CHEMBIOCHEM

Supporting Information

CHEMBIOCHEM

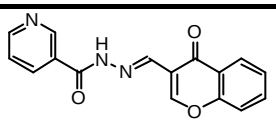
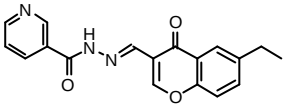
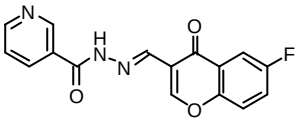
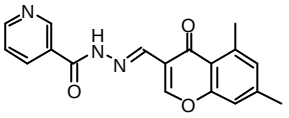
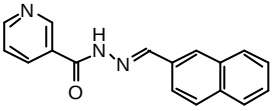
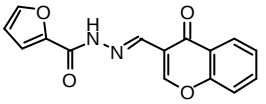
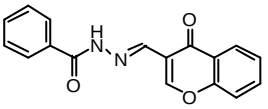
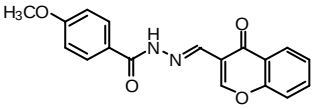
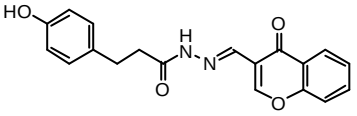
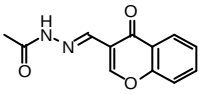
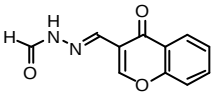
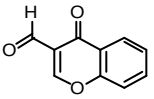
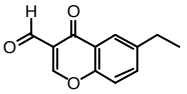
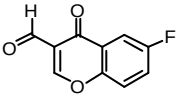
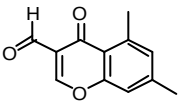
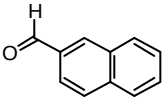
Supporting Information

for

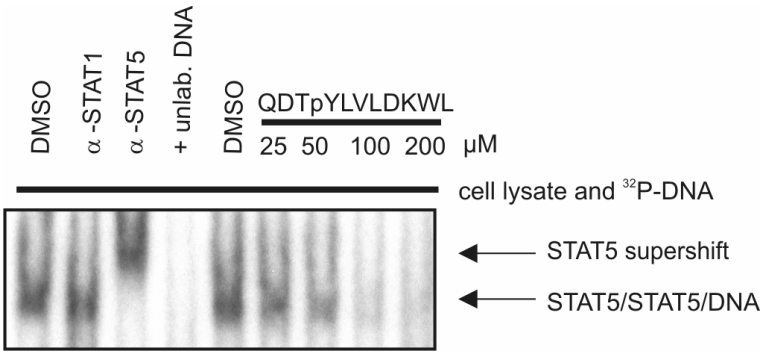
Discovery of Chromone-Based Inhibitors of the Transcription Factor STAT5

Judith Müller, Bianca Sperl, Wolfgang Reindl, Anke Kiessling, and Thorsten Berg*

Table S1. Overview on the origin of the test compounds.

No.	Structure	Synthesized (S) or purchased (P)	If purchased: supplier and compound code
1		S, P	Maybridge RF 00099
2		S	
3		S	
4		S	
5		S	
6		P	Maybridge RF 00098
7		S	
8		P	Maybridge RF 00103
9		P	Maybridge RF 00102
10		S	
11		P	Maybridge RF 00096
12		P	Sigma-Aldrich 383449
13		P	Sigma-Aldrich 402133
14		P	Sigma-Aldrich 543624
15		P	Chempur 12012
16		P	Sigma-Aldrich N20-6

A)



B)

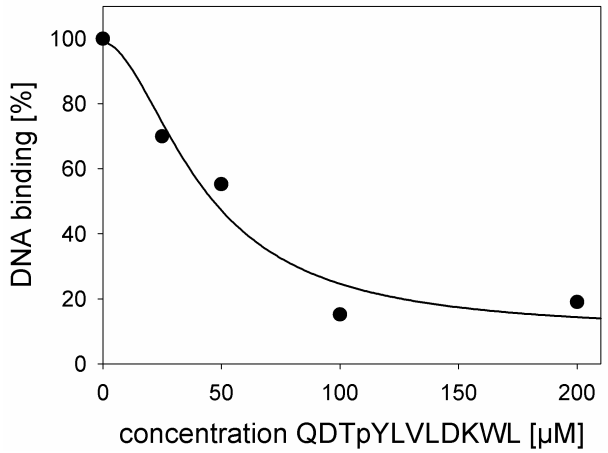


Figure S1. Electrophoretic mobility shift assay (EMSA) with a known high-affinity peptide ligand to the STAT5 SH2 domain. A) Lysates of K562 cells, which contain activated, i.e. tyrosine phosphorylated, STAT5 were incubated with the high-affinity STAT5 SH2 domain ligand QDTpYLVLDKWL at the indicated concentration or DMSO for 1 h. Subsequently, a ^{32}P-labeled, double-stranded oligonucleotide comprising the STAT5 binding motif was added, and the mixture was separated on a non-denaturing polyacrylamide gel. The identity of the STAT5/STAT5/DNA band is confirmed by a) its disappearance in the presence of an excess of unlabeled DNA (lane 4), b) its shift to a higher molecular weight in the presence of an antibody to STAT5 (lane 3), c) but not in the presence of an antibody to STAT1 (lane 2). Lanes 1 and 5 both contain DMSO instead of the peptide (final DMSO concentration in any case: 10%). In the presence of the peptide QDTpYLVLDKWL a dose-dependent decrease in DNA-binding activity of STAT5 is observed. B) Quantification of the EMSA shown in A).

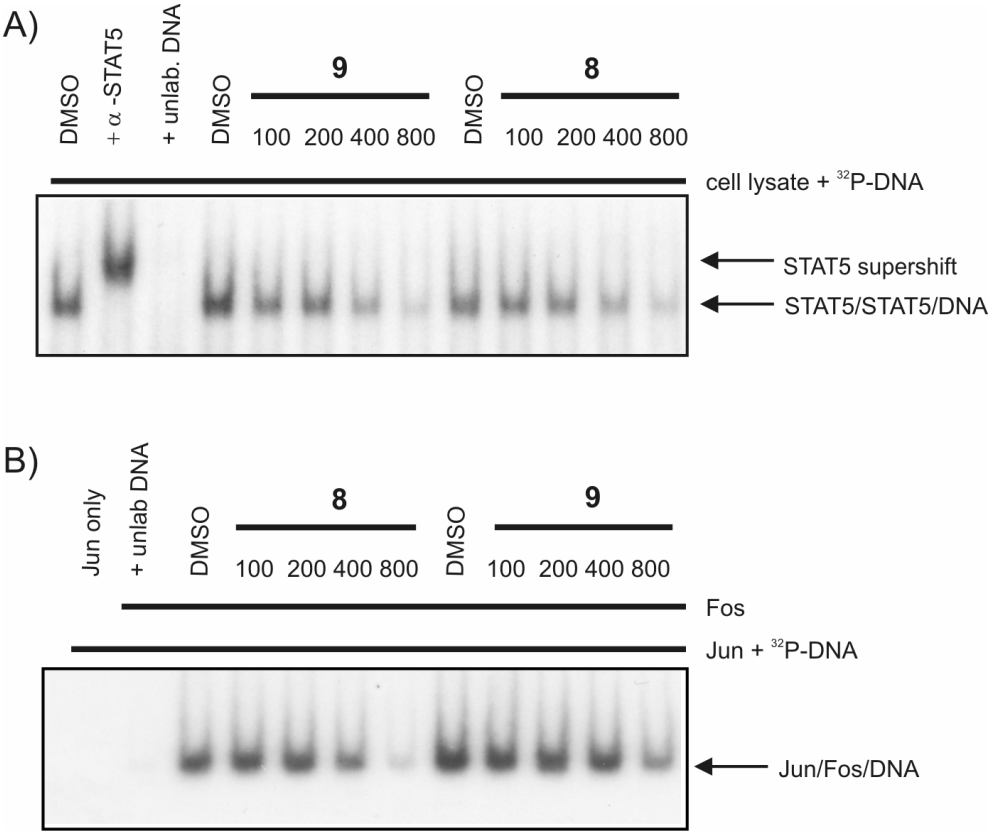


Figure S2. Effect of acyl hydrazones 8 and 9 on DNA binding of STAT5 and Jun/Fos as analyzed by electrophoretic mobility shift assay (EMSA). A) Effect of acyl hydrazones **8** and **9** on DNA binding of STAT5. Lysates of K562 cells, which contain activated, i.e. tyrosine phosphorylated, STAT5 were incubated with the test compounds at the indicated concentrations for 1 h. Subsequently, a ^{32}P-labeled oligonucleotide comprising the STAT5 binding site was added, and the mixtures were separated on a non-denaturing polyacrylamide gel. An antibody against STAT5 in the second lane increases the molecular weight of the STAT5/STAT5/DNA complex and thus causes a reduced mobility of the complex, thereby confirming the identity of the STAT5/STAT5/DNA band. An excess of unlabeled DNA in the third lane competes with the ^{32}P-labeled DNA for the proteins, and thereby abolishes the signal. Both compounds **8** and **9** decrease the concentration of the STAT/STAT5/DNA complex in a dose-dependent manner. B) DNA binding of Jun/Fos is partially inhibited by **8** and **9** in EMSA. All lanes contain ^{32}P-labeled DNA comprising the Jun/Fos binding site, Jun, and Fos, except for the first lane, which lacks Fos and thereby excludes the possibility that Jun/Jun homodimers are bound to DNA. Fos cannot bind DNA on its own. The second lane contains an excess of unlabeled competitor DNA to verify the identity of the band.

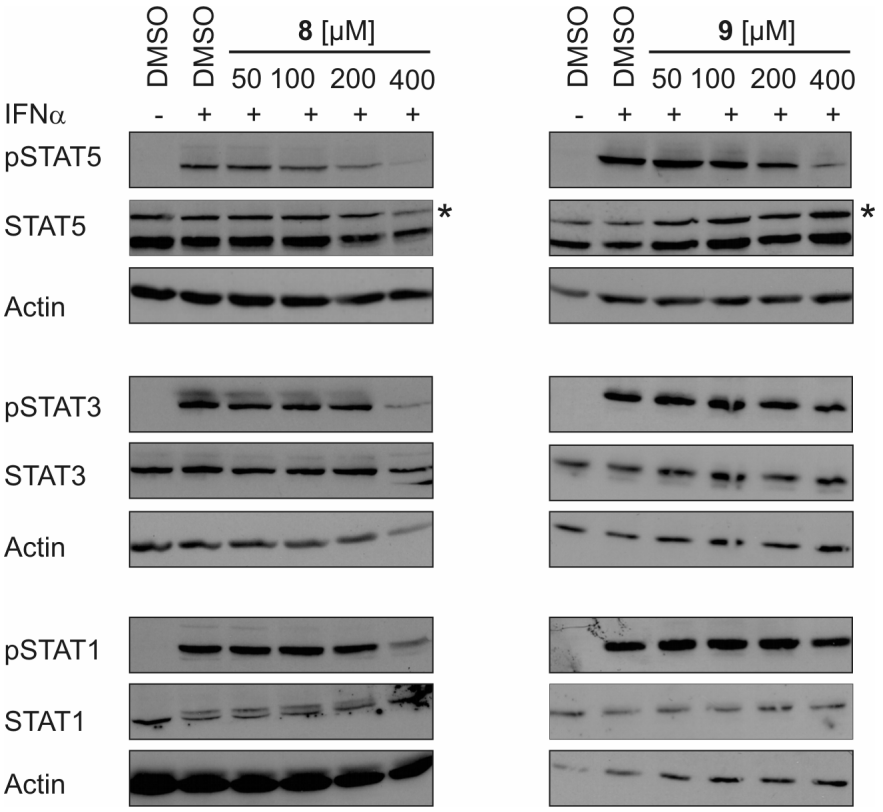


Figure S3. Effect of acyl hydrazones 8 and 9 on tyrosine phosphorylation of STAT5, STAT3, and STAT1 assayed by Western blot analysis. Lysates of lymphoma cells (Daudi) which had been stimulated with IFN-α in the presence or absence of acyl hydrazones **8** and **9** were separated on a polyacrylamide gel under denaturing conditions and transferred to a nitrocellulose membrane. Lysates of unstimulated Daudi cells are shown as negative control in the first lane. In all other lanes, cells were incubated with the indicated concentrations of compounds for 1 h before IFN-α stimulation, and lysed after stimulation. STAT phosphorylation was monitored using phospho-specific antibodies which only recognize the respective STAT proteins when the conserved tyrosine residue at the C-terminus of their SH2 domain is phosphorylated. Reblots using antibodies against STATs regardless of their phosphorylation state, and antibodies against actin were performed to control for equal loading of the lanes. The STAT5 antibodies do not discriminate between STAT5 isoforms. * denotes an unspecific protein recognized by the STAT5 antibody.

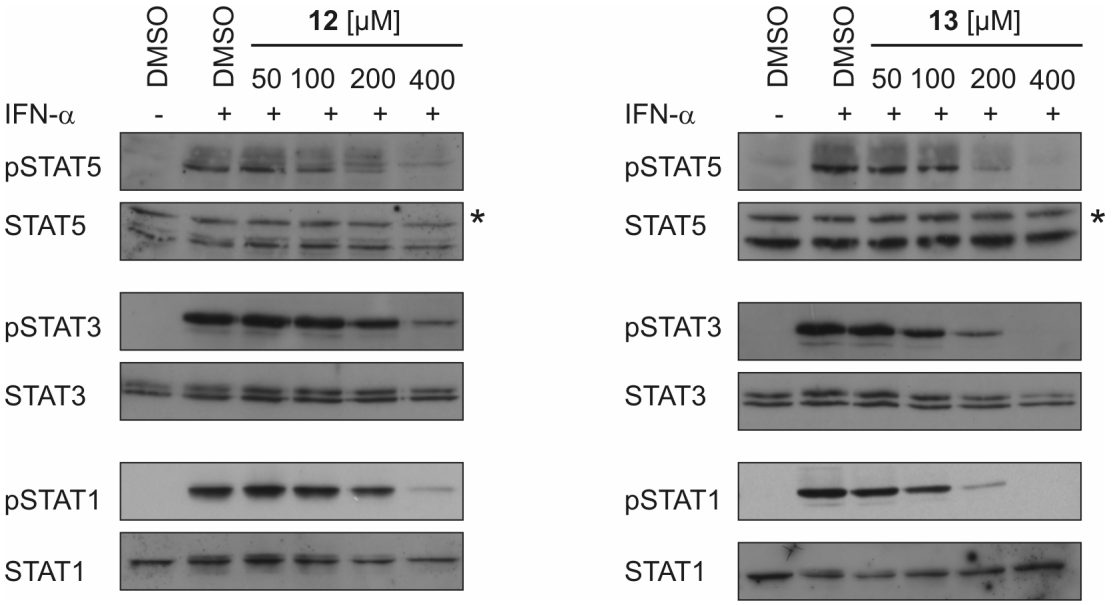


Figure S4. Effect of chromone aldehydes 12 and 13 on tyrosine phosphorylation of STAT5, STAT3, and STAT1 assayed by Western blot analysis. Lysates of lymphoma cells (Daudi) which had been stimulated with IFN-α in the presence or absence of aldehydes **12** and **13** were separated on a polyacrylamide gel under denaturing conditions and transferred to a nylon membrane. Lysates of unstimulated Daudi cells are shown as negative control in the first lane. In all other lanes, cells were incubated with the indicated concentrations of compounds for 1 h before IFN-α stimulation, and lysed after stimulation. STAT phosphorylation was monitored using phospho-specific antibodies which only recognize the respective STAT proteins when the conserved tyrosine residue at the C-terminus of their SH2 domain is phosphorylated. Reblots using antibodies against STATs regardless of their phosphorylation state, and antibodies against actin were performed to control for equal loading of the lanes. The STAT5 antibodies do not discriminate between STAT5 isoforms. * denotes an additional protein recognized by the STAT5 antibody.

Experimental procedures

Chemical synthesis. Acyl hydrazones were synthesized from the corresponding aldehydes and hydrazides according to the published procedure.[1] Equimolar amounts of aldehydes and hydrazides were mixed in ethanol / water (95:5) (70 mL / mmol of reagents). Upon addition of a catalytic amount of glacial acetic acid, the mixtures were refluxed for 5 h. The solvent was partially evaporated under reduced pressure, and the crystalline reaction products were isolated by filtration. The acyl hydrazones can be recrystallized from ethanol.

1: N'-((4-oxo-4H-chromen-3-yl)methylene)nicotinohydrazide was synthesized from 3-formyl chromone (870 mg, 5 mmol) and nicotinic hydrazide (685 mg, 5 mmol). Yield: 855 mg (58%); ^{1}H NMR (400 MHz, [D$_6$]DMSO): d = 12.08 (s, 1H), 9.07 (s, 1H), 8.85 (s, 1H), 8.76 (d, J = 4 Hz, 1H), 8.62 (s, 1H), 8.26 (d, J = 8 Hz, 1H), 8.13 (d, J = 7.5 Hz, 1H), 7.86 (t, J = 7.5 Hz, 1H), 7.73 (d, J = 8Hz, 1H), 7.56 ppm (m, 2H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): d = 175.0, 161.5, 155.7, 154.7, 152.3, 148.6, 141.0, 135.4, 134.7, 128.9, 126.1, 125.2, 123.6, 123.3, 118.7, 118.1 ppm; MS $C_{16}H_{11}N_3O_3$ calcd: 294.0879 [M+H], found: 294.0832.

2: N'-((6-ethyl-4-oxo-4H-chromen-3-yl)methylene)nicotinohydrazide was synthesized from 6-ethyl-3-formylchromone (404 mg, 2 mmol) and nicotinic hydrazide (274 mg, 2 mmol). Yield: 287 mg (45%); ^{1}H NMR (400 MHz, [D$_6$]DMSO): d = 12.09 (s, 1H), 9.07 (s, 1H), 8.84 (s, 1H), 8.76 (d, J = 4 Hz, 1H), 8.62 (s, 1H), 8.26 (d, J = 7.5 Hz, 1H), 7.93 (s, 1H), 7.72 (d, 1H), 7.66 (d, J = 7.5 Hz, 1H), 7.57 (d, J = 7 Hz, 1H), 2.75 (q, J = 7 Hz, 2H), 1.22 ppm (t, 3H, J = 7 Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): d = 175.0, 161.5, 154.6, 154.2, 152.3, 148.6, 142.0, 141.2, 135.4, 134.8, 128.9, 123.6, 123.2, 123.1, 118.7, 118.0, 27.5, 15.4 ppm; MS $C_{18}H_{15}N_3O_3$ calcd: 322.1192 [M+H], found: 322.1179.

3: N'-((6-fluoro-4-oxo-4H-chromen-3-yl)methylene)nicotinohydrazide was synthesized from 6-fluorochromone-3-carboxaldehyde (384 mg, 2 mmol) and nicotinic hydrazide (274 mg, 2 mmol). Yield: 210 mg (34%); ^{1}H NMR (400 MHz, [D$_6$]DMSO): d = 12.09 (s, 1H), 9.07 (s, 1H), 8.87 (s, 1H), 8.76 (d, J = 3.5 Hz, 1H), 8.60 (s, 1H), 8.26 (d, J = 7.5 Hz, 1H), 7.70 – 7.95 (m, 3H), 7.56 ppm (m, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): d = 174.4, 161.5, 160.5, 158.0, 155.0, 152.3, 148.6, 140.7, 135.4, 128.9, 124.6, 124.5, 123.6, 122.9, 122.7, 121.6, 121.6, 117.6, 109.9, 109.7 ppm; MS $C_{16}H_{10}FN_3O_3$ calcd: 312.0784 [M+H], found: 312.0771.

4: N'-((5,7-dimethyl-4-oxo-4H-chromen-3-yl)methylene)nicotinohydrazide was synthesized from 5,7-dimethyl-3-formylchromone (404 mg, 2 mmol) and nicotinic hydrazide (274 mg, 2 mmol). Yield: 188 mg (29%); ^{1}H NMR (400 MHz, [D$_6$]DMSO): d = 12.07 (s, 1H), 9.05 (s, 1H), 8.75 (d, J = 4 Hz, 1H), 8.67 (s, 1H), 8.55 (s, 1H), 8.25 (d, J = 8 Hz, 1H), 7.56 (m, 1H), 7.33 (s, 1H), 7.11 (s, 1H), 2.49 (s, 3H), 2.40 (s, 3H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): d = 176.7, 171.2, 161.5, 157.3, 153.1, 152.3, 148.6, 144.5, 141.6, 139.9, 135.4, 129.7, 129.0, 123.6, 118.9, 116.3, 22.3, 21.0 ppm; MS $C_{18}H_{15}N_3O_3$ calcd: 322.1192 [M+H], found: 322.1176.

5: N'-(naphthalen-2-ylmethylene)nicotinohydrazide was synthesized from 2-naphthaldehyde (312 mg, 2 mmol) and nicotinic hydrazide (274 mg, 2 mmol). Yield: 336 mg (61%); ^{1}H NMR (400 MHz, [D$_6$]DMSO): d = 12.08 (s, broad, 1H), 9.09 (s, 1H), 8.77 (d, J = 4 Hz, 1H), 8.60 (s, 1H), 8.28 (d, J = 7.5 Hz, 1H), 8.17 (s, 1H), 7.95 – 8.10 (m, 4H), 7.57 ppm (m, 3H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): d = 161.7, 152.2, 148.6, 148.3, 135.4, 133.8, 132.8, 131.9, 129.2, 128.9, 128.5, 128.3, 127.7, 127.2, 126.7, 123.6, 122.6 ppm; MS $C_{17}H_{13}N_3O$ calcd: 276.1137 [M+H], found: 276.1122.

7: *N'*-((4-oxo-4H-chromen-3-yl)methylene)benzohydrazide was synthesized from 3-formyl chromone (348 mg, 2 mmol) and benzoic hydrazide (272 mg, 2 mmol). Yield: 113 mg (19%); ^{1}H NMR (400 MHz, [D$_6$]DMSO): d = 11.95 (s, 1H), 8.84 (s, 1H), 8.62 (s, 1H), 8.13 (d, J = 8 Hz, 1H), 7.8 – 8.0 (m, 3H), 7.73 (d, J = 8.5 Hz, 1H), 8.5 – 8.7 (m, 4H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): d = 175.1, 162.9, 155.8, 154.5, 147.9, 140.3, 134.7, 133.2, 131.8, 128.5, 127.6, 126.1, 125.2, 123.3, 118.7, 118.4 ppm; MS $C_{17}H_{12}N_2O_3$ calcd: 293.0926 [M+H], found: 293.0916.

10: *N'*-((4-oxo-4H-chromen-3-yl)methylene)acetohydrazide was synthesized from 3-formyl chromone (870 mg, 5 mmol) and acetic hydrazide (370 mg, 5 mmol). Yield: 831 mg (72%); ^{1}H NMR (400 MHz, [D$_6$]DMSO): Two isomers in a ratio of 2:1. Major isomer: d = 11.30 (s, 1H), 8.79 (s, 1H), 8.28 (s, 1H), 8.10 (m, 1H), 7.83 (m, 1H), 7.69 (m, 1H), 7.52 (m, 1H), 2.18 (s, 3H). Minor isomer: d = 11.45 (s, 1H), 8.73 (s, 1H), 8.10 (m, 1H), 8.09 (s, 1H), 7.83 (m, 1H), 7.69 (m, 1H), 7.52 (m, 1H), 2.18 (s, 3H); ^{13}C NMR (100 MHz, [D$_6$]DMSO) (both isomers): d = 175.0, 174.9, 171.9, 165.4, 155.7, 154.2, 154.1, 138.1, 134.9, 134.6, 134.6, 126.0, 125.2, 123.3, 118.7, 118.4, 118.3, 21.5, 20.2 ppm; MS $C_{12}H_{10}N_2O_3$ calcd: 231.0770 [M+H], found: 231.0737.

Plasmid construction and protein expression. Nucleotides coding for amino acids 127-722 of murine STAT3 (kindly provided by Dr. C. Müller, EMBL Grenoble, France)

(100% identical to human STAT3 on the protein level) were cloned into the *NdeI*/*Eco*RI sites of pET28a as published previously.[2] Nucleotides coding for human STAT1 amino acids 135–712 (kindly provided by Dr. M. H. Heim, University Hospital Basel, Switzerland)[3] were cloned into the NdeI/SalI sites of pET28a. Nucleotides coding for amino acids 136–703 of human STAT5b were PCR-amplified from placenta cDNA and cloned into the *FseI*/*AscI* sites of a modified pQE70 vector. Plasmid pGEX-LckSH2, coding for the Lck amino acids 121–226 fused to the C terminus of GST was kindly provided by Dr. B. Biesinger (University of Erlangen, Germany).

High-throughput screening and fluorescence polarization assays. 8,298 compounds from Chemical Diversity and 9,000 compounds from Maybridge were tested in a fluorescence polarization assay which analyzes binding of small molecules to the STAT5b SH2 domain. The specificity of screening hits was validated in analogous assays for binding of the test compounds to the SH2 domains of STAT3[2], STAT1[4], and Lck[2]. The final concentration of buffer components used for all FP assays was 10 mM Hepes, pH 7.5, 1 mM EDTA, 0.1% Nonidet P-40, 50 mM NaCl, 1 mM dithiothreitol, and 10% DMSO. The sequences of the peptides were: STAT5: 5-carboxyfluorescein-GY(PO_3H_2)LVLDKW; STAT3: 5-carboxyfluorescein-GY(PO_3H_2)LPQTV-NH_2; STAT1: 5-carboxyfluorescein-GY(PO_3H_2)DKPHVL; Lck: 5-carboxyfluorescein-GY(PO_3H_2)EEIP. Proteins were used at the following final concentrations: STAT5: 125 nM; STAT3 and STAT1: 105 nM; Lck: 30 nM. Proteins were incubated with test compounds in Eppendorf tubes at 22 °C for 60 minutes prior addition of the respective 5-carboxyfluorescein labeled peptides (final concentration: 10 nM), and fluorescence polarization was analyzed immediately thereafter. Binding curves and inhibition curves were fitted using SigmaPlot (SPSS Science Software GmbH). All competition curves were repeated three times in independent experiments.

Electrophoretic mobility shift analysis (EMSA). Nuclear lysates from K562 cells were prepared essentially as described[5]. 3 µL of nuclear protein (approximately 5 µg /µL) was added to 2 µL of 5x binding buffer (65 mM Hepes, pH 7.9, 0.75 mM EDTA, 40 % glycerol), 1 µL test compound dissolved in DMSO from a 10x stock, and 1 µg of poly(dI-dC) (Roche Applied Science). For competition binding assays, unlabeled oligonucleotide was added to the reaction in 1000-fold molar excess. For the supershift control, 0.2 µg anti-STAT5 antibody (Santa Cruz) was added to the reaction mixtures. Total reaction volume was 10 µL. All reactions were incubated at 37°C for 1 h prior to addition of the [γ-^{32}P]-ATP-labeled oligonucleotides [upper strand sequence: (MGFe):

5'-TTTCTAGGAATTCAATC-3'] for 20 minutes at room temperature. Protein–DNA complexes were resolved on a 4% acrylamide gel (45 mM Tris-borate, 1 mM EDTA), and gels were dried before autoradiography.

Western blot analysis. Daudi cells (ATCC number CCL-213) were grown in RPMI 1640 media according to ATCC recommendations, incubated for 1 h with the indicated concentration of compounds, and stimulated for 5 minutes with 5000 U of interferon-α_{2A}. The final DMSO concentrations for the test compounds were as follows: compounds **1**, **6**, **12**, **13**: 0.4% DMSO; compounds **8** and **9**: 2% DMSO; compound **4**: 1.6% DMSO. Unstimulated and stimulated control cells were exposed to the same DMSO concentrations. Cells were washed twice with ice-cold PBS and lysed with 80 µL of buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerine, 1% Triton X-100, 10 mM $Na_4P_2O_7$, 10 mM NaF, 20 µM Na_3VO_4, 10 µM PMSF and 100 ng/ml aprotinin). Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the relevant antibodies (anti-STAT5 pTyr694, anti-STAT3 pTyr705, anti-STAT1 pTyr701, anti-STAT5, anti-STAT1 (all rabbit) from Cell Signaling; rabbit anti-STAT3 from Santa Cruz; rabbit anti-actin from Sigma). Secondary horseradish peroxidase (HRP)-conjugated antibodies were from DakoCytomation.

References

[1] L. Tsao, V. Van, G. Sun, Y. Lyu, *Russ. J. Gen. Chem.* **2001**, *71*, 767-769.

[2] J. Schust, T. Berg, *Anal. Biochem.* **2004**, *330*, 114-118.

[3] M. Wiederkehr-Adam, P. Ernst, K. Muller, E. Bieck, F. O. Gombert, J. Ottl, P. Graff, F. Grossmuller, M. H. Heim, *J. Biol. Chem.* **2003**, *278*, 16117-16128.

[4] P. Wu, M. Brasseur, U. Schindler, *Anal. Biochem.* **1997**, *249*, 29-36.

[5] R. Garcia, C. L. Yu, A. Hudnall, R. Catlett, K. L. Nelson, T. Smithgall, D. J. Fujita, S. P. Ethier, R. Jove, *Cell Growth Differ.* **1997**, *8*, 1267-1276.