Inhibition of the Signal Transducer and Activator of Transcription-3 (STAT3) Signaling Pathway by 4-Oxo-1-Phenyl-1,4-Dihydroquinoline-3-Carboxylic Acid Esters

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The JAK-STAT3 pathway regulates genes that are important in cell proliferation and thus is a promising target for cancer therapy. A high-throughput screening (HTS) campaign using an Apo-ONE Homogenous Caspase 3/7 assay in U266 cells identified 4-oxo-1-phenyl-1,4-dihydroquinoline-3-carboxylic acid ethyl ester **4** as a potential STAT3 pathway inhibitor. Optimization of this HTS hit led to the identification of the 7-cyano analogue **8**, which inhibited STAT3-Y705 phosphorylation with an EC_{50} of 170 nM. Compound **8** also inhibited cytokine induced JAK activation but did not inhibit BCR-ABL activated STAT5 phosphorylation in K562 cells.

Introduction

Signal transducers and activators of transcription (STATs)[a] are an important family of molecules that mediate signal transduction in cells.[1,2] They are latent in the cytoplasm until activated by various extracellular signaling proteins such as cytokines and growth factors. Upon stimulation, STATs are activated by phosphorylation on specific tyrosine residues by Janus kinase (JAK), receptor tyrosine kinases such as PDGF, and nonreceptor tyrosine kinases such as the Src family members. JAK activation may also be required in the latter two activation pathways. Upon phosphorylation, STATs dimerize and translocate into the nucleus, where they function as transcription factors on their target genes.

Accumulating evidence indicates that STAT family members play important roles in carcinogenesis and, in particular, STAT3 has emerged as a good target for cancer therapy.[3–8] STAT3 regulates genes that are important in cell proliferation (cyclin D1, c-myc), cell survival (survivin, Bcl-$_{XL}$, Mcl-1), angiogenesis (VEGF, HIF-1),[6] and modulates p53 transcription.[9] STAT3 is constitutively activated in many forms of hemotopoitic and solid tumors including, breast, head and neck, prostate, lung, melanoma, multiple myeoloma, lymphomas, and leukemia. An artificially engineered and constitutively active mutant form of STAT3, STAT3C, induces transformation and cells transformed by this active STAT3 mutant form tumor in vivo.[10] Inhibition of the STAT3 pathway by the dominant negative form, antisense oligonucleotides and RNAi of STAT3, and JAK inhibitors has been shown to inhibit cell growth and induce apoptosis. Loss of expression of negative regulators of the JAK-STAT3 pathway has been linked with hepatocellular carcinoma (SOCS-1 and SOCS-3)[11,12] and anaplastic large cell lymphoma (PIAS3).[13] In addition, activating JAK2 (ie. Y617F) and JAK3 mutants have been recently reported in patients with myeloproliferative disorders (JAK2)[14,15] and acute megakaryoblastic leukemia (JAK3).[16] STAT pathways are important mediators in pathologic events, thus therapeutics that inhibit the JAK-STAT pathway may have potential for treating solid tumors and hematological malignancies. Recently, several inhibitors of STAT3 signaling including natural products,[20–24] peptidomimetics,[17] platinum compounds,[18,19] and small molecules (**1**−**3** in Figure 1)[25–27] have been reported. The natural product Phaeosphaeride A has been shown to inhibit STAT3-dependent signaling,[20] while Flavopiridol disrupts STAT3/DNA interactions and attenuates STAT3-directed transcription[21] and Resveratrol blocks constitutive STAT3 protein activation in malignant cells by inhibiting upstream Src tyrosine kinase activity.[22] Cucurbitacin Q selectively inhibits the activation of STAT3 and induces apoptosis without inhibition of JAK2, Src, Akt, Erk, or JNK activation,[23] and Curcumin (diferuloylmethane) inhibits IL-6-induced STAT3 phosphorylation and consequent STAT3 nuclear translocation.[24] The synthetic terpenoid CDDO-Im (**1**) suppresses both constitutive and IL-6-induced STAT3 and STAT5 phosphorylation in human myeloma and lung cancer cells.[25] STA-21 (**2**) inhibits

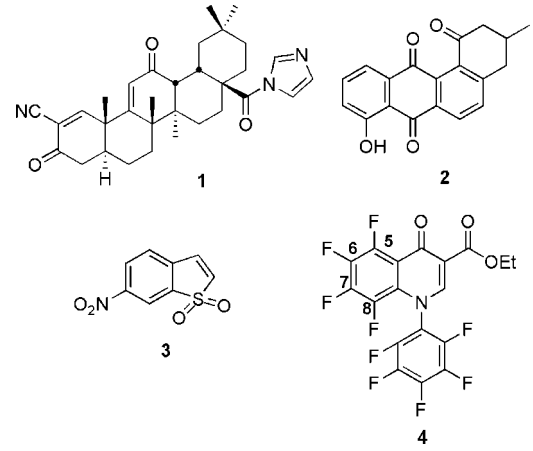

Figure 1. Structures of STAT3 pathway inhibitors.

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[a] Abbreviation: STAT3, signal transducer and activator of transcription 3; JAK2, Janus kinase 2; BCR, breakpoint cluster region; PDGF, platelet-derived growth factor; Bcl, B-cell lymphoma; Mcl-1, myeloid cell leukemia-1; VEGF, vascular endothelial growth factor; HIF-1, hypoxia-inducible factor 1; SOCS, suppressors of cytokine signaling; PIAS3: protein inhibitor of activated STAT3; AKT, protein kinase B; mTOR, mammalian target of rapamycin; IL-6, interleukin-6; INF-α, interferons-α; TYK2, tyrosine kinase 2; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase.

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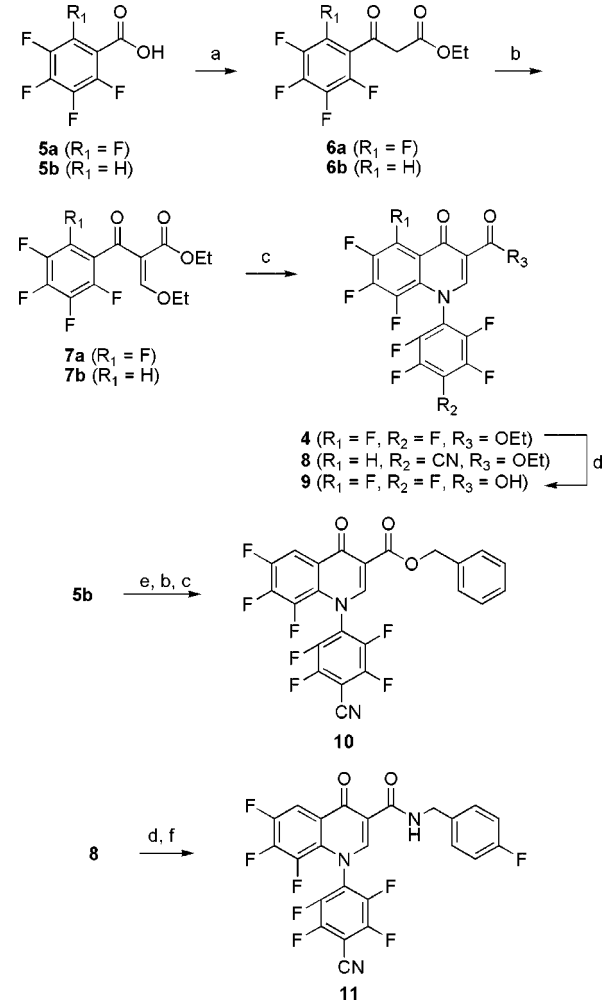
STAT3 dimerization, DNA binding, and luciferase activity in breast cancer cells that express constitutively active STAT3.[26] Also recently, Stattic (**3**) has been reported to selectively inhibit activation, dimerization, and nuclear translocation of STAT3 and increases the apoptosis of STAT3-dependent breast cancer cell lines.[27]

In the human myeloma cell line U266, STAT3 is constitutively activated through an IL-6 autocrine loop. Inhibition of the constitutive STAT3 pathway induces the cells into apoptosis.[28] Using an Apo-ONE Homogenous Caspase 3/7 assay (Promega) in the U266 cell line, we conducted a high-throughput screen (HTS) for compounds that inhibit the STAT3 pathway and cause cell apoptosis. Ethyl 5,6,7,8-tetrafluoro-4-oxo-1-(pentafluorophenyl)-1,4-dihydroquinoline-3-carboxylate (**4**) (Figure 1) induced Apo-ONE signal at low micromolar concentration in U266 and was also shown to inhibit the STAT3-Y705 phosphorylation by Western blot analysis. Further characterizations revealed that activation of the upstream activators of STAT3, the Janus kinases (JAKs), were also inhibited. Previously, quinolone derivatives, especially C-7 amine derivatives, have been shown to possess antibacterial activity by inhibiting DNA gyrase and topoisomerase IV[29,30] and have also been reported as anticancer or antitumor agents.[31] Herein we report the characterization and optimization of the quinolinone class of JAK-STAT3 pathway inhibitors.

Chemistry

The synthesis of the 1,4-dihydroquinoline derivatives was carried out using previously reported literature procedures[32,33] as shown in Scheme 1. Treatment of 2,3,4,5,6-pentafluorobenzoic acid (**5a**) or 2,3,4,5-tetrafluorobenzoic acid (**5b**) with thionyl chloride in the presence of catalytic amount of DMF gave the corresponding acid chlorides, which were reacted with the dianion of monoethyl malonate to give the corresponding β-keto esters **6a** and **6b** in high yield (~90%). These esters were reacted with triethyl orthoformate in acetic anhydride at 140 °C to provide the enol ether intermediates **7a** and **7b**, which were used in the next step without purification. Reactions of enol ether **7a** with 2,3,4,5,6-pentafluoroaniline and enol ether **7b** with 4-amino-2,3,5,6-tetrafluorobenzonitrile in the presence of NaH afforded dihydroquinoline carboxylates **4** and **8**, respectively, in good yield (~80%). Ethyl ester **4** was hydrolyzed under acidic or basic conditions to provide the corresponding dihydroquinoline carboxylic acid **9**. Benzyl ester **10** was also prepared in a similar manner as described for the synthesis of **8** except using monobenzyl malonate. Ethyl ester **8** was hydrolyzed to the corresponding dihydroquinoline carboxylic acid. The carboxylic acid was activated by ethyl chloroformate, and the resulting anhydride reacted with 4-fluorobenzylamine to yield benzyl amide **11** in high yield (85%).

Scheme 1[a]



[a] (a) (i) $(SOCl)_2$, DMF, 10 h; (ii) *n*-BuLi, −78 °C, $CH_2CO_2Et(COOH)$, 2 h; (iii) HCl, 90%; (b) triethyl orthoformate, Ac_2O, 140 °C, 5 h, 98%; (c) (i) $ArNH_2$ (2,3,4,5,6-pentafluoroaniline or 4-amino-2,3,5,6-tetrafluorobenzonitrile), EtOH, rt, 12 h; (ii) K_2CO_3, DMF, rt, 12 h, or NaH, THF, 40 °C, 80%; (d) H_2SO_4, 100 °C, 12 h or LiOH/THF-MeOH, rt, 12 h, 100%; (e) (i) $(SOCl)_2$, DMF, 10 h; (ii) *n*-BuLi, −78 °C, $CH_2CO_2Bn(COOH)$, 2 h; (iii) HCl, 90%; (f) ethyl chloroformate, $N(C_2H_5)_3$, THF-CH_2Cl_2 (v/v′ = 2:1), 0 °C, 30 min; (iii) 4-fluorobenzylamine, 0 °C, 30 min, 85%.

Results and Discussion

Constitutively active STAT3 pathways have been implicated in many types of cancer. We surveyed many cultured cell lines for steady state level of STAT3-Y705 phosphorylation as an indicator of the constitutively active pathway because phosphorylation of this site is required for its transcription activities. Among the cell lines tested, human multiple myeloma cell line U266 showed the highest level of STAT3-Y705 phosphorylation (data not shown) and thus was chosen as our model cell line. U266 cells have an IL-6 autocrine loop and inhibition of the IL-6-JAK-STAT3 pathway at various points lead to apoptosis.[28] We identified compound **4** via HTS using an Apo-ONE Homogenous Caspase 3/7 assay. The compound induced Apo-ONE signal at low micromolar concentration in U266 but did not induce Apo-ONE signal in the A2780 cell line in which STAT3-Y705 is not constitutively phosphorylated.[34] Western blot analysis on U266 cell lysates confirmed that compound **4** induced apoptosis through inhibiting the STAT3 pathway. As shown in Figure 2A, compound **4** inhibited STAT3-Y705 phosphorylation in a dose dependent manner after 1 h treatment with an EC_{50} of 4.6 μM (Table 1).

Analogue synthesis showed that while removal of the labile C-5 fluorine only led to a slight reduction in potency, removal of the C-6, C-7, and C-8 fluorine atoms led to a more significant loss in activity (data not shown). Also, hydrolysis of the ethyl ester yielded the corresponding carboxylic acid analogue **9** (Scheme 1), which was 10-fold less active (EC_{50} = 46 μM) than ethyl ester **4** in inhibiting STAT3-Y705 phosphorylation (Table 1 and Figure 2B). Array synthesis varying the aniline moiety led to the identification of the 4-cyanophenyl analogue **8**, with a 30-fold increase in potency (EC_{50} = 170 nM). The benzyl ester **10** and benzyl amide **11** showed similar potency with EC_{50}'s of 130 nM and 200 nM, respectively. As shown in Figure 3, compound **8** inhibited steady state STAT3-Y705

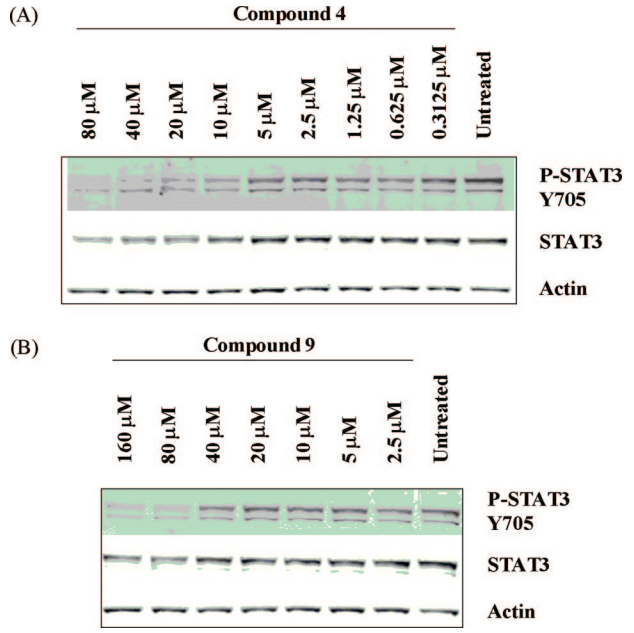
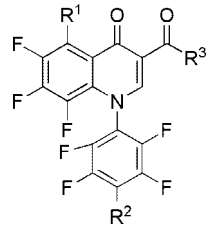


Figure 2. STAT3-Y705 phosphorylation affected by compound **4** (A) and compound **9** (B) in U266 cells.

Table 1. Inhibition of STAT3-Y705 Phosphorylation by Compounds **4**, **8**−**11**



compound	R^1	R^2	R^3	STAT3-P[a] EC_{50} (μM)
4	F	F	OEt	4.6
9	F	F	OH	46
8	H	CN	OEt	0.17
10	H	CN	OBn	0.13
11	H	CN	p-F-BnNH	0.20

[a] Inhibition of STAT3-Y705 phosphorylation as estimated based on Western blot assay, repeated at least 3 times for each compound.

phosphorylation (P-STAT3-Y705) with an EC_{50} of 170 nM while the total level of STAT3 was not affected at this concentration.[35] The inhibitory effects were very fast, being observed within 30 min, thus excluding indirect secondary effects by **8** on the pathway. However, **8** did not inhibit the AKT-mTOR pathway as indicated by the phosphorylation status of the p70 S6 kinase (P-p70 S6 kinase-T389) and S6 protein (P-S6-S240/244) nor the MAPK pathway as indicated by the MAPK phosphorylation (P-p44/42 MAPK-T202/204) status. Interestingly, STAT3-Ser727 phosphorylation, which has been reported to be mediated by serine/theonine kinases involving the MAPK pathway, AKT-mTOR, and PKC,[36–41] was not inhibited. Compound **8** induced apoptosis in U266 cells, but the caspase activation detected with the Apo-ONE Homogenous Caspase 3/7 assay did not occur until at least 6 h after compound treatment. Furthermore, apoptosis could be halted with a general antioxidant *N*-acetyl-L-cysteine, while inhibition of STAT3 phosphorylation was not affected (data not shown), suggesting pathway inhibition is not a result of apoptosis.

Compound **8** also inhibited STAT3-Y705 phosphorylation in serum starved U266 cells activated by IL-6 (data not shown) and IFN-α cytokines (Figure 4). These cytokines use different

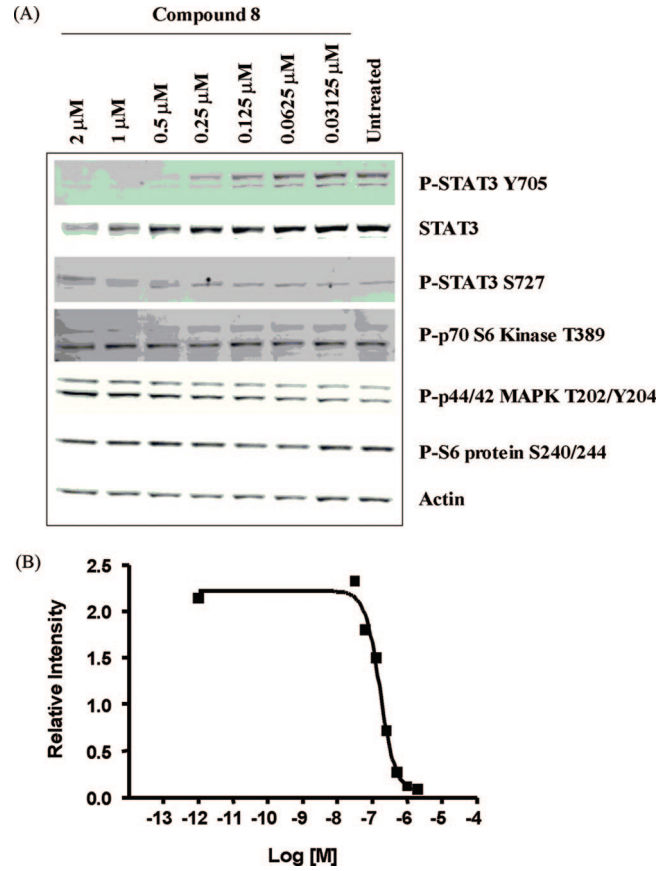


Figure 3. Compound **8** potently and specifically inhibits STAT3 phosphorylation in U266 cells (A). Western blot data was quantified by GraphPad Prism software (B).

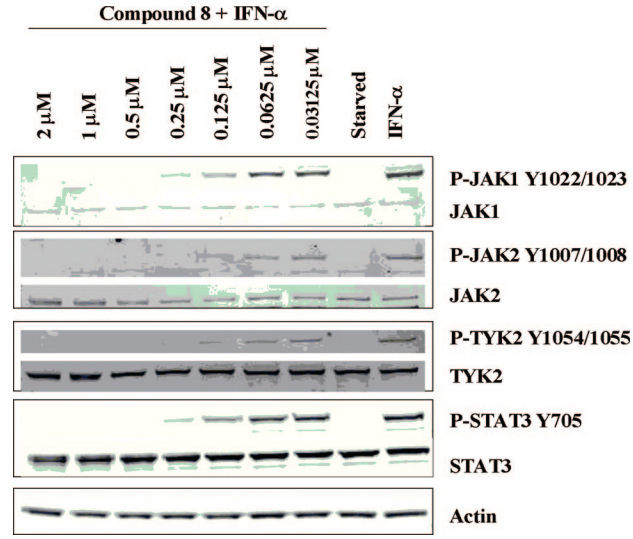


Figure 4. Compound **8** inhibits cytokines induced phosphorylation of JAK1, JAK2, and Tyk2 in U266 cells.

receptors but converge on Janus kinase family members (JAKs) to activate STAT3. Thus we tested whether the compounds would inhibit JAK function. The steady state levels of phosphorylated JAKs were very difficult to detect using available antibodies, so we used cytokine treatment to increase the phospho-JAK levels. Compound **8** inhibited cytokine induced phosphorylation of JAK1, JAK2, and Tyk2 (P-JAK1-Y1022/1023, P-JAK2-Y1007/1008, P-Tyk2-Y1054/1055), while the total protein amounts were not affected (Figure 4). JAK3

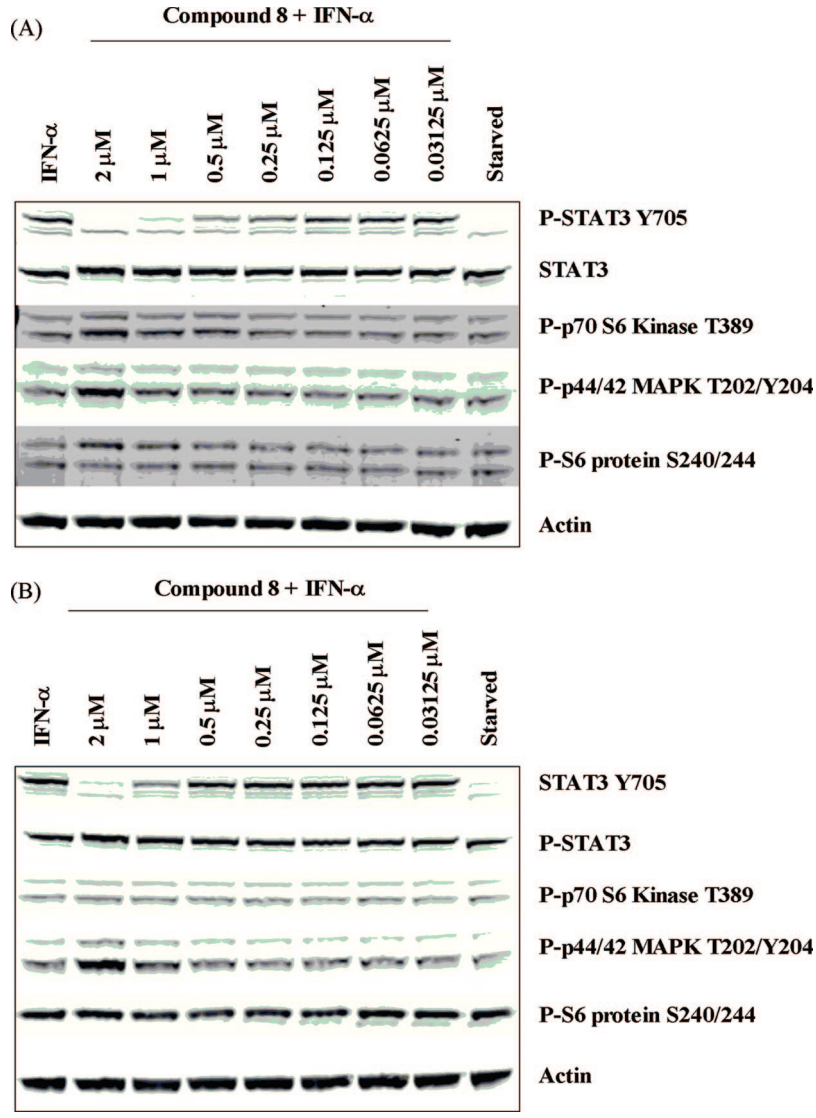


Figure 5. Compound **8** potently and specifically inhibits STAT3 phosphorylation in HeLa (A) and DU145 (B) cells.

phosphorylation still remained undetectable. Furthermore, this inhibition of JAK activation correlated well with inhibition of STAT3 activation. However, compound **8** did not inhibit JAK1, JAK2, and JAK3 enzyme activity in an in vitro kinase assay at the concentration of 1 and 10 μM.

We further tested the inhibition of STAT3 target gene transcription by compound **8** using microarray analysis. U266 cells were treated with compound **8** (0.5 μM, $\sim EC_{90}$) and 100 μM of compound **12** (AG490: ((*E*)-2-cyano-3-(3,4-dihydrophenyl)-*N*-(phenylmethyl)-2-propenamide), a well-characterized inhibitor of the JAK/STAT pathway[42] as a control, for 6 h and RNA samples were extracted for microarray analysis. Using a 1.5 fold as cutoff for significant changes, several STAT3 target genes, including cyclin D1, myeloid cell leukemia sequence 1 (MCL1), and Bcl2-like 1, were down regulated to a similar extent by both **8** and **12**.

Compound **8** also inhibited steady state (data not shown) and cytokine induced STAT3-Y705 phosphorylation in HeLa and DU145 cells, while the phosphorylation status of the AKT-mTOR and MAPK pathway markers were not affected (Figure 5A,B). Cytokine induced STAT3 nuclear translocation was inhibited when visualized in a High-content screening assay,[43] indicating the inhibitory effects demonstrated by these compounds were not restricted to a particular cell line.

The specificity of compound **8** for inhibition of the JAK-STAT3 pathway was further tested in K562 and A431 cells. In K562 cells, STAT5 is constitutively activated by a different signaling pathway, the oncogene BCR-ABL.[44] As expected and in contrast to a control Src kinase inhibitor, compound **8** did not inhibit STAT5-Y694/699 phosphorylation (Figure 6A). When both U266 and K562 cells were treated with compound **8** for 4 days, growth of U266 cells was much more inhibited than that of K562 cells (Figure 6B). Further, compound **8** did not inhibit EGF stimulated activation of MAPK in A431 cells, a cell line widely used for studying the EGFR pathway (data not shown).

Conclusion

In summary, we used a cell based apoptosis assay to identify the potential JAK-STAT3 pathway inhibitor **4**. Lead optimization led to the identification of compound **8** in which cell apoptosis induction correlates well with inhibition of steady state and cytokine induced JAK and STAT3 activation. Compound **8** inhibited activation of JAKs but did not inhibit JAK1, JAK2, and JAK3 enzyme activity in in vitro kinase assay. Biochemical evidence suggests compound **8** was selective for the JAK-STAT pathway, and it inhibited many STAT3 target genes. Further investigation of the specific molecular target is underway.

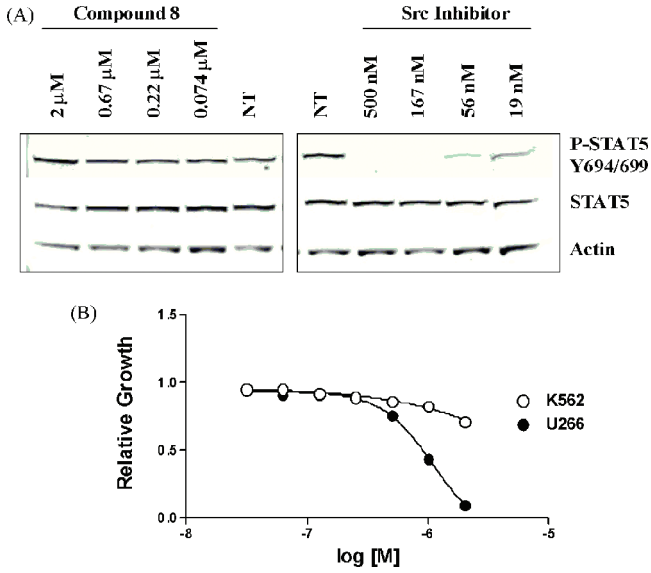


Figure 6. Compound **8** does not inhibit BCR-ABL activated STAT5 phosphorylation in K562 cells (A) but inhibits growth of U266 cells selectively over K562 cells (B).

Emerging evidence continues to implicate the JAK-STAT pathway participation and crosstalk with other oncogenic pathways for tumor growth and survival, thus JAK-STAT inhibitors may be promising therapeutics. Compound **8** may be a potentially useful tool in elucidating the inhibition of JAK-STAT signaling pathway in cancer or tumor cell lines.

Experimental Section

Cell Culture and Cell Treatment. All cell lines were obtained from American Type Culture Collection (ATCC). U266 and K562 cells were cultured in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and Penicillin-Streptomycin (Pen-Strep). For steady state phosphorylation, exponentially growing cells (1×10^6 cells at density 2×10^5 cells/mL for U266 and 1×10^6 cells at density 1×10^6 cells/mL for K562) were treated with compound for 1 h before being harvested for Western blot analysis. For cytokine induction, cells were starved in serum free medium for 2 h before compound treatment. One hour after compound treatment, human interferon alpha α (IFN-α) was added at 20000 units/mL. Cells were harvested 20 min after cytokine induction for Western blot analysis. Cells were lysed in 100 μL of PhosphoSafe extraction reagent (Novagen) with protease inhibitor cocktail after being washed once with PBS. Then 13 μL for U266 and 16 μL for K562 of lysate were loaded for each sample for Western blot analysis. HeLa and DU145 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and Penicillin-Streptomycin (Pen-Strep). For cytokine induction, ~80% confluent cells were starved in serum free medium overnight before compound treatment. One hour after compound treatment, IFN-α was added for cytokine induction at 20000 units/mL. Cells were harvested 20 min after cytokine induction for Western blot analysis. Cells from each 12-well plate were collected in 100 μL of 1.1X NuPAGE LDS sample buffer (Invitrogen) after being washed once with PBS and were then sonicated. Then 18 microliter of lysates were loaded for each sample for Western blot analysis. IFN-α was purchased from PBL Biomedical Laboratories.

Western Blot Analyses. Cell lysates were separated on NuPAGE gels (Invitrogen). Proteins were transferred to nitrocellulose membranes (Invitrogen) and then blocked for 1 h in Odyssey blocking buffer (LI-COR Biosciences). Membranes were incubated with specific antibodies for 2 h and then secondary antibodies for 1 h at room temperature in Odyssey blocking buffer with 0.1% Tween-20, except 5% nonfat milk in PBS was used for blocking and primary antibody incubation for JAK family antibodies. Specific antibodies for phosphorylated STAT3 (Tyr705 and Ser727), STAT3, phosphorylated p70 S6 kinase (Thr389), phosphorylated p44/42 MAPK (Thr202/Tyr204), phosphorylated S6 ribosomal protein (Ser240/Ser244), phosphorylated JAK2 (Tyr1007/Tyr1008), and phosphorylated TYK2 (Tyr1054/Tyr1055) were purchased from Cell Signaling Technology. Antibodies for phosphorylated JAK1 (Tyr1022/Tyr1023) and JAK2 were from Biosource. Antibodies for JAK1, TYK2, and phosphorylated STAT5 (Tyr694/Tyr699) were from Upstate. Antibodies for STAT5, JAK3, and phosphorylated JAK3 (Tyr980) were from Santa Cruz Biotechnology. Antibody for actin was from Chemicon. All antibodies were diluted 1:1000 with the exception of the JAK family member antibodies, which were diluted 1:500, and the actin antibody, which was diluted 1:10000. Secondary antibodies Alexa Fluor 680 conjugated goat antirabbit IgG and goat antimouse IgG were obtained from Invitrogen. IRDye 800 conjugated goat antimouse IgG secondary antibody was from Rockland Immunochemicals. Western analyses were developed using the Odyssey infrared imaging system (LI-COR Biosciences) for pictures.

STAT3-Y705 Phosphorylation Analyses. Western blots were quantified using the Odyssey infrared imaging system (LI-COR Biosciences). Actin levels were used for normalization. Quantified data were analyzed using GraphPad Prism software. An arbitrary 10^{-12} M was used for no compound treatment for plotting purpose. A representative Western blot analysis was shown from several repeated experiments.

Proliferation Assay. Cells were seeded at a density of 2×10^5 cells per mL, and compounds were dosed at various doses. Four days later, viable cells were stained using Guava ViaCount reagent and counted using Guava PCA-96 flow cytometer (Guava Technologies Inc.). Data were analyzed and plotted using GraphPad Prism software.

RNA Expression Analysis. U266 cells were seeded at the density of 1×10^6 cells per mL and treated with 0.5 μM compound **8** or 100 μM compound **12** for 6 h in triplicate. Cells were collected and RNA purified using an RNeasy kit (QIAGEN). mRNA levels were determined by microarray analysis. Briefly, 2−5 μg of total RNA from each individual sample was used to generate biotin-labeled cRNA using an oligo T7 primer in a reverse transcription reaction followed by an in vitro transcription reaction with biotin-labeled UTP and CTP. Then 10 μg of cRNA were fragmented and hybridized to a human genome array (U133A; Affymetrix). Hybridized arrays were stained and scanned according to the manufacturer's protocols (Fluidics Station 450 and Affymetrix scanner 3000; Affymetrix). We determined signal values using the operating system software (GCOS 1.0; Affymetrix). For each array, all probe sets were normalized to a mean signal intensity value of 100. The default GCOS statistical values were used for all analysis. Signal values and absolute detection calls were imported into Expressionist (GeneData) for analysis.

General Methods. All reactions were performed under N_2 atmosphere using anhydrous solvents. Purification of the products was carried out by flash chromatography using EM silica gel 60 (230−400 mesh). Semipreparative HPLC was used under the conditions: A = 0.02% TFA in water, B = 0.02% TFA in acetonitrile, 10−95% B in 8 min, 34 mL/min, 50 °C, 215 nm detection, Waters Xterra 20 mm × 50 mm column.

Ethyl 3-oxo-3-(2,3,4,5,6-Pentafluoro-phenyl)-propanoate (6a). Ester **6a** was prepared in a similar manner as described for the synthesis of **6b** except using 2,3,4,5,6-pentafluorobenzoic acid. ^{1}H NMR spectroscopy data was confirmed by reported data. ^{1}H NMR (400 MHz, $CDCl_3$): δ (two sets of signals) 4.23 (q, J = 7.6 Hz, 2H), 3.91 (s, 2H), 1.26 (t, J = 7.6 Hz, 3H); 12.35 (s, OH), 5.42 (s, 1H), 4.39 (q, J = 7.6 Hz, 2H), 1.38 (t, J = 7.6 Hz, 3H).

Ethyl 3-oxo-3-(2,3,4,5-Tetrafluorophenyl)-propanoate (6b). To a solution of 2,3,4,5-tetrafluorobenzoic acid (1 g, 5.15 mmol) in thionyl chloride (10 mL) were added 2 drops of anhydrous DMF at room temperature. The reaction mixture was stirred for 12 h at room temperature. The mixture was concentrated, dissolved in anhydrous THF (10 mL), and saved as an acyl chloride solution. To a solution of malonic acid half-ethyl ester (1.21 mL, 10.3 mmol)

in THF (20 mL) at 0 °C was added a solution of *n*-BuLi (8.23 mL, 2.5 M in hexanes, 20.6 mmol) and the mixture was stirred for 1 h at room temperature. The mixture was cooled to −78 °C, and a solution of acyl chloride, prepared previously, was added to the mixture slowly. The mixture was warmed to room temperature and quenched with 1N HCl (30 mL). The solution was extracted with ethyl ether (3 × 30 mL), and the organic layer was washed with saturated $NaHCO_3$ (2 × 50 mL) and then water. The organic solution was dried over Na_2SO_4 and concentrated. The residue was purified by short column chromatography with CH_2Cl_2 to give 1.22 g (90%) of **6b** as a yellow solid. ^{1}H NMR spectroscopy data was confirmed by reported data. ^{1}H NMR (400 MHz, $CDCl_3$): δ (two sets of signals) 7.63 (m, 1H), 4.22 (q, J = 7.6 Hz, 2H), 3.97 (s, 1H), 3.96 (s, 1H), 1.27 (t, J = 7.6 Hz, 3H); 12.73 (s, OH), 7.53 (m, 1H), 5.84 (s, 1H), 4.30 (q, J = 7.6 Hz, 2H), 1.35 (t, J = 7.6 Hz, 3H).

(*Z*)-Ethyl 3-Ethoxy-2-(2,3,4,5,6-pentafluoroben-zoyl)acrylate (7a). (*Z*)-Ethyl 3-ethoxy-2-(2,3,4,5,6-pentafluorobenzoyl)acrylate (**7a**) was prepared in a similar manner as described for the synthesis of **7b** and the product was used for further reaction without purification.

(*Z*)-Ethyl 3-Ethoxy-2-(2,3,4,5-tetrafluorobenzoyl)-acrylate (7b). The mixture of **7b** (1.20 g, 4.54 mmol), triethylorthoformate (1.13 mL, 6.81 mmol), and Ac_2O (1.85 mL, 19.4 mmol) was heated at 140 °C for 5 h. The mixture was concentrated to give crude **7b**.

Ethyl 1-(4-Cyano-2,3,5,6-tetrafluorophenyl)-6,7,8-trifluoro-4-oxo-1,4-dihydroquino-line-3-carboxylate (8). To a solution of **7b** (1.45 g, 4.54 mmol) in THF (30 mL) were added NaH (174 mg, 60% in oil, 4.54 mmol) and 4-amino-2,3,5,6-tetrafluorobenzonitrile (863 mg, 4.54 mmol). The mixture was stirred for 1 h at room temperature then stirred for 1 h at 40 °C. The reaction was quenched with water (10 mL), and the mixture was extracted with ethyl acetate (3 × 30 mL). The organic solution was washed with brine, dried over Na_2SO_4, and concentrated. The residue was purified by column chromatography with 50% ethyl acetate in hexanes to give 1.31 g (65%) of **8** as an orange solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.19 (m, 1H), 8.18 (s, 1H), 4.41 (q, J = 7.6 Hz, 2H), 1.40 (t, J = 7.6 Hz, 3H). Anal. ($C_{18}H_7F_4N_3O_4$) C, H, N.

Ethyl 5,6,7,8-Tetrafluoro-4-oxo-1-(pentafluoro-phenyl)-1,4-dihydroquinoline-3-carboxylate (4). Known compound **4**[32] was prepared in a similar manner as described for the synthesis of **8** except using **5a** instead of **5b** and using 2,3,4,5,6-pentafluoroaniline instead of 4-amino-2,3,5,6-tetrafluorobenzonitrile. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.64 (s, 1H), 4.25 (q, J = 7.2 Hz, 2H), 1.27 (t, J = 7.2 Hz, 3H). HRMS (ESI) $[M + H]^+$ calcd for $C_{18}H_7F_9NO_3$, 456.0277; found, 456.0286.

5,6,7,8-Tetrafluoro-4-oxo-1-(pentafluoro-phenyl)-1,4-dihydroquinoline-3-carboxylic acid (9). Known acid **9**[32] was prepared from the corresponding known ethyl ester **4**. A mixture of **4** (1.0 g, 2.34 mmol) in 25 mL of 2N H_2SO_4 was stirred at 100 °C for 20 h. After cooled to room temperature, the mixture was extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4 and concentrated to give **9** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.93 (s, 1H). HRMS (ESI) $[M + H]^+$ calcd for $C_{16}H_3F_9NO_3$, 427.9970; found, 427.9975.

Benzyl 1-(4-Cyano-2,3,5,6-tetrafluorophenyl)-6,7,8-trifluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (10). Benzyl ester **10** was prepared in a similar manner as described for the synthesis of **8** except using monobenzyl malonate instead of monoethyl malonate. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.79 (s, 1H), 8.13 (m, 1H), 7.48 (m, 2H), 7.39 (m, 2H), 7.34 (m, 1H), 5.33 (s, 2H). HRMS (ESI) $[M + H]^+$ calcd for $C_{24}H_{10}F_7N_2O_3$, 507.0574; found, 507.0568.

1-(4-Cyano-2,3,5,6-tetrafluorophenyl)-6,7,8-trifluoro-*N*-(4-fluorobenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxamide (11). A mixture of **8** (1.0 g, 2.25 mmol) in 25 mL of 2N H_2SO_4 was stirred at 100 °C for 20 h. After cooled to room temperature, the mixture was extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4 and concentrated to give 1-(4-cyano-2,3,5,6-tetrafluoro-phenyl)-6,7,8-trifluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.00 (s, 1H), 8.24 (ddd, J = 10.0, 8.4, 2.0 Hz, 1H). HRMS (ESI) $[M + H]^+$ calcd for $C_{17}H_4F_7N_2O_3$, 417.0111; found, 417.0117.

To a solution of 1-(4-cyano-2,3,5,6-tetrafluorophenyl)-6,7,8-trifluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (35 mg, 0.08 mmol) in a mixture of THF (2 mL) and DCM (1 mL) was added triethylamine (50 μL, 0.4 mmol) and then the mixture was cooled to 0 °C. To the mixture was added ethyl chloroformate (20 μL, 0.21 mmol). The solution was stirred for 30 min at 0 °C and then 4-fluorobenzylamine (13 μL, 0.11 mmol) was added. The solution was stirred for 30 min, upon which LC/MS revealed the reaction was complete. The solvent was removed under vacuum, and the residue was purified by semipreparative HPLC to give **11** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.76 (t, J = 6.0 Hz 1H), 8.91 (s, 1H), 8.20 (m, 1H), 7.40 (m, 2H), 7.16 (m, 2H), 4.56 (d, J = 6.0 Hz, 2H). HRMS (ESI) $[M + H]^+$ calcd for $C_{24}H_{10}F_8N_3O_2$, 524.0640; found, 524.0641.

Supporting Information Available: Table S1 for the inhibition of STAT3 target genes by compound **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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