 (s, 1H), 6.56 (s, 1H), 6.52 (d, *J* = 4.0 Hz, 1H), 6.24 (d, *J* = 4.0 Hz, 1H), 4.15 (t, *J* = 8.0 Hz, 2H), 1.67 (m, 2H), 1.37 (m, 2H), 1.30–1.10 (m, 18H), 0.86 (t, *J* = 8.0 Hz, 3H) ppm; ¹³C NMR (100 MHz, acetone-*d*₆) δ 183.7, 183.1, 165.4, 165.1, 165.0, 163.7, 163.5, 162.0, 160.1, 158.9, 155.4, 132.7, 129.3, 128.6, 123.3, 123.1, 121.0, 117.4, 116.8, 105.6, 105.55, 105.4, 104.3, 103.7, 99.8, 96.9, 94.9, 69.9, 32.7, 26.9, 23.4, 14.4 ppm; Maldi-TOF-HRMS: Calcd for C₄₃H₄₄O₁₀ [M + H]⁺: 721.3006 Found: 721.3007.

8-(5-(5,7-Dihydroxy-4-oxo-4H-chromen-2-yl)-2-hydroxyphenyl)-7-(hexadecyloxy)-5-hydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one (1e). 16% yield over 2 steps. ¹H NMR (400 MHz, acetone-*d*₆) δ 13.30 (s, 1H), 13.03 (s, 1H), 9.60 (br s, 1H), 9.20 (br s, 1H), 8.09 (d, *J* = 4.0 Hz, 1H), 8.03 (dd, *J* = 8.0, 4.0 Hz, 1H), 7.68 (d, *J* = 8.0 Hz, 2H), 7.25 (d, *J* = 8.0 Hz, 1H), 6.85 (d, *J* = 8.0 Hz, 2H), 6.72 (s, 1H), 6.67 (s, 1H), 6.56 (s, 1H), 6.52 (d, *J* = 4.0 Hz, 1H), 6.24 (d, *J* = 4.0 Hz, 1H), 4.15 (t, *J* = 8.0 Hz, 2H), 1.67 (m, 2H), 1.35 (m, 2H), 1.30–1.10 (m, 24H), 0.87 (t, *J* = 8.0 Hz, 3H) ppm; ¹³C NMR (100 MHz, acetone-*d*₆) δ 183.7, 183.1, 165.4, 165.1, 164.9, 163.7, 163.5, 162.0, 160.1, 158.9, 155.4, 132.7, 129.3, 128.6, 123.3, 123.1, 121.0, 117.4, 116.8, 105.6, 105.56, 105.5, 104.3, 103.7, 99.8, 96.9, 94.9, 69.9, 32.7, 26.9, 23.4, 14.4 ppm; Maldi-TOF-HRMS: Calcd for C₄₆H₅₀O₁₀ [M + H]⁺: 763.3476 Found: 763.3474.

7-(Hexyloxy)-8-(5-(7-(hexyloxy)-5-hydroxy-4-oxo-4H-chromen-2-yl)-2-hydroxyphenyl)-5-hydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one (1f). 20% yield over 2 steps. ¹H NMR (400 MHz, acetone-*d*₆) δ 13.32 (s, 1H), 12.99 (s, 1H), 8.12 (d, *J* = 4.0 Hz, 1H), 8.06 (dd, *J* = 8.0, 4.0 Hz, 1H), 7.69 (d, *J* = 8.0 Hz, 2H), 7.26 (d, *J* = 8.0 Hz, 1H), 6.85 (d, *J* = 8.0 Hz, 2H), 6.76 (s, 1H), 6.69 (s, 1H), 6.66 (d, *J* = 4.0 Hz, 1H), 6.58 (s, 1H), 6.30 (d, *J* = 4.0 Hz, 1H), 4.16 (t, *J* = 8.0 Hz, 2H), 4.09 (t, *J* = 8.0 Hz, 2H), 1.82–1.75 (m, 2H), 1.72–1.65 (m, 2H), 1.50–1.43 (m, 2H), 1.39–1.33 (m, 6H), 1.27–1.14 (m, 4H), 0.89 (t, *J* = 8.0 Hz, 3H), 0.74 (t, *J* = 8.0 Hz, 3H) ppm; ¹³C NMR (100 MHz, acetone-*d*₆) δ 184.1, 183.7, 166.5, 165.8, 164.1, 163.9, 163.5, 162.4, 160.7, 159.2, 155.9, 133.2, 129.8, 129.2, 123.7, 123.5, 121.5, 117.9, 117.3, 106.4, 106.1, 106.0, 104.8, 104.2, 99.6, 97.4, 94.2,

70.3, 69.9, 32.7, 32.6, 26.9, 26.8, 23.8, 23.7, 14.8, 14.7 ppm; Maldi-TOF-HRMS: Calcd for C₄₂H₄₂O₁₀ [M + H]⁺: 707.2851 Found: 707.2824.

5-Hydroxy-8-(2-hydroxy-5-(5-hydroxy-7-(octyloxy)-4-oxo-4H-chromen-2-yl)phenyl)-2-(4-hydroxyphenyl)-7-(octyloxy)-4H-chromen-4-one (1g). 17% yield over 2 steps. ¹H NMR (400 MHz, acetone-*d*₆) δ 13.31 (s, 1H), 12.98 (s, 1H), 8.11 (d, *J* = 4.0 Hz, 1H), 8.04 (dd, *J* = 8.0, 4.0 Hz, 1H), 7.69 (d, *J* = 8.0 Hz, 2H), 7.26 (d, *J* = 8.0 Hz, 1H), 6.86 (d, *J* = 8.0 Hz, 2H), 6.76 (s, 1H), 6.68 (s, 1H), 6.66 (d, *J* = 4.0 Hz, 1H), 6.57 (s, 1H), 6.30 (d, *J* = 4.0 Hz, 1H), 4.15 (t, *J* = 8.0 Hz, 2H), 4.08 (t, *J* = 8.0 Hz, 2H), 1.82–1.75 (m, 2H), 1.71–1.64 (m, 2H), 1.50–1.42 (m, 2H), 1.36–1.11 (m, 18H), 0.88 (t, *J* = 8.0 Hz, 3H), 0.77 (t, *J* = 8.0 Hz, 3H) ppm; ¹³C NMR (100 MHz, acetone-*d*₆) δ 184.0, 183.5, 166.4, 165.7, 165.6, 164.0, 163.6, 163.2, 162.3, 160.5, 159.2, 155.8, 133.2, 129.7, 129.1, 123.8, 123.5, 121.4, 117.8, 117.2, 106.4, 106.1, 106.0, 104.8, 104.2, 99.6, 97.3, 94.2, 70.3, 69.9, 33.04, 33.01, 27.3, 27.1, 23.8, 23.7, 14.9, 14.8 ppm; Maldi-TOF-HRMS: Calcd for C₄₆H₅₀O₁₀ [M + H]⁺: 763.3476 Found: 763.3474.

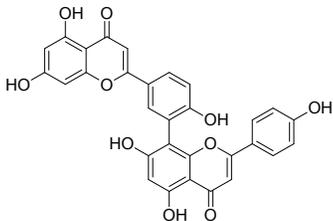
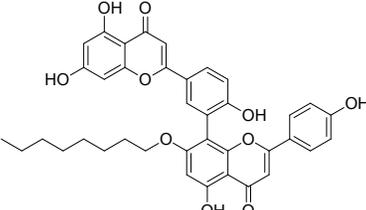
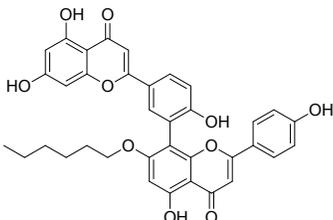
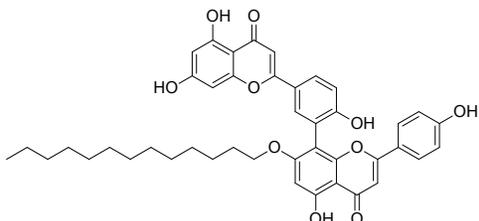
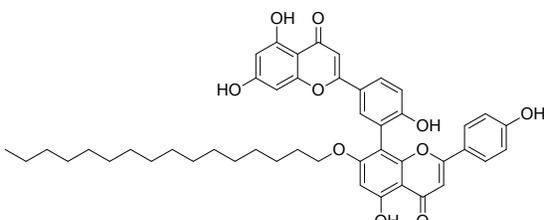
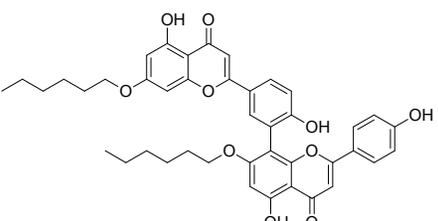
7-(Decyloxy)-8-(5-(5,7-dihydroxy-4-oxo-4H-chromen-2-yl)-2-hydroxyphenyl)-5-hydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one (1h). 15% yield over 2 steps. ¹H NMR (400 MHz, acetone-*d*₆) δ 13.32 (s, 1H), 13.04 (s, 1H), 8.12 (d, *J* = 4.0 Hz, 1H), 8.04 (dd, *J* = 8.0, 4.0 Hz, 1H), 7.70 (d, *J* = 8.0 Hz, 2H), 7.26 (d, *J* = 8.0 Hz, 1H), 6.86 (d, *J* = 8.0 Hz, 2H), 6.74 (s, 1H), 6.69 (s, 1H), 6.58 (s, 1H), 6.53 (d, *J* = 4.0 Hz, 1H), 6.25 (d, *J* = 4.0 Hz, 1H), 4.16 (t, *J* = 8.0 Hz, 2H), 1.72–1.65 (m, 2H), 1.40–1.12 (m, 14H), 0.83 (t, *J* = 8.0 Hz, 3H) ppm; ¹³C NMR (100 MHz, acetone-*d*₆) δ 184.1, 183.6, 165.8, 165.5, 165.4, 164.1, 163.9, 162.4, 160.5, 159.3, 155.8, 133.2, 129.8, 129.1, 123.8, 123.6, 121.4, 117.8, 117.2, 106.0, 105.99, 105.9, 104.7, 104.2, 100.2, 97.4, 95.3, 70.3, 33.1, 27.4, 23.8, 14.9 ppm; Maldi-TOF-HRMS: Calcd for C₄₀H₃₈O₁₀ [M + H]⁺: 679.2538 Found: 679.2511.

8-(5-(5,7-Dihydroxy-4-oxo-4H-chromen-2-yl)-2-hydroxyphenyl)-5-hydroxy-2-(4-hydroxyphenyl)-7-(nonadecyloxy)-4H-chromen-4-one (1i). 12% yield over 2 steps. ¹H NMR (400 MHz, acetone-*d*₆) δ 13.32 (s, 1H), 13.04 (s, 1H), 8.10 (d, *J* = 4.0 Hz, 1H), 8.04 (dd, *J* = 8.0, 4.0 Hz, 1H), 7.70 (d, *J* = 8.0 Hz, 2H), 7.26 (d, *J* = 8.0 Hz, 1H), 6.86 (d, *J* = 8.0 Hz, 2H), 6.73 (s, 1H), 6.69 (s, 1H), 6.58 (s, 1H), 6.53 (d, *J* = 4.0 Hz, 1H), 6.25 (d, *J* = 4.0 Hz, 1H), 4.16 (t, *J* = 8.0 Hz, 2H), 1.72–1.65 (m, 2H), 1.60–1.53 (m, 4H), 1.43–1.12 (m, 28H), 0.87 (t, *J* = 8.0 Hz, 3H) ppm; ¹³C NMR

(100 MHz, acetone-*d*₆) δ 184.1, 183.6, 165.8, 165.5, 165.4, 164.1, 163.9, 162.4, 160.5, 159.3, 155.8, 133.2, 129.7, 129.0, 123.8, 123.6, 121.4, 117.8, 117.2, 106.0, 105.99, 105.9, 104.7, 104.2, 100.2, 97.3, 95.3, 70.3, 33.1, 27.3, 23.8, 14.8 ppm; Maldi-TOF-HRMS: Calcd for C₄₉H₅₆O₁₀ [M + H]⁺: 805.3946 Found: 805.3921.

8-(5-(5,7-Dihydroxy-4-oxo-4H-chromen-2-yl)-2-hydroxyphenyl)-5-hydroxy-2-(4-hydroxyphenyl)-7-(((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)oxy)-4H-chromen-4-one (1j). 11% yield over 2 steps. ¹H NMR (400 MHz, acetone-*d*₆) δ 13.28 (s, 1H), 13.01 (s, 1H), 8.06 (d, *J* = 4.0 Hz, 1H), 7.99 (br d, *J* = 8.0, 1H), 7.66 (d, *J* = 8.0 Hz, 2H), 7.25 (d, *J* = 8.0 Hz, 1H), 6.84 (d, *J* = 8.0 Hz, 2H), 6.67 ((d, *J* = 8.0 Hz, 2H), 6.59 (s, 1H), 6.53 (br s, 1H), 6.24 (br s, 1H), 5.42 (t, *J* = 8.0 Hz, 2H), 5.08–5.00 (m, 2H), 4.78–4.68 (m, 2H), 2.09–2.00 (m, 4H), 1.97–1.95 (m, 2H), 1.91–1.85 (m, 2H), 1.75 (br s, 3H), 1.64–1.57 (m, 6H), 1.52 (d, *J* = 8.0 Hz, 3H) ppm; ¹³C NMR (100 MHz, acetone-*d*₆) δ 184.0, 183.5, 165.8, 165.6, 165.5, 163.8, 163.76, 163.7, 162.4, 160.5, 159.3, 155.8, 142.7, 136.3, 133.0, 132.0, 129.6, 129.0, 125.6, 124.9, 123.6, 123.5, 121.4, 120.4, 117.8, 117.2, 106.2, 106.0, 105.7, 104.6, 104.0, 100.2, 97.8, 95.3, 67.2, 40.8, 40.5, 27.8, 27.2, 26.2, 18.1, 17.2, 16.5 ppm.

Table S1. Chemical structures of the amentoflavone analogues and their corresponding ICM scores.

Compound	Chemical structure	ICM score
1a		-29.58
1b		-31.59
1c		-35.73
1d		-27.25
1e		-22.59
1f		-35.12

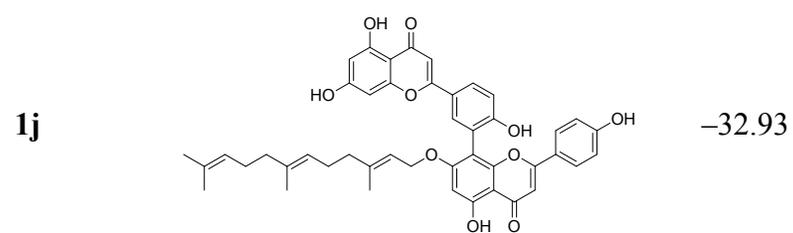
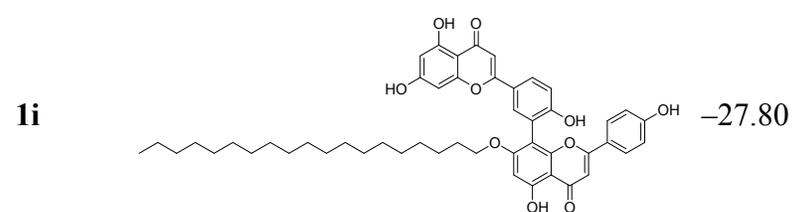
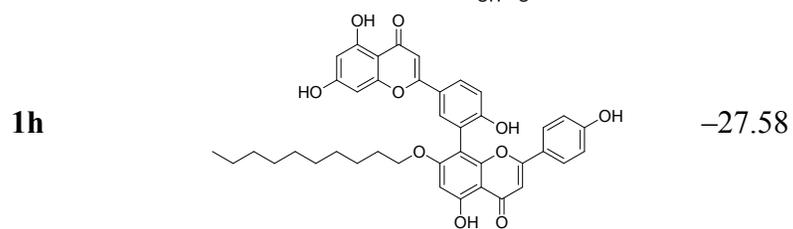
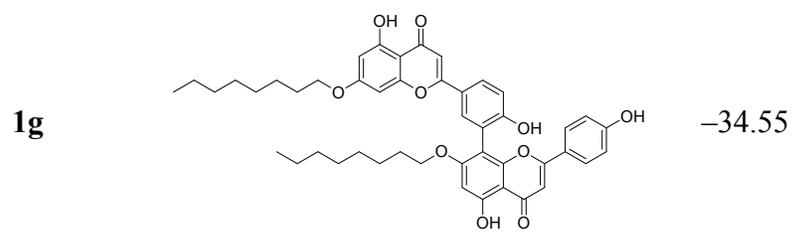
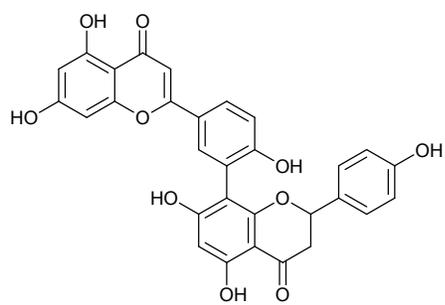


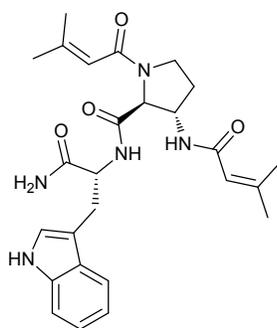
Table S2. IC₅₀ values of compounds in the MTT assay against HEL cells. Results are representative of three independent experiments.

Compound	HEL
1a	>100
1b	>100
1c	0.62
1d	>100
1e	>100
1f	64.34
1g	>100
1h	25.19
1i	>100
1j	1.40
JAK2 Inhibitor II	98.65

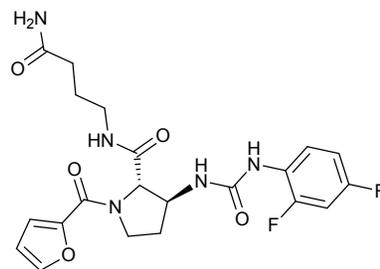
Fig. S1 The chemical structure of top eleven highest-scoring compounds from the initial high-throughput virtual screening campaign.



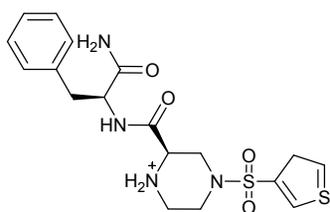
1a



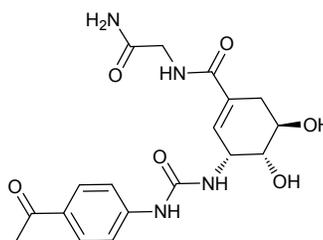
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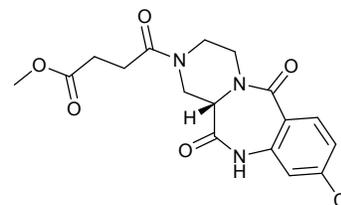
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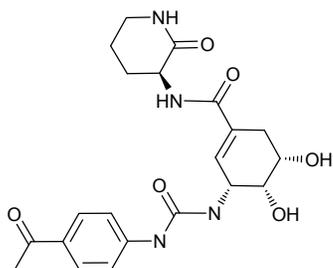
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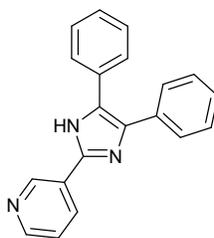
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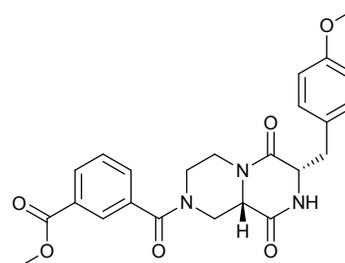
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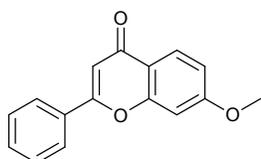
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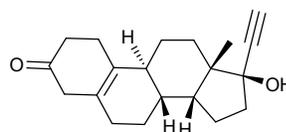
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10



11

Fig. S2 Dose-dependent inhibition of JAK2 enzyme activity by compound **1a** as determined by ELISA. Estimated IC_{50} value: 5 μ M. Three independent experiments were performed.

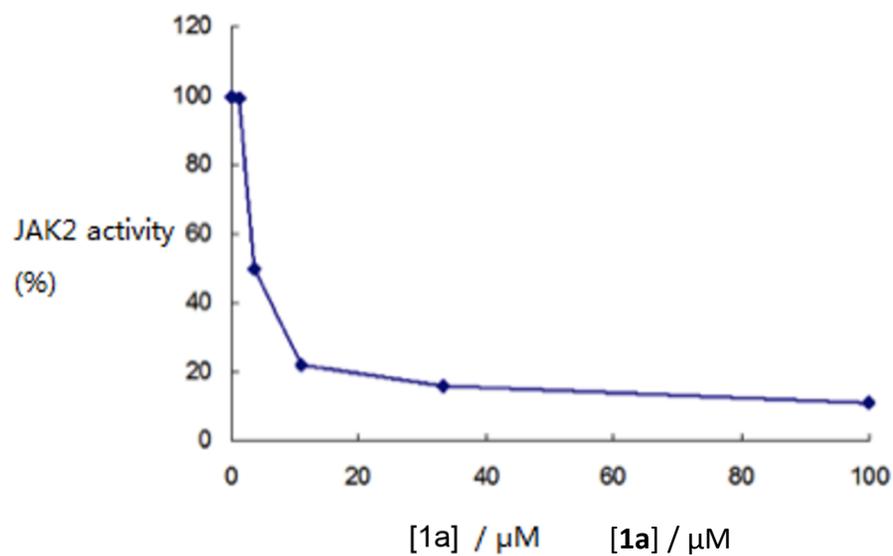


Fig. S3 Inhibition of HEL cell proliferation as determined by an MTT assay. HEL cells were incubated with the indicated concentrations of compounds for 72 h. This assay was repeated three times independently. Error bars represent the standard deviation of triplicate results.

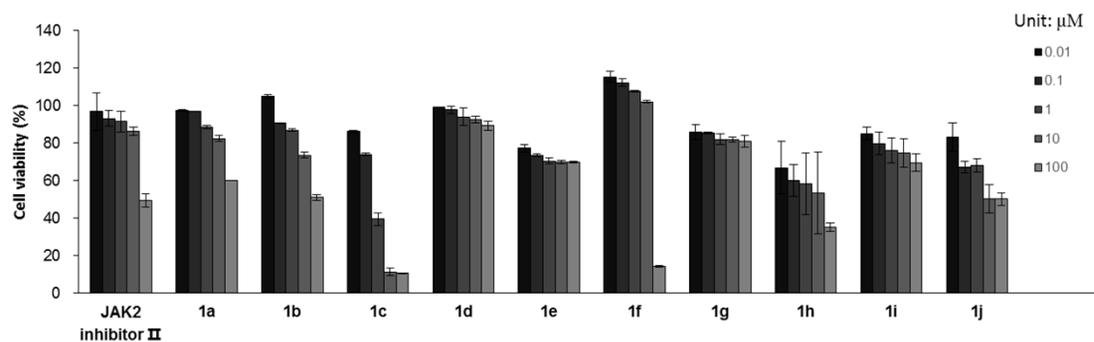


Fig. S4 Dose-dependent inhibition of JAK2 enzyme activity by compound **1b** as determined by ELISA. Estimated IC_{50} value: 3.9 μ M. Results are representative of three independent experiments.

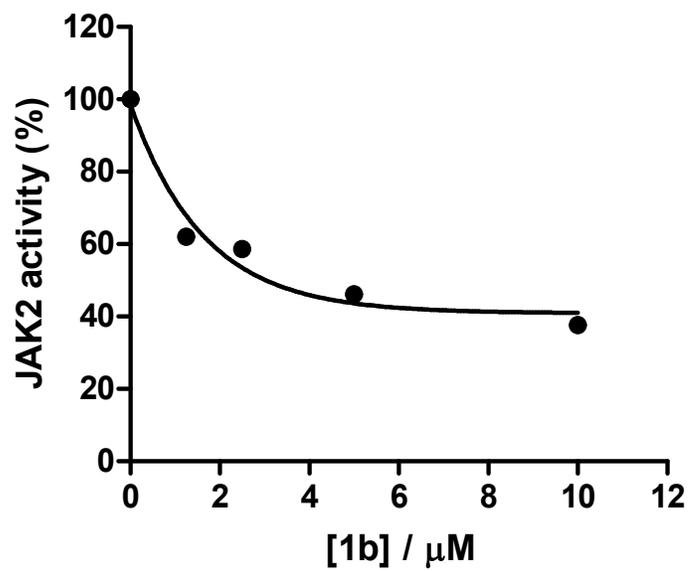


Fig. S5 Western blot analysis of the effect of compounds on JAK2 autophosphorylation *in cellulo*. HEL cells were incubated with the compounds (5 μ M) for 16 h and protein lysates were electrophoresed and immunoblotted using anti-phospho-JAK2 Y1007/Y1008 antibody (upper panel) or anti-JAK2 antibody (middle panel). Equal protein loading was confirmed by total β -actin levels (lower panel). Results are representative of three independent experiments.

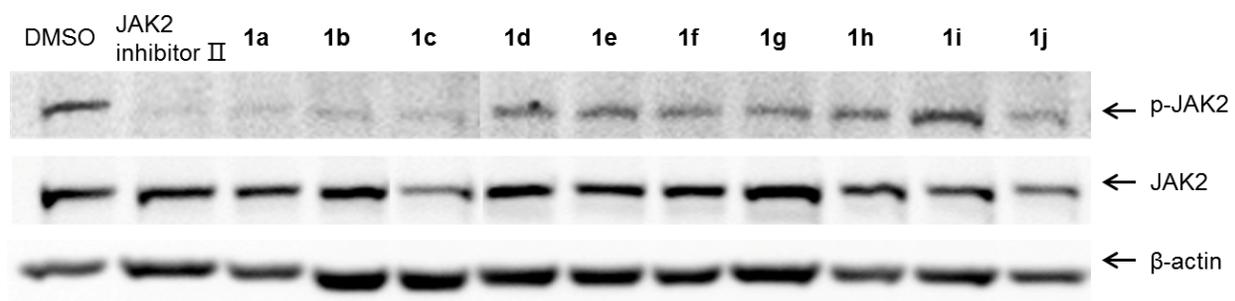
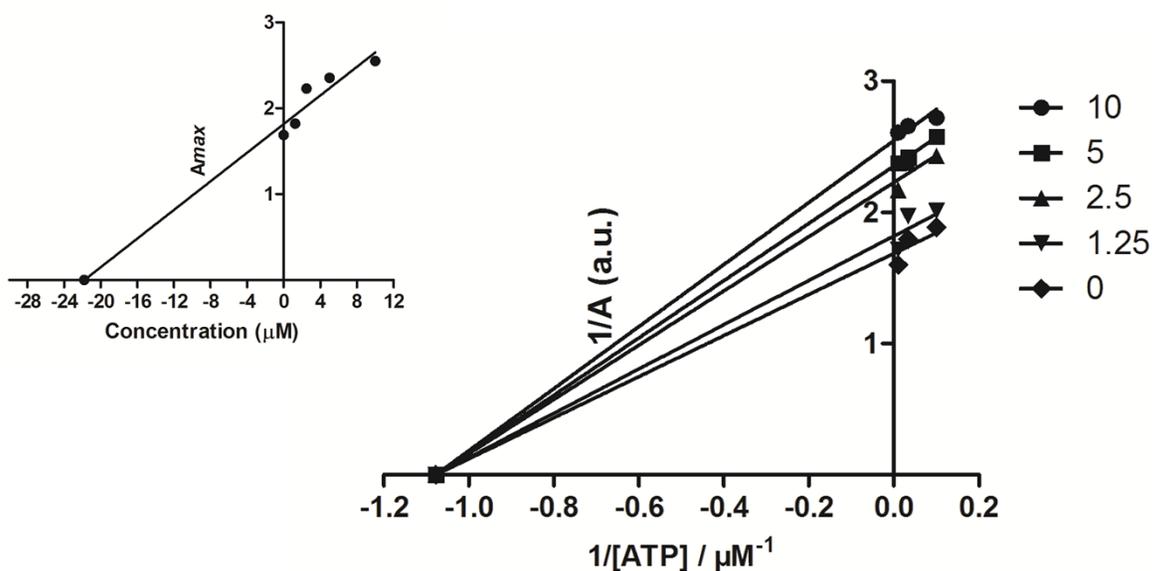


Fig. S6 Lineweaver-Burk analysis of JAK2 inhibition by NVP-BBT594 using ELISA. Recombinant JAK2 was incubated with the indicated concentrations of NVP-BBT594 and biotinylated peptide substrate at room temperature for 30 min. Peptide phosphorylation was detected with anti-phosphotyrosine primary and horseradish peroxidase-conjugated secondary antibody. Inset: Dixon analysis to estimate the K_i value. Results are representative of three independent experiments.



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