Abstract

**Purpose:** Signal transducer and activator of transcription 3 (Stat3) proteins have important roles in cancer cell survival and proliferation. Recent studies show that aberrant Stat3 activation promotes tumor growth and survival in several human cancers, and thus, presents an attractive pathway for the development of targeted anticancer therapy. Stat3 is a DNA-binding transcription factor, and thus, its function depends on cytoplasmic to nuclear translocation. To discover novel inhibitors of the Stat3 signaling pathway, we designed a cell-based screening assay capable of identifying compounds that inhibit Stat3 nuclear translocation and activity.

**Experimental Design:** Cell-based fluorescence microscope screening and quantitative measurement of enhanced green fluorescent protein – Stat3 nuclear translocation assays were used to identify novel Stat3 inhibitors. The effects of identified Stat3 inhibitors on Janus kinase (Jak), Stat3 expression, and activation were determined by Western blotting and kinase in vitro autophosphorylation assay. The effects of identified Stat3 inhibitors on cell growth was evaluated by cell proliferation assay and apoptosis assay.

**Results:** Among the National Cancer Institute Diversity set, a 2,000-member library of bioactive small molecules, we identified SD-1029 as a micromolar inhibitor of IL-6 or oncostatin-induced Stat3 nuclear translocation. Biochemical analysis shows that SD-1029 inhibits tyrosyl phosphorylation of Stat3 implicating SD-1029 as an inhibitor of Jak. Further analysis shows that this compound inhibits tyrosyl phosphorylation of the Jak2 isoenzyme. The antiapoptotic proteins Bcl-XL and survivin, target proteins of activated Stat3, are down-regulated by SD-1029 resulting in the induction of apoptosis in several human breast and ovarian cancer cell lines. SD-1029 also enhances apoptosis induced by paclitaxel in ovarian cancer cells.

**Conclusions:** These results show that SD-1029 directly abrogates the Jak-Stat3 signaling pathway in human cancer cells expressing constitutively active Stat, and add to the growing literature that validates this pathway as a viable target for further drug development. Finally, SD-1029 may represent a suitable prototype for structural optimization and exploration as a therapeutic lead.

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Many studies have shown the activation of the interleukin 6 (IL-6) and signal transducer and activator of transcription 3 (Stat3) pathway in a variety of human cancers (1–4). In particular, this pathway has been implicated in the malignant phenotype of numerous solid tumors, including epithelial tumors of the ovary, breast, and prostate, as well as in hematologic malignancies such as multiple myeloma, leukemia, and lymphoma (5–9). In these systems, Stat3 activation is evidenced by increased phosphorylation of Tyr705 followed by dimerization, nuclear translocation, and DNA binding. In vitro models and clinical studies show that Stat3 pathway activation is associated with high-grade tumors, drug resistance, and induction of antiapoptotic proteins such as survivin and Bcl-XL (3, 4, 7, 10). Recently, constitutive activation of Janus kinases (Jak) has been described in a variety of myeloproliferative disorders, and these observations may provide a mechanistic explanation for constitutive Stat activation (11, 12) witnessed in other malignancies. Additionally, cytokine-mediated autocrine or paracrine stimulation of upstream activators may provide an alternative mechanistic explanation of pathway activation. Further evidence for the direct role of Stat3 in...
supporting the malignant phenotype derives from in vitro and in vivo experiments showing the oncogenic potential of Stat3 overexpression (13–15). In corroboration, both inhibition of the Stat3 signaling pathway via the known tool compound and Jak inhibitor, AG490, and transfection of cells with a dominant-negative Stat3 expression construct significantly suppress the growth of ovarian and breast cancer cell lines harboring constitutively active Stat3 (16, 17). Several investigators have additionally provided evidence that inhibition of the Stat3 pathway in several models of human malignancies induces growth arrest and apoptosis (2, 18, 19).

IL-6 serves as a ligand and activates a heterodimeric receptor consisting of two membrane-bound glycoproteins: an 80 kDa IL-6-binding subunit, IL-6Rα, and gp130. The latter molecule serves as the subunit responsible for stabilization of the α-chain ligand complex and subsequent downstream signal transduction. The binding of IL-6 to IL-6R triggers the dimerization of the α-chain to gp130 with subsequent phosphorylation of gp130 by Janus kinases (Jak1, Jak2, Jak3, or Tyk2) leading to the activation of Stat3 (20). Immediately after stimulation of cell surface receptors (such as IL-6R), Stat3 is recruited to activated receptors by an interaction between its Src homology 2 domain and phosphotyrosine docking sites on the receptors’ intracellular domains. Subsequently, Stat3 is phosphorylated (pStat3) at a single tyrosine residue (Tyr705), either directly by the receptor kinase or indirectly by a receptor-associated Jak kinase. Following phosphorylation, Stat3 forms a homodimer via paired Src homology 2 domains. Dimeric pStat3 then translocates from the cytoplasm to the nucleus where it serves a critical role as a transcription factor. This occurs through binding of the dimer to a canonical 8- to 10-bp inverted repeat element with the consensus sequence 5′-TT(N4-6)AA-3′, commonly referred to as an IFN-γ-activated sequence element. Engagement of pStat3 dimers with this element then initiates an increase in the transcription of a number of genes including the apoptotic regulatory genes Bcl-XL, survivin, MCL-1, c-myc, and Fas (21, 22). Stat3 has been identified as the primary target transcription factor mediating IL-6-dependent cell growth, differentiation, and survival. Evidence for this central role is derived from experiments showing that transfection of dominant-negative Stat3 completely inhibits the antiapoptotic effect of IL-6 in carcinoma cells (23). More recent data also supports the role of IL-6 in tumor angiogenesis and the drug-resistant phenotype (24, 25).

Based on these observations supporting Jak-Stat as a target pathway in human cancers, several investigators have worked to develop novel pathway antagonists. Previously, a small molecule was identified which inhibits the growth of breast cancer cells expressing constitutively active Stat3 (26). A second study identified indirubin derivatives which inhibit Stat3 signaling and induce apoptosis in human cancer (19). These and other approaches have identified small molecules that either bind to Stat3 or inhibit Stat3 activation (27–30). None of the prior approaches have used cell-based systems targeting Stat3 nuclear translocation. Cell-based screening techniques have the advantage of identifying small molecule hits that are available to intracellular targets in the presence of serum, and interact with intact signaling pathways. Thus, we established a cell-based, Stat3 nuclear translocation screen to identify novel Stat3 pathway inhibitors. Additionally, this system proved amenable to high-throughput screening techniques. Using this approach, we have identified SD-1029, a small molecule that inhibits the activation and translocation of Stat3 in several human breast and ovarian cancer cell lines. For the purpose of quantitatively discriminating assay positives, we have also developed an image-based, high-content immunofluorescent assay that allows the quantitative detection of nuclear translocation induced or inhibited by a small molecule. This assay allows for dose-ranging measurements for defining IC50 and is compatible with 384-well plate format for possible downstream utility in high-throughput screening.

**Materials and Methods**

**Plasmids, cell lines, antibodies, and small molecules.** The Stat3 and enhanced green fluorescent protein (EGFP) fusion protein expression vector pCORON1000 EGFP-Stat3 (abbreviated as pEGFP-Stat3) was purchased from Amersham Biosciences (Buckinghamshire, United Kingdom). This pEGFP-Stat3 vector was generated by fusing Stat3 to the COOH terminus of EGFP. The expressed EGFP fusion protein contains a full-length functional Stat3. A hamster kidney cell line (BHK-21), human osteosarcoma cell line (U2-OS), and human ovarian cell line (OVCAR8) were stably transfected with pEGFP-Stat3 through selection with G418. Stat3, pstat3, plak1, psrc, Bcl-XL, MCL-1, and survivin antibodies were obtained from Cell Signaling Technologies (Cambridge, MA). Jak2 and plak2 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibody to human actin was obtained from Sigma-Aldrich (St. Louis, MO). AG490 was purchased from Calbiochem (La Jolla, CA).

**Cell culture.** The human ovarian cancer cell lines SKOV-3, SW626, and CaOV-3, the human breast cancer cell lines MDA-MB-468, MDA-MB-435, MDA-MB-231, and MCF-7, and the human osteosarcoma cell line, U2-OS, were obtained from the American Type Culture Collection (Rockville, MD). Dr. Patricia Donahoe (Massachusetts General Hospital, Boston, MA) provided the OV1063, IGROV-1, and OVCAR8 human ovarian cancer cell lines. Paxilaxis-resistant cell lines were established in this laboratory as described previously (31–33). The cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin (all obtained from Invitrogen, Carlsbad, CA). The murine pre-B cell BA/F3 cell line was transformed to cytokine-independent growth by transduction with both the erythropoietin receptor and the constitutively active JAK2V617F kinase (34) or with various TEL-Jak fusions including TEL-Jak1, TEL-Jak2, and TEL-Jak.4 Each of these fusions removed the amino terminal inhibitory domain found in the corresponding Jak, thereby constitutively activating these enzymes.

**National Cancer Institute Diversity set.** The Structural Diversity Set is a library of 1,990 small molecules derived from the almost 140,000 compounds available on plates through the National Cancer Institute (NCI). Detailed information on the selection, structures, and activities of these diversity set compounds can be found on the NCI Developmental Therapeutics Program web site (http://dtp.nci.nih.gov).

**Screening for inhibition of phospho-Stat3 nuclear translocation assays.** The pEGFP-Stat3 vector was stably introduced into hamster kidney BHK-21 cells, human osteosarcoma U-2OS cells, or human ovarian cell OVCAR8. BHK-21, U2-OS, or OVCAR8-derived EGFP-Stat3–expressing cells were seeded at a density of 4,000 cells per well in 96-well plates and incubated overnight at 37°C. The cells were then treated with DMSO alone or a 10 μmol/L concentration of a single NCI Diversity Set compound. After a 4-hour incubation, human recombinant IL-6 (R&D Systems, Minneapolis, MN) was added to the wells to a final concentration of 30 ng/mL for an additional hour of incubation. Human recombinant oncostatin (R&D Systems) was added to the wells

4 R. Levine and G. Gilliland, unpublished data.
of OVCAR8-derived EGFP-Stat3–expressing cells to a final concentration of 60 ng/mL for an additional hour of incubation. IL-6 or oncostatin-dependent nuclear translocation of EGFP-Stat3 was analyzed using an Olympus 1X71 fluorescence microscope and the data were captured as digital images using IPLab Software from Scanalytics (Rockville, MD). AG490 (20 μmol/L) was used in all assays as a positive control. Compounds that inhibit the nuclear redistribution of EGFP-Stat3 after IL-6 treatment were considered as candidate Stat3 pathway inhibitors and were subjected to additional analyses.

Quantitative measurement of EGFP-Stat3 nuclear translocation in 384-well plate format. Stably transfected EGFP-Stat3 BHK-21 cells were seeded at a density of 2,000 cells/well in black, clear-bottomed 384-well clear-bottomed plates from Costar (Cambridge, MA) and incubated overnight at 37°C. The cells were then treated with DMSO alone or a 10 μmol/L concentration of a single NCI Diversity Set compound. After a 4-hour incubation, human recombinant IL-6 was added to the wells to a final concentration of 30 ng/mL following an incubation of 1 hour, cells were fixed with 3.7% formaldehyde, washed, counterstained with Hoechst, and visualized by epifluorescence microscopy (ImageXpress 5000A, Axiom Laboratories) with automated image acquisition. Analysis was done using an analysis script built within the ImageXpress software suite. Individual cells were identified using a Region Detect primary mask to identify the EGFP-rich regions. Nuclei were then identified using the texture ATH secondary mask function to mask the Hoechst-rich regions within each EGFP-rich locus. The analysis script captures average EGFP intensity within both of these regions, along with their respective areas. An average cytoplasmic/nuclear (C:N) EGFP intensity ratio for each cell was calculated, and the mean C:N ratio across all cells was reported as a summary statistic for an individual well.

Western blotting. Protein lysates from cells were generated through lysis with 1× radioimmunoprecipitation assay buffer (Upstate Biotechnology, Charlottesville, VA) and protein concentration was determined by DC Protein assay (Bio-Rad, Hercules, CA). Twenty-five micrograms of total protein was resolved on NuPage 4% to 12% Bis-Tris gel (Invitrogen) and immunoblotted with specific antibodies. Primary antibodies were incubated in TBS (pH 7.4) with 0.1% Tween 20 or a combination of the two drugs for an additional 24 hours. The cells were then lysed by an additional 10 μL of 10% NP40 per well, following the manufacturer's instructions for apoptosis assay.

Cytotoxicity assay. In vitro cytotoxicity was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in the antibodies supplier's instructions. Horseradish peroxidase–conjugated secondary antibodies were incubated in TBS (pH 7.4) with 5% nonfat milk and 0.1% Tween 20, at 1:2,000 dilution for 1 hour at room temperature with gentle agitation. Positive immunoreactions were detected by using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Jak2 kinase in vitro autophosphorylation assay. The Jak2 autophosphorylation kinase assay was done using recombinant Jak2 protein (Upstate Biotechnology). The assay was done as described in the manufacturer's protocol. Briefly, 15 μL of the Jak2 agarose was washed twice with kinase assay buffer, resuspended, and mixed with either DMSO control, SD-1029, or AG490 for 1 hour at room temperature with constant, gentle agitation. Following the addition of 1 mmol/L of ATP and an additional 60 minutes of incubation at room temperature, the reaction was halted using a stop buffer. Inhibition of autophosphorylation of Jak2 kinase was evaluated by Western blot as described above using antiphosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology).

Kinase profile of lead compound SD-1029. The specificity of SD-1029 for Jak kinase was evaluated by commercial Kinase Profiler Selectivity Screening Service from Upstate Biotechnology (http://www.upstate.com/discovery/services/kp_overview.q./Kinase+Profiler). This analysis includes 49 different activated serine/threonine and tyrosine kinase domains by a radiometric filter-binding screening assay.

Apoptosis assay. Whole-cell lysates were immunoblotted with specific antibodies to PARP (Cell Signaling Technologies) and its cleavage products. Positive immunoreactions were detected by using Super Signal West Pico Chemiluminescent Substrate. Apoptosis was also evaluated using the M30-Apoptosense ELISA assay kit (Peviva AB, Bromma, Sweden) according to the manufacturer's instructions. For the paclitaxel and AG490 treated, OVCAR8 cells were seeded at 8,000 cells/well in a 96-well plate for 24 hours before treatment. The cells were then treated with 0.01 μmol/L of paclitaxel, 20 μmol/L of AG490, or a combination of the two drugs for an additional 24 hours. The cells were then lysed by an additional 10 μL of 10% NP40 per well, following the manufacturer's instructions for apoptosis assay.

Western blot analysis of EGFP-Stat3 nuclear translocation in BHK-21 and U-2OS cells. BHK-21 or U-2OS cells which stably express the EGFP-Stat3 fusion protein were incubated for 4 hours with DMSO (control), SD-1029 (10 μmol/L), or AG490 (20 μmol/L) followed immediately thereafter with the addition of IL-6 to a final concentration of 30 ng/mL. Subcellular localization of the fusion protein was assessed by fluorescence microscopy. A, BHK-21-derived pEGFP-Stat3 expression cells; B, U-2OS-derived pEGFP-Stat3 expression cells; C, SD-1029 structure.
previously described (31). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Sigma-Aldrich. Briefly, 2 × 10^3 cells per well were plated in 96-well plates. Cells were plated in RPMI 1640 containing increasing concentrations of paclitaxel and SD-1029 at final concentrations of 3, 5, and 10 μmol/L, respectively. After 7 days of culture in paclitaxel, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/mL in PBS) was added to each well and the plates were incubated for 4 hours. The resulting formazan product was dissolved with acid-isopropanol and the absorbance at a wavelength of 490 nm (A₄₉₀) was read on a BT 2000 Microkinetics Reader. The absorbance values were normalized, assigning the value of the parent line in medium without drug to 1.0, and the value of the no-cell control to 0. Experiments were done in duplicate.

**BA/F3 cells bearing TEL-Jak fusion proteins.** The BA/F3 cell line, an IL-3-dependent murine pro–B cell line was stably transduced with MSCV retroviral supernatants containing either TEL-Jak1, TEL-Jak2, or TEL-Jak3-IRES GFP supernatants as previously described (35). The BA/F3-EPOR-Jak3V617F cell line was derived by stable transduction with MSCV-EPOR-Neo, selected in G418/Neomycin (1 mg/mL) and then transduced with retroviral supernatant containing JakV617F-Puromycin and then selected in both neomycin and puromycin (2 μg/mL). These lines, unlike the parental line, are transformed to cytokine-independent growth and result in constitutive activation of Stat3 and Stat5. For dose-response curves, different Jak-transfected and Stat5-activated BA/F3 cells were incubated for 72 hours in the presence of different concentrations of SD-1029 or AG490. Viable cells were determined with the CellTiter 96 AQueous One solution proliferation assay (Promega, Madison, WI). Dose-response curves were fitted with the use of GraphPad PRISM 4 software (GraphPad Software, San Diego, CA).

**Results**

**Real-time analysis of Stat3 nucleocytoplasmic translocation and identification of SD-1029.** In an attempt to identify novel compounds that interrupt IL-6-dependent Stat3 nuclear translocation, a novel real-time cell-based method was developed to image an EGFP-Stat3 chimera in the nucleus and cytoplasm in the hamster kidney cell line BHK-21, human osteosarcoma cell line U-2OS, and the human ovarian cancer cell line OVCAR8. In resting cells, the majority of EGFP-Stat3 was cytoplasmic [Fig. 1A(a) and B(a)], until the addition of human IL-6, which promptly induced the translocation of fluorescent Stat3 molecules to the nucleus in BHK-21 and U-2OS cells [Fig. 1A(b) and B(b)]. Similar results were also seen in the human ovarian cancer cell line OVCAR8 transfected with pEGFP-Stat3 and treated with oncostatin (data not shown). Pretreatment of the cells with the Jak inhibitor AG490 (20 μmol/L) blocked IL-6-dependent translocation of EGFP-Stat3 [Fig. 1A(c) and B(c)]. Preliminary time and dose titration experiments determined...
that the greatest fluorescent intensity of nuclear EGFP-Stat3 was achieved between 30 and 60 minutes after stimulation with IL-6 at a concentration of 30 to 60 ng/mL (data not shown). We evaluated a 1,992-member library of diverse bioactive small molecules for agents capable of blocking Stat3 nuclear translocation using this screening assay as described in Materials and Methods. Visual analysis of the screening results indicated that several compounds inhibited Stat3 nuclear translocation. The most potent of these compounds was SD-1029 (NCI identifier: NSC 371488; Fig. 1C), which suppressed EGFP-Stat3 nuclear translocation at a concentration of 10 μM/L in both BHK-21 and U2-OS cells ([Fig. 1A(d) and B(d)].

Quantitative assessment of Stat3 nuclear translocation and confirmation of SD-1029 inhibition. Although the initial screen was done manually using visual inspection to determine assay positives, we became interested in quantitatively measuring the effect of small molecules on translocation. An unbiased, semiautomated high-throughput approach was developed. First, the above screen of BHK-21 cells was miniaturized and found to be highly compatible with the 384-well plate format. An optimized methodology was then developed for cell seeding, adherence, fixation, nuclear staining, and washing. Protocols for cell-by-cell, two-channel fluorescent detection and capture of nuclear (Hoechst) and total cellular EGFP-Stat3 signals were developed using automated epifluorescent microscopy. Acquisition and analysis scripts for the detection and quantitative assessment of nuclear and cytoplasmic EGFP-Stat3 signal were developed using the ImageXPress software suite (Molecular Devices, Sunnyvale, CA). This technique was optimized using experimentally derived demonstrative images of nuclear translocation after IL-6 treatment (data not shown). With this protocol, as many as 4,000 cells are assessed for nuclear translocation in a single well of a 384-well plate, and quantitative data are obtained using automated analysis masks (Fig. 2A). A composite cytoplasmic/nuclear score across the field of cells is then determined.

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\frac{C : N = \frac{[T^*AT] - (N^*AN)}{(AT - AN)}}
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where C is the average cytoplasmic EGFP intensity; N, average nuclear EGFP intensity; T, average total cell EGFP intensity; AT, area total cell; and AN, the area of nucleus. Using this methodology, we determined that increasing concentrations of SD-1029 or AG490 increased the cytoplasmic/nuclear EGFP-Stat3 signal following IL-6 treatment. This is consistent with the inhibition of IL-6-dependent Stat3 nuclear translocation. Dose-ranging studies confirmed a dynamic range of this assay compatible with IC50 determination and comparative stratification of ligands. Maximum inhibition of nuclear translocation was seen at a concentration of 20 μM/L of AG490 and 5 μM/L for SD-1029 (Fig. 2B). Notably, significant inhibition of Stat3 nuclear translocation was witnessed at 1.25 μM/L of SD-1029. At higher concentrations of SD-1029, cell death accompanied by decreased adherence and aberrant morphology resulted in a declining cytoplasmic/nuclear signal, illustrating an advantageous feature of high-content imaging in the biological annotation and screening of small molecules.

SD-1029 suppresses pStat3 levels in human breast and ovarian cancer cell lines. After the identification of SD-1029 as an inhibitor of Stat3 nuclear translocation in BHK-21 cells, the effect of SD-1029 on Stat3 phosphorylation was examined in human cancer cell lines. First, human ovarian and breast cancer cell lines with constitutively high levels of pStat3 were identified by immunoblot (Fig. 3A): MDA-MB-468 and MDA435 (breast cancer), OVI1063 (ovarian cancer), and the paclitaxel-resistant ovarian cancer daughter lines, SKOV-3-TR and OVCAR8-TR. In each of these cell lines, incubation in 10 μM/L of SD-1029 for 24 hours led to reduced levels of pStat3 (Fig. 3B). To evaluate the time- and dose-dependent inhibition of Stat3 activation, the MDA-MB-468 and OVCAR8-TR cell lines were treated with SD-1029 (10 μM/L) for varying time periods, or alternatively with varying doses (1, 5, and 10 μM/L) for 24 hours. The pStat3 level decreased as early as 30 minutes after the addition of 10 μM/L of SD-1029. With a longer 24-hour treatment, significant inhibition of Stat3 phosphorylation was witnessed when cultured in 1 μM/L of SD-1029 (Fig. 3C), consistent with the derived phenotypes and quantitative methods of the high-content imaging protocol. Importantly, SD-1029 had no significant effect on the total amount of cellular Stat3 protein as determined qualitatively by immunoblot (Fig. 3B and C). We further examined whether or not SD-1029 could inhibit the AKT signaling pathway; SD-1029 did not significantly affect the level of phosphorylation of AKT (data not shown).

In addition, further biochemical analysis of SD-1029 was done by a commercial kinase profile service using 49 different kinases. This study evaluated the ability of 10 μM/L of SD-1029 to inhibit activated kinase domains including Jak2. Analysis showed that SD-1029 did not produce a significant inhibition of any of the activated kinases at this single
concentration point. The discrepancy between the profile result and the in vitro autophosphorylation kinase assay on Jak2 may be explained by the use of the activated Jak2 domain in the commercial kinase profiling assay whereas whole inactivated Jak2 protein was used in the autophosphorylation kinase assay. These data suggest that SD-1029 may selectively bind inactivated Jak2 protein kinase.

**SD-1029 inhibits Stat3-mediated antiapoptotic protein expression.** Stat3 phosphorylation and nuclear translocation are required for Stat3 transcriptional activity. We hypothesized that inhibition of nuclear transport should suppress transcription and subsequent translation of Stat3-dependent genes. Therefore, we examined whether the exposure of cell lines to SD-1029 resulted in decreased expression of the antiapoptotic proteins Bcl-XL, MCL-1, and survivin. Incubation in SD-1029 for 24 hours significantly down-regulated Bcl-XL and survivin expression in both OVCAR-8TR and MDA-MB-468 cells (Fig. 4). During this incubation period, the expression of MCL-1 protein expression also decreased in OVCAR-8TR but did not change in MDA-MB-468 cells (Fig. 4).

**SD-1029 suppresses phosphotyrosine levels of Jak2.** SD-1029 suppresses pStat3 levels suggesting that this compound might interfere with the function of one or more of the upstream tyrosine kinases such as Jak or Src. Evaluation of the effect of SD-1029 on the phosphotyrosine levels of Jak1, Jak2, and Src in the OVCAR-8TR and MDA-MB-468 cell lines in vitro showed the significant suppression of tyrosine phosphorylated Jak2 levels. Tyrosine phosphorylation of Jak1 and Src was unaffected in comparison to the inhibition of Jak2 tyrosine phosphorylation (Fig. 5A). To more directly evaluate the effects of SD-1029 on Jak2, a cell-free in vitro assay was done by incubating recombinant Jak2 with SD-1029. Analysis by Western blot showed that SD-1029 is a more effective inhibitor of Jak2 autophosphorylation compared with AG490 (Fig. 5B).

**Effect of SD-1029 on cell growth in cells with activated Stat pathways.** To confirm that the Jak-Stat pathway is a target of SD-1029, we tested the effect of SD-1029 on the growth of cytokine-independent BA/F3 cells expressing different TEL-Jak fusion proteins or expressing JAK2V617F. The proliferation of BA/F3 cells expressing activated Jak1, Jak2, and Jak3 was efficiently inhibited by SD-1029 or AG490 (Fig. 6A).

**SD-1029 induces apoptosis in human cancer cells.** The effect of SD-1029 on the induction of apoptosis was assessed by evaluating PARP cleavage (Fig. 6B) and by M-30-Apoptosense ELISA (Fig. 7). PARP cleavage was detected 6 hours following incubation with 10 μmol/L of SD-1029. A dose-response analysis revealed the appearance of PARP cleavage products in the presence of 1 μmol/L of SD-1029 when the cells were allowed to incubate for 24 hours (Fig. 6B).
SD-1029 enhances apoptosis induced by paclitaxel in human cancer cells. Constitutively activated Stat3 may contribute to the survival advantage of human cancer cells, in part through the induction of antiapoptotic regulatory proteins. We hypothesized that inhibition of this pathway and its dependent apoptotic proteins would lower the apoptotic threshold and increase chemotherapy sensitivity. To investigate this, several pStat3-activated cell lines were treated with either SD-1029 (5 or 10 μmol/L) alone, paclitaxel alone, or the combination of paclitaxel and SD-1029 for 24 hours. Apoptosis was scored using the M30-Apoptosense ELISA assay. SD-1029 treatment resulted in a marked, 20-fold induction of apoptosis in the OVCAR8TR cells that express constitutively activated Stat3 (Fig. 7A). The combination of paclitaxel and SD-1029 resulted in significantly greater cell death as compared with paclitaxel or SD-1029 alone. Additionally, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assay also showed that SD-1029 has synergic effect on paclitaxel-induced cell death (Fig. 7B).

**Discussion**

Nuclear translocation of activated pStat3 is a crucial event for its transcriptional function (20, 36). Blocking the phosphorylation and translocation of Stat3 using small molecules is a rational approach for the inhibition of the Stat3 signaling pathway in human cancer. In this study, we report that SD-1029 is the first inhibitor of Stat3 signaling selected by a cell-based assay of Stat3 nuclear translocation. SD-1029 inhibits the growth of several different human cancer cell lines including those that are dependent on constitutive Jak expression. The inhibition of Jak2 phosphorylation in cell culture and in cell-free autophosphorylation in vitro assays suggests that the principal mechanism of SD-1029 is Jak inhibition. The inhibition of a panel of BA/F3 lines transfected with constitutively active Jak genes suggests that SD-1029 is likely a pan-Jak inhibitor. This is supported in part by cell culture data that shows modest Jak1 inhibition at higher concentrations of the drug. Further biochemical analysis of SD-1029 will be required to determine its Jak isoenzyme and global kinase inhibitory profile. Interestingly, SD-1029 inhibits the proliferation of BA/F3 cells transfected with the TEL-Jak2 mutant or the JakV617F mutant. JakV617F is the preferred pro-growth mutant found in many myeloproliferative diseases, most notably polycythemia vera. As anticipated, in the incubation of human ovarian cancer cell lines with SD-1029 down-regulates Stat3-dependent protein expression and cell proliferation, and induces apoptotic cell death. Lowering of the apoptotic threshold also increases the sensitivity of these cells to the cytotoxic effects of paclitaxel.

An important downstream effect of Stat3 activation is the Stat3-dependent regulation of several antiapoptotic genes including Bcl-XL, survivin, and MCL-1. Many studies have found these survival-promoting genes are highly expressed in human cancer, especially in high-grade tumors (3, 7). For example, we have recently shown that Stat3 and more notably pStat3 expression are elevated in recurrent tumors collected post-chemotherapy as compared with matched primary tumors collected prior to chemotherapy. The finding that SD-1029 induces apoptosis in cells that express constitutively activated Stat3 both as a single agent and when combined with paclitaxel suggests that SD-1029 or other agents that target the Stat3 pathway may be useful in the clinic. The finding that Bcl-XL and survivin expression are significantly reduced in the cancer cells treated with SD-1029 is consistent with the hypothesis that the decrease in Bcl-XL and survivin expression contributes to the induction of apoptosis (3, 4). This hypothesis is also supported by a recent study showing that the novel Stat3 target gene Bcl-XL inhibitor, ABT-737, significantly enhanced the activities of paclitaxel in lung cancer cells (37).

This study provides evidence that phenotypic screening using a cell-based assay is an effective strategy for identifying inhibitors of the Jak-Stat pathway. SD-1029 distinguishes itself from other inhibitors of the Jak-Stat pathway as it was identified through a cell-based screen and an ability to inhibit pStat3 nuclear translocation. More importantly, this biologically

![Fig. 6](image-url)
Materials and Methods.

Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in Materials and Methods. SD-1029 inhibits cell growth and induces apoptosis in OVCAR8OV ovarian cancer cells. OVCAR8OV cells were seeded at a density of 8,000 cells per well in a 96-well plate for 24 hours. Cells were then treated with different drugs for 24 hours. The cells were lysed with 10% NP40 and the M30-Apoptosense ELISA assay was done as described in Materials and Methods. Effect of varying concentrations of SD-1029 on paclitaxel-induced cell death in OVCAR8OV cells by 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide assay as described in Materials and Methods.

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Fig. 7. SD-1029 enhances apoptosis induced by paclitaxel in human cancer cells. A, SD-1029 inhibits cell growth and induces apoptosis in OVCAR8OV ovarian cancer cells. OVCAR8OV cells were seeded at a density of 8,000 cells per well in a 96-well plate for 24 hours. Cells were then treated with different drugs for 24 hours. The cells were lysed with 10% NP40 and the M30-Apoptosense ELISA assay was done as described in Materials and Methods. B, effect of varying concentrations of SD-1029 on paclitaxel-induced cell death in OVCAR8OV cells by 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide assay as described in Materials and Methods.
SD-1029 Inhibits Signal Transducer and Activator of Transcription 3 Nuclear Translocation

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