Supporting Information

Selective inhibitors of Janus kinase 3 modify responses to lipopolysaccharides by increasing the Interleukin-10 to Tumor Necrosis Factor Alpha ratio

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Contents of Supporting Information

Synthesis of 4

General chemistry

Unless stated otherwise, solvents, reagents and other materials were of commercial quality and used without further purification. Flash chromatography was performed using an Interchim PuriFlash 5.020 automated flash chromatography system with pre-packed Interchim columns containing either 15 µm or 50 µm silica. Gradients for flash chromatography were calculated automatically via the accompanying "TLC to Flash and Prep Chromatography" software. TLC was performed using Merck TLC Silica gel 60 F_{254} plates. For detection, we used UV light at 254 nm or cerium molybdate staining solution. The purity and t_{ret} of intermediates and final compounds were determined on a Varian ProStar210 system coupled with a SEDEX LT-ELSD 80 LT and using a Dr. Maisch ReproSil-Pur120 C18-Aq column (75 x 3 mm, 5 µm). The mobile phase was composed of water containing 0.05 % formic acid (eluent A) and methanol containing 0.05 % formic acid (eluent B). Two different gradients were used depending on the analyte: 20 % B for 5 min, to 100 % B in 20 min, 100 % B for 4 min, to 20 % B in 1 min, 20 % B for 5 min (method A), or: 5 % B for 5 min, to 100 % B in 20 min, 100 % B for 4 min, to 5 % B in 1 min, 5 % B for 5 min (method B). The flow rate for both methods was 1.3 ml/min. Nitrogen gas was used for the nebulization and evaporation of the mobile phase. Pressure was set at 3.3 bar and the drift tube temperature of the ELSD was 75 °C. NMR spectra were either recorded on a Bruker Avance 400 Mhz, a Bruker Avance III HDX 400 Mhz or a Bruker Avance III 300 Mhz. Chemical shifts are reported in ppm relative to TMS and calibrated against the residual proton peak of the respective solvent. Standard mass spectra were obtained as ESI-MS (pos. mode) from a Thermo Finnigan LCQ Deca XP system (settings: ESI voltage 3.0 kV, capillary voltage 9 V, capillary temperature 275 °C, gas flow 7 l/min).

HRMS measurements were made using a Bruker maXis 4G ESI-TOF from Daltonik at the Institute of Organic Chemistry, Eberhard-Karls-University Tuebingen, using ESI+ mode and the following settings: Capillary voltage 4.5 kV, source temperature 200 °C, gas flow 6 l/min, nebulizer gas pressure 1.2 bar, end plate offset – 0.5 kV and an *m/z* range of 80 to 1350 *m/z*. All final compounds show ≥ 95 % purity according to analytical HPLC. In case of *E/Z* mixtures obtained from Knoevenagel condensations, purity is calculated from the sum of both isomer peak areas.

*Synthesis of benzyl (2-((2-(((2S,3S,4R,6R)-2-(((2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10-trihydroxy-13-(((2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-3,5,6,8,10,12,14-heptamethyl-15-oxo-1-oxa-6-azacyclopentadecan-11-yl)oxy)-4-hydroxy-6-methyltetrahydro-2H-pyran-3-yl)(methyl)amino)ethyl)(methyl)amino)-2-oxoethyl)(methyl)carbamate (**9**):*

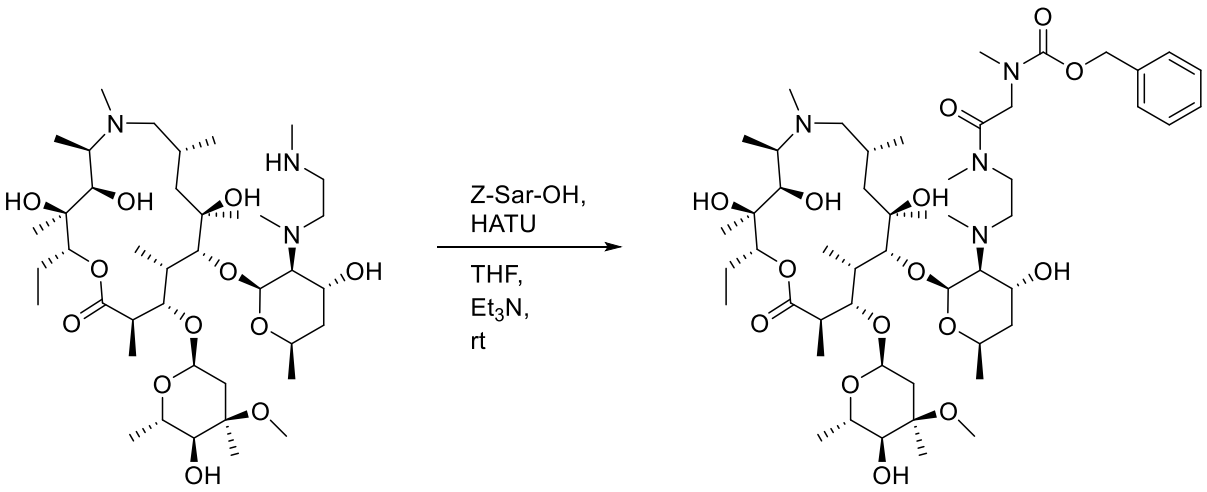

Figure S1. Synthesis of intermediate compound **9**.

579 mg (2.59 mmol, 1.05 equiv.) of Z-Sar-OH, 986 mg (2.59 mmol, 1.05 equiv) of HATU and 361 µl (2.59 mmol, 1.05 equiv) of triethylamine were dissolved in 13 ml of dry THF and stirred for 1 h at ambient temperature. Then, 1.96 g (2.47 mmol, 1.0 equiv) of (2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10-trihydroxy-13-(((2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-11-(((2S,3S,4R,6R)-4-hydroxy-6-methyl-3-(methyl(2-(methylamino)ethyl)amino)tetrahydro-2H-pyran-2-yl)oxy)-3,5,6,8,10,12,14-heptamethyl-1-oxa-6-azacyclopentadecan-15-one[1] were added and stirring was continued overnight. The solution was concentrated under reduced pressure, taken up in DCM and sat. $NaHCO_3$ solution and extracted several times with DCM. The organic phase was dried over Na_2SO_4, evaporated under reduced pressure and the crude product was purified by flash chromatography (EtOAc/MeOH, automatic gradient). Yield: 1.81 g (73 %) as beige foam. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.25 (m, 5H), 5.11 – 5.04 (m, *J* = 4.7 Hz, 3H), 4.96 – 4.88 (m, *J* = 15.1, 4.4 Hz, 1H), 4.67 (dd, *J* = 9.9, 2.1 Hz, 1H), 4.32 – 4.27 (m, *J* = 1.4 Hz, 1H), 4.09 – 4.04 (m, *J* = 9.9, 4.6 Hz, 2H), 4.02 (d, *J* = 4.2 Hz, 1H), 3.94 – 3.82 (m, *J* = 12.5, 9.1 Hz, 2H), 3.80 – 3.72 (m, 1H), 3.64 – 3.56 (m, 2H), 3.31 – 3.26 (m, 3H), 3.22 (s, 1H), 3.16 – 3.01 (m, 2H), 2.99 – 2.95 (m, *J* = 7.4 Hz, 5H), 2.91

(s, 1H), 2.89 (d, *J* = 5.4 Hz, 1H), 2.71 – 2.61 (m, *J* = 6.9, 4.1 Hz, 3H), 2.55 (d, *J* = 4.4 Hz, 2H), 2.53 – 2.49 (m, *J* = 12.7 Hz, 1H), 2.47 – 2.41 (m, *J* = 9.3 Hz, 1H), 2.31 (s, 1H), 2.29 (s, 3H), 2.03 – 1.97 (m, *J* = 9.7 Hz, 2H), 1.92 – 1.82 (m, 2H), 1.76 (dd, *J* = 14.6, 6.8 Hz, 1H), 1.58 – 1.51 (m, *J* = 6.2 Hz, 2H), 1.50 – 1.38 (m, 2H), 1.33 – 1.27 (m, 6H), 1.21 (t, *J* = 7.1 Hz, 2H), 1.17 (d, *J* = 2.9 Hz, 3H), 1.14 (d, *J* = 5.8 Hz, 5H), 1.07 – 1.02 (m, *J* = 9.0 Hz, 6H), 0.92 – 0.82 (m, *J* = 14.9, 7.5 Hz, 9H). ^{13}C-NMR (101 MHz, $CDCl_3$) δ 178.74, 168.39, 168.11, 157.02, 136.74, 128.48, 127.95, 127.72, 100.44, 95.04, 94.87, 78.23, 77.92, 74.31, 74.12, 74.00, 73.75, 72.88, 72.78, 71.14, 70.57, 70.08, 67.40, 67.21, 66.34, 65.70, 64.47, 62.60, 60.36, 54.89, 50.96, 50.52, 49.29, 48.47, 47.04, 45.55, 42.94, 42.24, 42.05, 41.84, 40.80, 38.62, 36.95, 36.33, 35.67, 35.51, 35.23, 34.84, 34.02, 27.74, 26.71, 22.18, 21.71, 21.55, 21.30, 21.00, 18.30, 16.34, 15.02, 14.85, 14.22, 11.22, 9.19, 9.04, 7.39. MS (ESI) m/z: 997.87 $[M + H]^+$; HPLC t_{ret} = 14.3 min (method B).

*Synthesis of N-(2-(((2S,3S,4R,6R)-2-(((2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10-trihydroxy-13-(((2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-3,5,6,8,10,12,14-heptamethyl-15-oxo-1-oxa-6-azacyclopentadecan-11-yl)oxy)-4-hydroxy-6-methyltetrahydro-2H-pyran-3-yl)(methyl)amino)ethyl)-N-methyl-2-(methylamino)acetamide (**10**):*

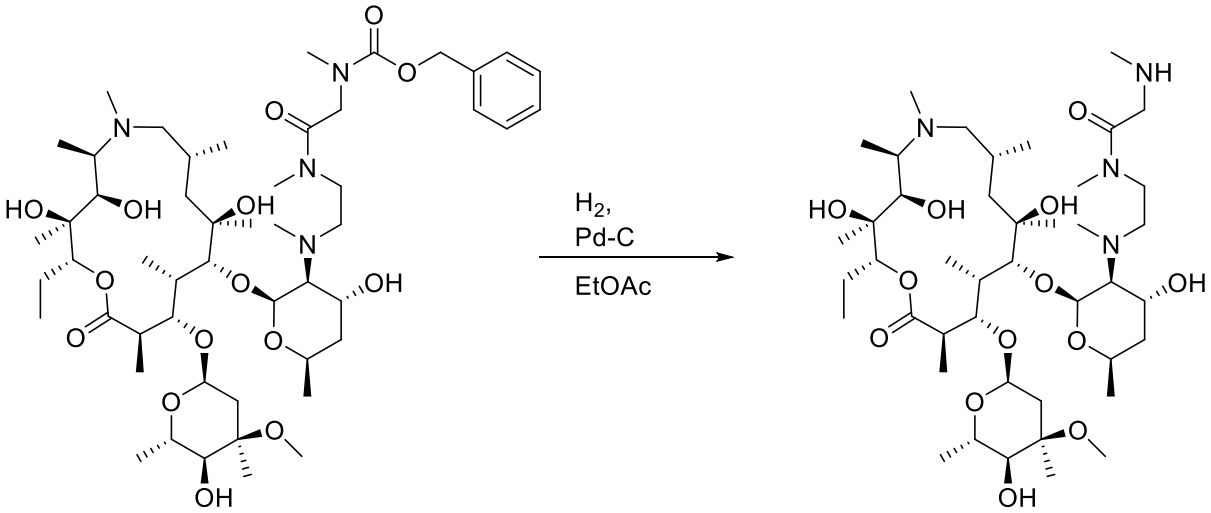

Figure S2. Synthesis of intermediate compound **10**.

1.5 g (1.5 mmol, 1.0 equiv.) of benzyl (2-((2-(((2S,3S,4R,6R)-2-(((2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10-trihydroxy-13-(((2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-3,5,6,8,10,12,14-heptamethyl-15-oxo-1-oxa-6-azacyclopentadecan-11-yl)oxy)-4-hydroxy-6-methyltetrahydro-2H-pyran-3-

yl)(methyl)amino)ethyl)(methyl)amino)-2-oxoethyl)(methyl)carbamate (**9**) were dissolved in 45 ml of EtOAc and stirred. The mixture was purged with argon before addition of 150 mg of Pd/C (3 %), then purged with H_2 and left to stir overnight. Leftover hydrogen gas was removed from the closed apparatus by another argon purge, then the mixture was filtrated over a Celite pad to remove solids. As impurities were detected, the crude was subjected to flash chromatography (EtOAc/MeOH, automatic gradient). Yield: 630 mg (49 %) as white solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.98 (t, *J* = 5.2 Hz, 1H), 4.95 – 4.92 (m, 1H), 4.65 (d, *J* = 9.1 Hz, 1H), 4.29 (d, *J* = 5.0 Hz, 1H), 4.12 – 3.95 (m, 2H), 3.92 – 3.83 (m, 2H), 3.66 – 3.56 (m, 3H), 3.41 (d, *J* = 6.8 Hz, 1H), 3.39 (s, 1H), 3.33 (d, *J* = 2.1 Hz, 1H), 3.28 (d, *J* = 3.5 Hz, 3H), 3.25 – 3.11 (m, 3H), 2.96 (dd, *J* = 9.3, 4.2 Hz, 1H), 2.92 (d, *J* = 2.4 Hz, 3H), 2.87 – 2.79 (m, 1H), 2.72 (dd, *J* = 14.4, 7.2 Hz, 3H), 2.67 – 2.59 (m, 2H), 2.53 (d, *J* = 8.3 Hz, 3H), 2.48 (d, *J* = 10.6 Hz, 1H), 2.39 (d, *J* = 4.2 Hz, 3H), 2.30 (s, 1H), 2.28 (s, 3H), 2.03 – 1.94 (m, 2H), 1.89 – 1.81 (m, 2H), 1.76 (dd, *J* = 14.5, 5.9 Hz, 1H), 1.60 – 1.39 (m, 4H), 1.30 (s, 3H), 1.26 (d, *J* = 6.2 Hz, 3H), 1.24 – 1.19 (m, 1H), 1.17 (s, 3H), 1.15 (s, 2H), 1.14 (s, 3H), 1.13 – 1.11 (m, 2H), 1.07 – 1.00 (m, 6H), 0.91 – 0.82 (m, 9H). ^{13}C-NMR (101 MHz, $CDCl_3$) δ 178.56, 178.51, 170.79, 170.61, 100.50, 100.40, 95.37, 95.26, 85.76, 85.27, 78.56, 78.35, 78.21, 78.13, 74.41, 73.68, 73.60, 73.01, 72.94, 70.70, 70.49, 70.19, 66.41, 66.31, 65.79, 65.72, 64.75, 64.12, 63.35, 62.37, 54.60, 54.48, 52.28, 51.68, 49.36, 48.40, 46.70, 46.16, 45.46, 43.10, 41.95, 41.74, 41.59, 41.02, 36.83, 36.58, 36.49, 35.07, 34.88, 33.98, 27.68, 26.77, 25.45, 25.27, 22.26, 22.20, 21.75, 21.60, 21.25, 21.22, 18.39, 18.35, 16.42, 15.44, 15.33, 11.24, 10.45, 9.50, 9.44, 7.63. MS (ESI) m/z: 863.62 $[M + H]^+$; HPLC t_{ret} = 10.1 min (method B).

*Synthesis of 2-cyano-N-(2-((2-(((2S,3S,4R,6R)-2-(((2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10-trihydroxy-13-(((2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-3,5,6,8,10,12,14-heptamethyl-15-oxo-1-oxa-6-azacyclopentadecan-11-yl)oxy)-4-hydroxy-6-methyltetrahydro-2H-pyran-3-yl)(methyl)amino)ethyl)(methyl)amino)-2-oxoethyl)-N-methylacetamide (**11**):*

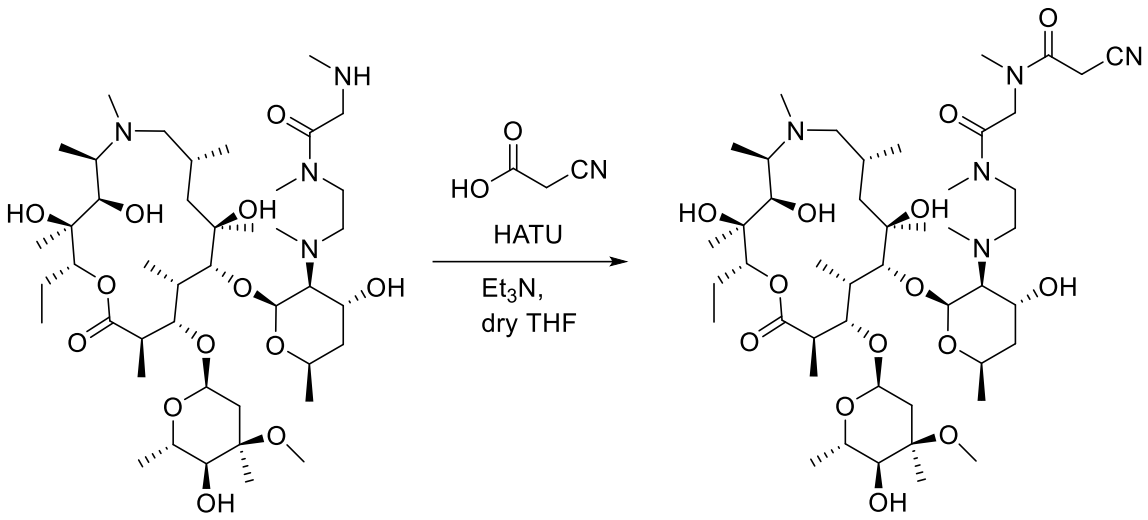

Figure S3. Synthesis of intermediate compound **11**.

53 mg (0.62 mmol, 1.0 equiv) of cyanoacetic acid, 247 mg (0.65 mmol, 1.05 equiv.) of HATU and 91 µl triethylamine (0.65 mmol, 1.05 equiv.) were dissolved in 8 ml dry THF and stirred at ambient temperature for 20 min. Then, 535 mg (0.62 mmol, 1.0 equiv.) of N-(2-(((2S,3S,4R,6R)-2-(((2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10-trihydroxy-13-(((2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-3,5,6,8,10,12,14-heptamethyl-15-oxo-1-oxa-6-azacyclopentadecan-11-yl)oxy)-4-hydroxy-6-methyltetrahydro-2H-pyran-3-yl)(methyl)amino)ethyl)-N-methyl-2-(methylamino)acetamide (**10**), dissolved in a few ml of dry THF, were added to the mixture and left to stir overnight. The mixture was concentrated under reduced pressure, taken up in DCM and half-saturated $NaHCO_3$ solution and extracted four times with DCM. The combined organic phases were dried over Na_2SO_4 and evaporated under reduced pressure. HPLC control indicated that no further purification steps were necessary before the next step. Yield: 460 mg (80 %) as off-white solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.06 – 4.99 (m, 1H), 4.93 (dd, *J* = 10.9, 2.3 Hz, 1H), 4.66 (d, *J* = 9.7 Hz, 1H), 4.31 – 4.25 (m, 1H), 4.15 – 4.01 (m, *J* = 15.3, 14.0, 7.2 Hz, 2H), 3.94 – 3.84 (m, 2H), 3.67 – 3.54 (m, 4H), 3.51 – 3.43 (m, 1H), 3.29 (d, *J* = 6.3 Hz, 3H), 3.25 – 3.11 (m, *J* = 18.5, 16.3, 4.8 Hz, 2H), 3.07 (d, *J* = 6.3 Hz, 3H), 3.01 – 2.96 (m, 3H), 2.95 – 2.90 (m, 3H), 2.73 – 2.66 (m, *J* = 12.5, 7.0 Hz, 2H), 2.65 – 2.60 (m, *J* = 8.7 Hz, 1H), 2.59 – 2.56 (m, *J* = 5.8 Hz, 1H), 2.53 (d, *J* = 7.2 Hz, 3H), 2.37 – 2.32 (m, 1H), 2.29 (s, 3H), 2.10 – 1.93 (m, *J* = 26.0, 13.9 Hz, 2H), 1.91 – 1.80 (m, 2H), 1.78 – 1.70 (m, *J* = 14.2, 7.0 Hz, 1H), 1.60 – 1.40 (m, 4H), 1.31 (s, 3H), 1.27 (d, *J* = 6.2 Hz, 3H), 1.23 – 1.20 (m, 1H), 1.18 – 1.11 (m, 9H), 1.09 – 1.02 (m, 6H), 0.92 – 0.81 (m, *J* = 11.6, 7.4 Hz, 9H). ^{13}C-NMR (101 MHz,

$CDCl_3$) δ 178.68, 167.31, 166.93, 165.81, 163.44, 162.86, 125.53, 114.01, 113.89, 100.55, 100.40, 95.27, 95.12, 85.61, 84.84, 78.21, 78.11, 74.41, 73.77, 73.66, 72.93, 72.82, 71.00, 70.48, 69.99, 66.52, 66.35, 65.81, 65.72, 64.88, 63.46, 62.52, 54.91, 54.52, 50.14, 49.65, 49.40, 49.35, 48.66, 47.21, 45.48, 42.99, 42.03, 41.93, 41.69, 40.73, 38.65, 37.49, 37.44, 36.87, 36.54, 35.48, 34.96, 34.18, 31.29, 30.41, 27.66, 26.68, 25.06, 22.25, 22.19, 21.74, 21.57, 21.21, 18.38, 18.33, 16.40, 15.29, 15.17, 11.21, 9.40, 9.25, 7.64. MS (ESI) m/z: 930.73 $[M + H]^+$; HPLC t_{ret} = 12.1 min (method B).

*Synthesis of (E)-2-cyano-3-(5-(1-cyclohexyl-1,6-dihydroimidazo[4,5-d]pyrrolo[2,3-b]pyridin-2-yl)furan-2-yl)-N-(2-((2-(((2S,3S,4R,6R)-2-(((2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10-trihydroxy-13-(((2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-3,5,6,8,10,12,14-heptamethyl-15-oxo-1-oxa-6-azacyclopentadecan-11-yl)oxy)-4-hydroxy-6-methyltetrahydro-2H-pyran-3-yl)(methyl)amino)ethyl)(methyl)amino)-2-oxoethyl)-N-methylacrylamide (**4**):*

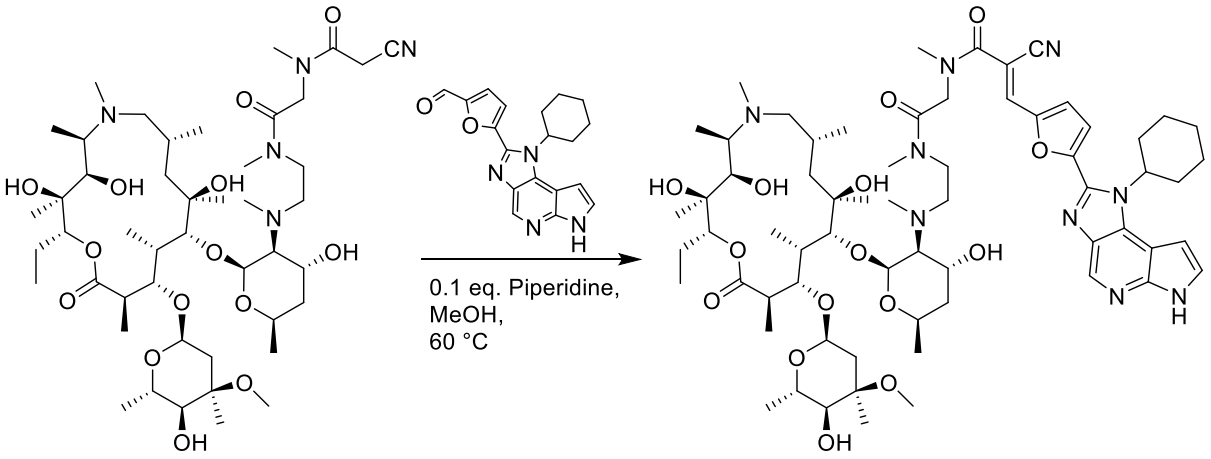

Figure S4. Synthesis of JAK3 inhibitor compound **4**.

70 mg (0.209 mmol, 1.0 equiv.) of 5-(1-cyclohexyl-1,6-dihydroimidazo[4,5-d]pyrrolo[2,3-b]pyridin-2-yl)furan-2-carbaldehyde[1], 195 mg (0.209 mmol, 1.0 equiv.) of 2-cyano-N-(2-((2-(((2S,3S,4R,6R)-2-(((2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10-trihydroxy-13-(((2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-3,5,6,8,10,12,14-heptamethyl-15-oxo-1-oxa-6-azacyclopentadecan-11-yl)oxy)-4-hydroxy-6-methyltetrahydro-2H-pyran-3-yl)(methyl)amino)ethyl)(methyl)amino)-2-oxoethyl)-N-methylacetamide (**11**) and 2.1 µl (0.021 mmol, 0.1 equiv.) of triethylamine were suspended in 1.5 ml of MeOH and stirred at 60 °C oil bath temperature

for 2 h, at which point TLC and MS indicated full consumption of starting materials. The crude product was purified by flash chromatography (EtOAc/MeOH, automatic gradient). Yield: 177 mg (68 %) as yellow solid. ^{1}H-NMR (700 MHz, $CDCl_3$) δ 11.32 (bs, 1H), 9.07 (bs, 1H), 8.75 (s, 1H), 7.70 (s, 1H), 7.40 (d, *J* = 21.5 Hz, 2H), 7.22 (d, *J* = 15.7 Hz, 1H), 6.81 (s, 1H), 5.07 – 4.98 (m, 2H), 4.95 – 4.88 (m, *J* = 21.3 Hz, 2H), 4.67 (bs, 1H), 4.34 – 4.20 (m, 2H), 4.09 – 4.03 (m, 1H), 3.95 – 3.83 (m, *J* = 46.4 Hz, 2H), 3.69 (bs, *J* = 35.1 Hz, 1H), 3.62 (s, 1H), 3.58 (bs, 1H), 3.26 (s, 6H), 3.15 (d, *J* = 8.0 Hz, 2H), 3.00 – 2.91 (m, 6H), 2.66 (bs, 3H), 2.55 – 2.47 (m, *J* = 22.3, 12.1 Hz, 4H), 2.44 – 2.38 (m, 2H), 2.30 – 2.24 (m, 4H), 2.03 – 1.93 (m, *J* = 19.3, 12.6 Hz, 6H), 1.87 – 1.73 (m, 4H), 1.55 – 1.44 (m, *J* = 29.1, 11.9 Hz, 6H), 1.34 (s, 1H), 1.32 – 1.23 (m, *J* = 29.7 Hz, 9H), 1.14 – 1.09 (m, 9H), 1.02 (s, 6H), 0.89 – 0.77 (m, 10H). ^{13}C-NMR (151 MHz, $CDCl_3$) δ 178.78, 178.69, 167.21, 166.95, 164.26, 164.18, 150.04, 149.56, 144.82, 141.39, 138.10, 136.90, 135.83, 134.06, 123.44, 121.15, 116.35, 115.83, 105.16, 102.10, 102.00, 101.33, 100.67, 100.54, 95.10, 94.95, 85.54, 84.68, 78.18, 78.13, 77.71, 74.33, 74.09, 73.86, 73.74, 72.96, 72.82, 70.51, 69.95, 67.16, 66.56, 66.46, 65.77, 65.73, 64.97, 62.74, 62.66, 57.15, 54.68, 54.48, 50.78, 50.34, 49.39, 48.57, 46.88, 46.11, 45.52, 42.94, 42.85, 42.13, 41.94, 40.93, 38.73, 36.94, 36.77, 36.40, 34.86, 34.81, 34.14, 30.62, 29.76, 27.79, 26.99, 26.68, 25.73, 24.89, 22.32, 22.26, 21.78, 21.60, 21.28, 18.36, 18.33, 16.44, 15.19, 15.02, 11.25, 9.33, 9.14, 7.52. ESI-HRMS: $[M + H]^+$ calculated for $C_{65}H_{99}N_9O_{15}$: 1246.73334, found 1246.73426; HPLC t_{ret} = 15.3 and 15.4 min (*E*/Z mixture, method B).

Pharmacokinetics of 4

Experimental animals

All animal experiments were carried out in accordance with German law. Seven to eight weeks old BALB/cJ female mice were purchased from Janvier Labs and maintained in our dedicated specific-pathogen-free (SPF) animal facility. They were kept for seven days after arrival for acclimatization. To reduce the number of animals per pharmacokinetics experiment, compounds were given as cassettes of three to five substances per animal, and *n* was chosen to be 3 per group. Body weights were approximately 20 g per mouse, with each mouse being weighed right before treatment to ensure equal doses for all animals.

Formulations for pharmacokinetic studies

Treatment solutions were freshly prepared before the start of each study. For i.v. treatment, compounds were dissolved in DMSO and then diluted 10-fold in BALB/c female serum (final DMSO concentration 10 %) for application at 5 ml/kg to reach a dose of 2.4 µmol/kg per substance. The vehicle for p.o. treatment comprised a solution in DMSO that was diluted 10-fold in 0.5 % citric acid (final DMSO concentration 10 %) with each compound being administered at a dose of 12 µmol/kg. The solutions were thoroughly homogenized via vortex mixer and ultrasonic bath.

Collection of samples

To measure drug concentrations in plasma, mice were bled from the tail vein at eight time points after treatment. Animals were sacrificed after 8 h by CO_2 inhalation. We collected heart blood, bile, brain, ileum, liver, lung, kidneys and spleen. Blood was collected in heparinized tubes and centrifuged for 8 min at 8,000 rpm at 4 °C. The supernatant was used to determine plasma concentrations. Both plasma and organ samples were immediately stored at -25 °C until workup for analytics.

Sample workup for HPLC-MS

Plasma samples were diluted with ACN containing 5 nM sulfentrazone and 1 nM terbuthylazine (used as negative and positive internal standards respectively), homogenized in a FastPrep FP-120 instrument and then centrifuged at 14,000 rpm for 7 min at 4° C. Organ samples were treated with 1 µl proteinase K solution (0.5 mg/ml in 20mM phosphate buffer) per mg organ weight and then processed analogously to the plasma samples. Bile samples were diluted with deionized water, homogenized and then further diluted with ACN plus internal standards, followed by homogenization and centrifugation.

Sample analysis

Compound concentrations were measured using reverse-phase HPLC with MS detection. The procedure used a mobile phase comprised of 0.1 % formic acid in water (solvent A) and 0.1 % formic acid in acetonitrile

(solvent B). Method: 10 % B for 1 min, to 100 % B in 4 min, 100 % B for 2 min, to 10 % B in 1 min, 10 % B for 2 min, stop time 10 min, flow rate 500 µl/min, injection volume 6 µl. Using a thermostat, a constant column temperature of 45 °C was maintained, while the samples were kept at 6 °C.

Calculation of plasma half-lives

Half-lives of compounds in mice were calculated using the plasma concentrations measured with the aforementioned methods. To reduce the influence of distribution processes, plasma concentrations before t = 15 min were not included. Values outside the quantification limits were disregarded for the calculation of the elimination constants and resulting half-lives.

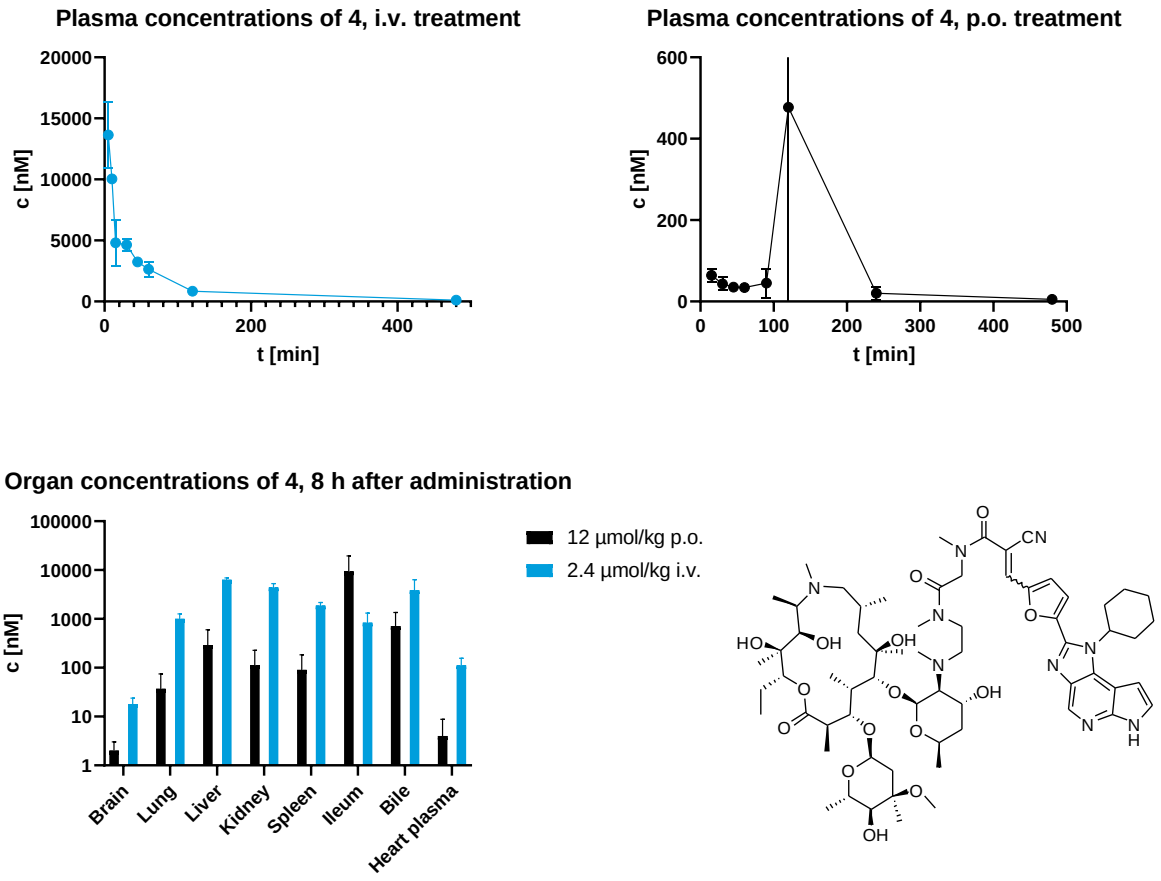

Figure S5. Plasma (top) and organ (bottom) concentrations ± standard deviation of **4** from pharmacokinetic studies in Balb/c female mice (n = 3).

The plasma half-life of **4** in mice was determined to be 88 min. Apparent oral bioavailability, calculated from the AUC values of plasma curves, was 2 %.

Plasma concentrations of 4 and 7 in the i.v. dose response study

Plasma was collected, worked up and compound concentrations in samples were determined by HPLC-MS as described above. The concentrations of compound **7** could not accurately be determined, as they were below the lower limit of quantification in all groups.

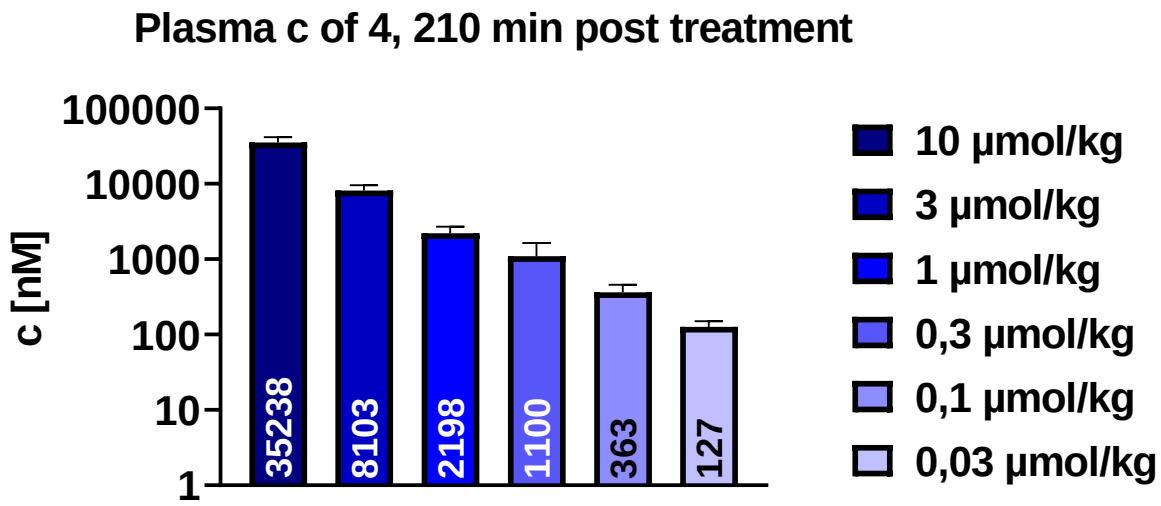

Figure S6. Heart plasma concentrations ± standard deviation of **4** from the dose-response study (n = 6). Plasma was taken 210 min after i.v. treatment. **7** is not depicted, as plasma concentrations in all groups were below the lower limit of quantification.

JAK3 inhibition assays

To determine JAK3 IC_{50} values, assays were as described previously[2]. A 96-well plate was fitted with artificial, tyrosine-rich peptides to act as phosphorylation targets. A fragment (amino acids 781 to 1124) of human JAK3 containing the active site was incubated with 1.4 µM ATP (twice the K_m value), leading to phosphorylation of the peptide substrate. By addition of a horseradish peroxidase-conjugated phosphotyrosine antibody followed by 3,3',5,5'-tetra-methylbenzidine, color development proportional to bound antibody was observed. The reaction was stopped after a set time via sulphuric acid and the optical density (OD) was determined at 450 nm. Through inclusion of potential inhibitors to the incubation step at varying concentrations, their inhibitory potencies could be determined by comparison of the resulting OD_{450} values to those of control reactions.

Table S1. JAK3 IC_{50} values of JAK3 inhibitors used in this work

Compound	JAK3 IC_{50} (nM)[a]
1	99 ± 6
2	122 ± 18
3	192 ± 12
4	39 ± 2
8	12 ± 1

IC_{50} values are calculated by ELISA.[2] [a]average ± SEM (n = 3).

JAK selectivity of 1 and 2

The JAK selectivity of **1** and **2** was assessed at Reaction Biology Europe, using a radiometric assay based on ^{33}P-ATP. Previously, selectivity data for **8** and related compounds were reported using a similar setup.[3]

Table S2. IC_{50} values of **1** and **2** for the four JAK family enzymes.

	IC_{50} [nM][a]			
No.	**JAK1**	**JAK2**	**JAK3**	**TYK2**
1	541 ± 101	5116 ± 86	12 ± 0,3	> 10 µM
2	> 10 µM	1965 ± 30	16 ± 1,5	9647 ± 0

[a]Obtained from a radiometric assay at Reaction Biology Europe, using a ten-point serial dilution (10 µM to 0,3 nM) and duplicate measurements. [ATP] = 1 µM (JAK1), 0,3 µM (JAK2) or 0,1 µM (JAK3, TYK2).

References

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(2) Bauer, S. M.; Gehringer, M.; Laufer, S. A. A Direct Enzyme-Linked Immunosorbent Assay (ELISA) for the Quantitative Evaluation of Janus Kinase 3 (JAK3) Inhibitors. **2014**, 8817–8822. https://doi.org/10.1039/c4ay01589d.

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