

8-Benzyl-4-oxo-8-azabicyclo[3.2.1]oct-2-ene-6,7-dicarboxylic Acid (SD-1008), a Novel Janus Kinase 2 Inhibitor, Increases Chemotherapy Sensitivity in Human Ovarian Cancer Cells

Zhenfeng Duan, James Bradner, Edward Greenberg, Ralph Mazitschek, Rosemary Foster, Jennifer Mahoney, and Michael V. Seiden

Department of Hematology/Oncology, Massachusetts General Hospital, Boston, Massachusetts (Z.D., R.S., J.M., M.V.S.); and Chemical Biology Program, Broad Institute of Harvard, and Massachusetts Institute of Technology, Cambridge, MA (J.D., E.G., R.M.)

Received May 14, 2007; accepted August 3, 2007

ABSTRACT

Interleukin 6 and the signal transducer and activator of transcription (STAT) 3 proteins have important roles in cancer cell survival and proliferation. Recent studies demonstrate that abnormal STAT3 activation promotes tumor growth and supports survival of many human cancers, and thus, this protein or the pathway responsible for its activation is a potential target for the new anticancer therapy. STAT3 is a DNA binding transcription factor, and therefore, its function depends on nuclear translocation. To discover inhibitors of the STAT3 pathway, we designed a cell-based screening assay capable of identifying small molecules that inhibit nuclear translocation. Among the 2000-compound National Cancer Institute Diversity set, we identified 8-benzyl-4-oxo-8-azabicyclo[3.2.1]oct-2-ene-6,7-dicarboxylic acid (SD-1008) as a micromolar inhibitor of interleukin-6 or oncostatin-induced STAT3 nuclear translocation. In

addition, SD-1008 inhibits tyrosyl phosphorylation of STAT3, Janus kinase 2 (JAK2), and Src. SD-1008 also reduces STAT3-dependent luciferase activity. Biochemical studies with recombinant JAK2 proteins demonstrate that high concentrations of SD-1008 directly inhibit JAK2 kinase autophosphorylation. Exposure of various cell lines to SD-1008 decreases levels of the STAT3-dependent proteins, Bcl-X_L and survivin, inducing apoptosis. SD-1008 also enhances apoptosis induced by paclitaxel in ovarian cancer cells. These results demonstrate that SD-1008 directly blocks the JAK-STAT3 signaling pathway in human cancer cells that express constitutively active Stat and add to the growing literature that identifies this pathway as a viable target for drug development. Finally, SD-1008 may be a suitable prototype for further chemical modification and exploration as a therapeutic agent.

Many recent studies have demonstrated expression and activation of the interleukin and signal transducer and activator of transcription (STAT) 3 pathway in a variety of human cancers (Gamero et al., 2004; Yu and Jove, 2004; Diaz et al., 2006; Gritsko et al., 2006; Barré et al., 2007). This path-

way seems to be active in numerous solid tumors, including tumors of the ovary, breast, and prostate, and in hematologic malignancies such as leukemia, multiple myeloma, and lymphoma (Catlett-Falcone et al., 1999; Bromberg and Darnell, 2000; Turkson and Jove, 2000; Syed et al., 2002; Silver et al., 2004). In these diseases, STAT3 activation is evidenced by increased phosphorylation of Tyr⁷⁰⁵ followed by nuclear translocation and DNA binding. Both in vitro models and clinical studies of a variety of tumors demonstrate that STAT3 pathway activation is associated with high-grade tumors, drug resistance, and induction of antiapoptotic proteins such as survivin and Bcl-X_L (Silver et al., 2004; Ikuta et al., 2005; Vigneron et al., 2005; Diaz et al., 2006; Duan et al., 2006b; Gritsko et al., 2006). A pivotal role of this pathway in promoting oncogenesis has been demonstrated through the identification of activating Janus kinase (JAK) point muta-

This project was supported by the Ovarian Cancer Specialized Program in Research Excellence at the Dana-Farber/Harvard Cancer Center, grant 1P50-CA105009 from the Ovarian Cancer Education and Awareness Network, and support through the Lana Vento Foundation. Z.D. is supported, in part, through a grant from the Marsha Rivkin Foundation. M.V.S. is supported through a Mid Career Development Award, 1K24-CA109416-01A1. J.B. is supported by grants from the Multiple Myeloma Research Foundation and the Clinical Investigator Training Program: Harvard-MIT Health Sciences and Technology–Beth Israel Deaconess Medical Center, in collaboration with Pfizer Inc. and Merck and Company, Inc.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.107.038117.

ABBREVIATIONS: STAT3, signal transducer and activator of transcription 3; IL-6, interleukin 6; JAK, Janus-activated kinase; NCI, National Cancer Institute; SD-1008, 8-benzyl-4-oxo-8-azabicyclo[3.2.1]oct-2-ene-6,7-dicarboxylic acid; DMSO, dimethyl sulfoxide; EGFP, enhanced green fluorescent protein; PARP, poly(ADP-ribose) polymerase; ELISA, enzyme-linked immunosorbent assay; AG490, α -cyano-(3,4-dihydroxy)-*N*-benzylcinnamide; SD-1029, 9-(3,4-dichlorophenyl)-2,7-bis(dimethylaminomethyl)-3,4,5,6,7,9-hexahydro-2*H*-xanthene-1,8-dione.

tions in a variety of myeloproliferative disorders, providing genetic evidence for observed, constitutive Stat activation (Levine et al., 2005a; Tefferi and Gilliland, 2005). Mechanisms including cytokine-mediated autocrine or paracrine stimulation of upstream activators such as Src have also been described and may provide alternative mechanistic explanations of pathway activation. Studies involving transgenic animals also support the direct role of STAT3 in the malignant phenotype (Cao et al., 1996; Bromberg et al., 1998; Bromberg, 2002). Inhibition of the STAT3 signaling pathway via the known tool compound and JAK2 inhibitor, AG490, and transfection of cells with a dominant-negative STAT3 expression construct significantly suppress the growth of ovarian and breast cancer cell lines with constitutively active STAT3 (Huang et al., 2000; Mora et al., 2002). Likewise, inhibition of the STAT3 pathway in several models of human malignancies induces growth arrest and apoptosis (Yu and Jove, 2004; Nam et al., 2005; Turkson et al., 2005). More recent experiments also support the role of IL-6 in tumor angiogenesis and the drug-resistant phenotype (Bellone et al., 2005; Nilsson et al., 2005).

IL-6 associated activation of the JAK-STAT3 pathway signals through a heterodimeric receptor (IL-6R) consisting of two membrane-bound glycoproteins: an 80-kDa IL-6-binding subunit, IL-6R α , and gp130. The gp130 component of the receptor is responsible for stabilization of the α -chain ligand complex and subsequent downstream signal transduction. The binding of IL-6 to its receptor triggers the association of the α -chain with gp130 and subsequent phosphorylation of gp130 by Janus-activated kinase (JAK1, JAK2, JAK3, or Tyk2), leading to activation of STAT3 (Schindler and Strehlow, 2000). STAT3 binds activated receptors through an interaction between its Src homology 2 domain and phosphotyrosine docking sites on the intracellular domains of activated receptors. JAK- or gp130-dependent STAT3 phosphorylation (pSTAT3) at a single tyrosine residue (Tyr⁷⁰⁵) induces homodimerization of STAT3 via paired Src homology 2 domains. Dimeric pSTAT3 then translocates from the cytoplasm to the nucleus, where it binds DNA as a transcription factor. STAT3 dimers bind a canonical 8- to 10-base pair inverted repeat element with a consensus sequence 5'-TT(N₄₋₆)AA-3', commonly referred to as an interferon- γ -activated sequence element. Engagement of pSTAT3 with DNA then initiates an increase in transcription of a number of genes, including the antiapoptotic regulatory genes Bcl-X_L, survivin, MCL-1, and c-myc (Darnell, 1997; Alvarez et al., 2005).

The credentialing of JAK-Stat as a target pathway in human cancers has led to a search for novel pathway antagonists. Previously our group and others have described STAT3-specific small interfering RNA or small molecules that inhibit cell growth and increase chemotherapy drug-induced apoptosis in cancer cell lines that express constitutively active STAT3 (Faderl et al., 2005; Nam et al., 2005; Song et al., 2005; Duan et al., 2006b). Several small molecules have been identified to inhibit STAT3 signaling by cell-free systems (Faderl et al., 2005; Nam et al., 2005; Song et al., 2005; Turkson et al., 2005; Pedrazzini et al., 2006; Schust et al., 2006). None of the prior approaches have used cell-based systems targeting STAT3 nuclear translocation. Our recently published study was the first to use a cell-based system targeting STAT3 nuclear translocation (Duan et al., 2006a). Cell-based screening techniques have the advantage of identifying small-molecule hits that are accessible to intracellular targets, active with intact signaling path-

ways, and in the presence of serum. This cell-based STAT3 nuclear translocation screen has identified a low micromolar inhibitor of STAT3 nuclear translocation and has proven amenable to high-throughput screening (Duan et al., 2006a). Here we describe the identification of a JAK kinase inhibitor, a polycyclic dicarboxylic acid belonging to the tropidine compound class, here referred to as SD-1008. This compound has been characterized as a potent inhibitor of IL-6, oncostatin, and STAT3 signaling in human breast and ovarian cancer cell lines. SD-1008 also enhances apoptosis induced by paclitaxel in human cancer cells.

Materials and Methods

Plasmids, Cell Lines, Antibodies, and Small Molecule Library. The STAT3 and enhanced green fluorescent protein (EGFP) fusion protein expression vector pCORON1000 EGFP-STAT3 (herein known as pEGFP-STAT3) was purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). This pEGFP-STAT3 vector was generated by fusing STAT3 to the C terminus of EGFP. The expressed EGFP fusion protein comprises 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, pJAK1, pSrc, Bcl-X_L, MCL-1, pAKT, and survivin antibodies were obtained from Cell Signaling Technology (Danvers, MA). JAK2 and pJAK2 antibodies were obtained from Upstate Biotechnology (Charlottesville, VA). Monoclonal antibody to human actin was obtained from Sigma (St. Louis, MO). AG490 was purchased from Calbiochem (La Jolla, CA). The human ovarian cancer cell line SKOV-3, human breast cancer cell lines MDA-MB-468 and MDA-MB-435, and human osteosarcoma cell line U-2OS were obtained from the American Type Culture Collection (Manassas, VA). Dr. Patricia Donahoe (Massachusetts General Hospital, Boston, MA) provided the OV1063 and OVCAR8 human ovarian cancer cell lines. The paclitaxel-resistant cell lines were established in this laboratory as described previously (Duan et al., 1999, 2005; Lamendola et al., 2003). The cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin (all obtained from Invitrogen, Carlsbad, CA). The Structural Diversity Set is a library of 1990 small molecules derived from the almost 140,000 compounds available on plates through the NCI. Detailed information on the selection, structures, and activities of these diversity set compounds can be found on the NCI Developmental Therapeutics Program website (available at <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, U-2OS, or OVCAR8-derived EGFP-STAT3-expressing cells were seeded at a density of 4000 cells/well in 96-well plates followed by an overnight incubation at 37°C. Cells were then incubated for an additional 4 h in the presence of either vehicle (DMSO) alone or a 10 μ M concentration of a single compound from the NCI Diversity Set. After 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. In separate experiments, human recombinant oncostatin (R&D Systems) was added to the wells of OVCAR8-EGFP-STAT3 cells to a final concentration of 60 ng/ml for an additional hour. IL-6 or oncostatin-dependent nuclear translocation of EGFP-STAT3 was analyzed using an Olympus 1 \times 71 fluorescence microscope (Olympus, Tokyo, Japan), and the data were captured as digital images using IPLab Software from Scanalytics (Rockville, MD). AG490 (20 μ M) was used in all assays as a positive control.

Quantitative Measurement of EGFP-STAT3 Nuclear Translocation in 384-Well Plate Format. Detailed description of the

quantitative measurement of EGFP-STAT3 nuclear translocation is described elsewhere (Duan et al., 2006b). In brief, stably transfected EGFP-STAT3 BHK-21 cells were seeded in black, clear-bottomed 384-well plates from Costar (Cambridge, MA). After incubation, cells were fixed, washed, counterstained with Hoechst, and visualized by epifluorescent microscopy using automated image acquisition. Analysis was performed using a modified script within the MetaXPress software suite (Molecular Devices, Sunnyvale CA). Individual cells were identified using a primary mask established by fluorescence intensity due to EGFP. Nuclei were then identified using a secondary mask established by fluorescence intensity due to Hoechst staining within identified EGFP-rich loci. Average EGFP intensity within both regions is captured along with the cytoplasmic and nuclear area. An average cytoplasmic/nuclear EGFP intensity ratio for each cell was calculated, and the mean cytoplasmic/nuclear 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) with protein concentration determined by Dc Protein assay (Bio-Rad Laboratories, Hercules, CA). Twenty-five micrograms of total protein was resolved on Nu-Page 4 to 12% Bis-Tris Gel (Invitrogen) and immunoblotted with specific antibodies. Primary antibodies were incubated in Tris-buffered saline, pH 7.4, with 0.1% Tween 20 and 5% nonfat milk (Bio-Rad) and overnight at 4°C as described in the antibody supplier's instructions. Signal was generated through incubation with horseradish peroxidase-conjugated secondary antibodies incubated in Tris-buffered saline, pH 7.4, with 5% nonfat milk and 0.1% Tween 20 at 1:2000 dilution for 1 h at room temperature. 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 performed using full-length recombinant JAK2 protein (Upstate Biotechnology) as described in the manufacturer's protocol. In brief, 15 μ l of JAK2 agarose was washed with kinase assay buffer, resuspended, and mixed with either DMSO control, SD-1008, or AG490 for 1 h at room temperature with constant, gentle agitation. After the addition of 1 mM ATP and an additional 60 min of incubation at room temperature, the reaction was concluded with a termination buffer. Relative autophosphoryla-

tion of JAK2 kinase was evaluated by Western blot as described above using antiphosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology).

Establishment of STAT3-Dependent Luciferase Reporter in Ovarian Cancer Cells. The pLucTKS3 vector (provided by Dr. Richard Jove, Lee Moffitt Cancer Center and Research Institute, Tampa, FL) contains seven copies of STAT3-binding sites, and its activation specifically depends on STAT3 status in the cell (Turkson et al., 1998). The pLucTKS3 and pIRESneo (Clontech, Mountain View, CA, for G418 resistance coselection) plasmids were cotransfected into OVCAR8_{TR} cell line by using Lipofectamine 2000 reagent and were selected with G418. Clones were selected for high luciferase activity and were used for evaluation. The selected clones were exposed to SD-1008 at varying concentrations, and luciferase activity was measured after 24 h of exposure by using a Promega Bright-Glo luciferase kit following the manufacturer's instructions.

Apoptosis Assays. Whole-cell lysates were immunoblotted with specific antibodies to PARP (Cell Signaling Technology) 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 drug exposure experiments, OVCAR8_{TR} cells were seeded at 8000 cells/well in a 96-well plate for 24 h before treatment. The cells were then treated with 0.01 μ M paclitaxel, 30 μ M AG490, 5 or 10 μ M SD-1008, or combinations of paclitaxel with either AG490 or SD-1008 drugs for additional 24 h.

Results

Analysis of STAT3 Nuclear Translocation and Identification of SD-1008. To identify novel compounds that interrupt IL-6-dependent STAT3 nuclear translocation, a novel real-time cell-based method was developed to image the EGFP-STAT3 chimera in the nucleus and cytoplasm in the hamster kidney cell line BHK-21, human osteosarcoma cell line U-2OS, and human ovarian cancer cell line OVCAR8. Resting cells demonstrated that the majority of EGFP-STAT3 was cytoplasmic (Fig. 1, Aa and Ba) until the

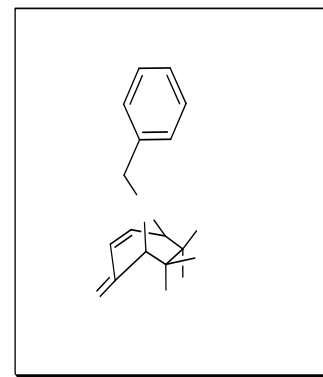
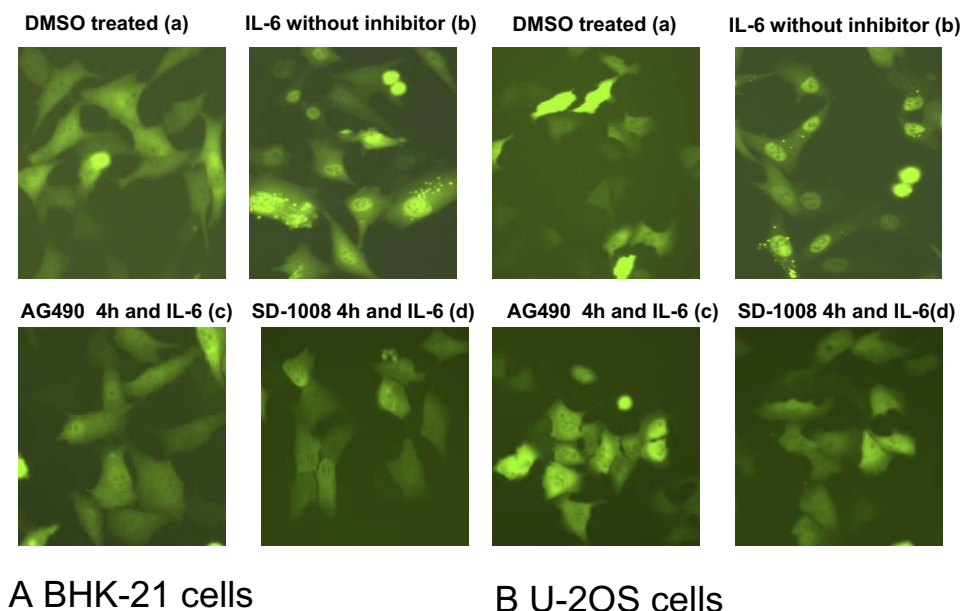


Fig. 1. SD-1008 inhibits EGFP-STAT3 nuclear translocation in BHK-21 and U-2OS cells. BHK-21 or U-2OS cells that stably express the EGFP-STAT3 fusion protein were incubated for 4 h with DMSO (control), SD-1008 (10 μ M), or AG490 (20 μ M) followed immediately thereafter with the addition of IL-6 to a final concentration of 30 ng/ml rIL-6. Cells were photographed 1 h later. 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-1008 structure.

addition of IL-6, which then promptly induced translocation of fluorescent STAT3 to the nucleus in BHK-21 and U-2OS cells (Fig. 1, Ab and Bb). 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 JAK2 inhibitor AG490 (20 μ M) blocked IL-6-dependent translocation of EGFP-STAT3 (Fig. 1, Ac and Bc). A screen of a 1992-member library of diverse bioactive small molecules identified several compounds that inhibited STAT3 nuclear translocation. One of the most potent of these compounds, SD-1008 (NCI identifier, NSC 360215; Fig. 1C), suppressed EGFP-STAT3 nuclear translocation at a concentration of 10 μ M in both BHK-21 and U-2OS cells (Fig. 1, Ad and Bd). Compound SD-1008 was analyzed by liquid chromatography/mass spectrometry to verify the identity and purity. We were surprised to find that the analysis yielded a 28 mass units greater molecular mass (329.04 atomic mass units) than predicted based on the reported structure (301.10 atomic mass units). For further structure elucidation, we analyzed the SD-1008 compound by one-dimensional and two-dimensional ^1H NMR. Based on this analysis, we determined the structure to correspond to the bis methyl ester of the reported structure. The vicinal coupling constants predict a relative stereochemistry of SD-1008 (Fig. 1C). In addition, we further analyzed and compared the SD-1008 structure with SD-1029 and AG490. The results demonstrated that there is no plausible structural similarity between the three classes of compounds; the only common motif that is shared by SD-1008 and SD-1029 is the aromatic substituent and the basic nitrogen. SD-1008 belongs to tropidine compound class, and SD-1029 belongs to xanthene compound class.

Quantitative Assessment of STAT3 Nuclear Translocation and Confirmation of SD-1008 Inhibition. In an attempt to generate a quantitative technique for scoring translocation, we developed and have recently described an unbiased, semiautomated, high-throughput approach to measure relative cytoplasmic to nuclear signal after IL-6 treatment. Maximum inhibition of nuclear translocation was seen at a concentration of 20 μ M AG490 and 10 μ M SD-1008 (Fig. 2). It is noteworthy that SD-1008 shows more robust inhibition of IL-6-dependent nuclear translocation than AG490.

SD-1008 Suppressed pSTAT3 Levels in Human Breast and Ovarian Cancer Cell Lines. After identification of SD-1008 as an inhibitor of STAT3 nuclear translocation in BHK-21 cells, the effect of SD-1008 on STAT3 phosphorylation was examined in human cancer cell lines. Studies focused on the breast cancer lines MDA-MB-468 and MDA435, the ovarian cancer cell line OV1063, and the paclitaxel-resistant ovarian cancer lines SKOV-3_{TR} and OVCAR8_{TR}, all of which express high levels of pSTAT3. In each of these cell lines, incubation in 10 μ M SD-1008 for 24 h led to reduced levels of pSTAT3 (Fig. 3A). To evaluate the time- and dose-dependent inhibition of STAT3 activation, the MDA-MB-468 and OVCAR8_{TR} cell lines were treated with SD-1008 (10 μ M) for varying time periods or alternatively with varying doses (1, 5, and 10 μ M) for 24 h. The pSTAT3 level decreased as early as 30 min after the addition of 10 μ M SD-1008 treatment. Lower concentrations of SD-1008 (1 μ M) inhibited STAT3 phosphorylation with longer 24-h incubations (Fig. 3B), consistent with the derived phenotypes and

quantitative methods of the high-content imaging protocol. It is noteworthy that SD-1008 had no effect on the total amount of cellular STAT3 protein, as determined qualitatively by immunoblot (Fig. 3, A and B).

SD-1008 Inhibited STAT3-Dependent Luciferase Reporter Activity in Ovarian Cancer Cells with Constitutive STAT3 Activation. The effect of SD-1008 on STAT3-dependent transcription was assessed using a luciferase reporter system. Starting with the OVCAR8_{TR} cell line, a cloned and stably transfected daughter line was isolated after transfection with the STAT3-dependent luciferase reporter, pLucTK3. Exposure of this line to SD-1008 or AG490 for 24 h significantly inhibited STAT3-dependent luciferase activity (Fig. 4). The STAT3-dependent luciferase activity was decreased by >3-fold after exposure to 50 μ M AG490 and by >8-fold after exposure to 50 μ M SD-1008. Similar inhibition was also observed in human osteosarcoma cell line U-2OS_{TR} transfected with pLucTK3 (data not shown).

SD-1008 Inhibited STAT3-Mediated Expression of Antiapoptotic Proteins. We next examined whether exposure of cell lines to SD-1008 resulted in decreased expression of the antiapoptotic proteins Bcl-X_L, MCL-1, and survivin. Incubation in SD-1008 for 24 h significantly down-regulated Bcl-X_L and survivin expression in both OVCAR8_{TR} and MDA-MB-468 cells (Fig. 5). After 24-h incubation, the expression of MCL-1 protein expression also decreased in OVCAR8_{TR} with minimal change in MDA-MB-468 cells (Fig. 5).

SD-1008 Suppressed Phosphotyrosine Levels of JAK2 and Src. SD-1008 suppresses pSTAT3 levels, suggesting that this compound may interfere with the function of one or more of the upstream tyrosine kinases such as JAK or Src. Evaluation of SD-1008 on the phosphotyrosine levels of pJAK1, pJAK2, and pSrc in the OVCAR8_{TR} and MDA-MB-468 cell lines in vitro demonstrated the suppression of levels

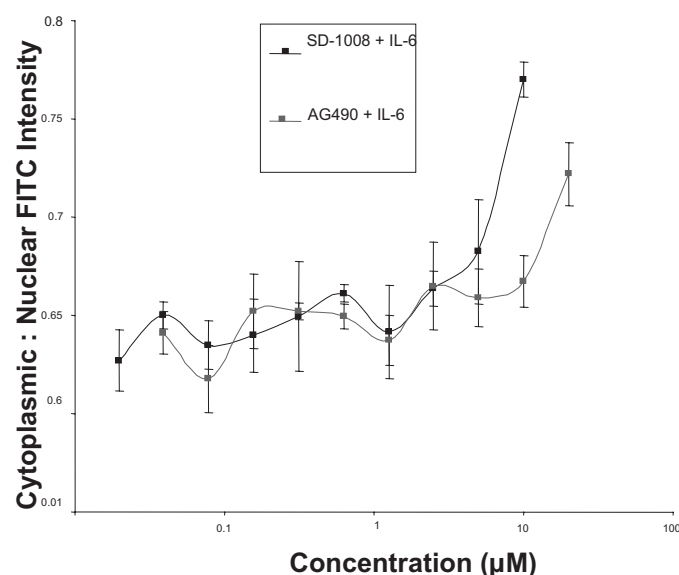


Fig. 2. Quantitative measurement of IL-6-induced STAT3 nuclear translocation. Stably transfected EGFP-STAT3 BHK-21 cells were incubated with AG490 followed by IL-6. After formaldehyde fixation and counterstaining with Hoechst, cells were visualized by epifluorescent microscopy with automated image acquisition. Average cytoplasmic/nuclear EGFP-STAT3 ratios are displayed in a dose-ranging experiment using SD-1008 and AG490. All experiments were performed in triplicate with error bars representing 1 S.D. from the mean cytoplasmic/nuclear ratio.

of pJAK2 and pSrc with much less inhibition of pJAK1 (Fig. 6A). To more directly evaluate the effects of SD-1008 on pJAK2, a cell-free in vitro assay was performed incubating recombinant JAK2 with SD-1008. Western blot demonstrated that SD-1008 inhibits autophosphorylation of JAK2 more effectively than AG490 (Fig. 6B), although evidenced at relatively high concentrations of the inhibitors. In separate control experiments, SD-1008 did not alter the level of phosphorylation of AKT (data not shown).

SD-1008 Induced Apoptosis in Human Cancer Cells.

The effect of SD-1008 on the induction of apoptosis was investigated by immunoblotting for PARP cleavage. PARP cleavage was detected after the incubation of OVCAR8_{TR} or MDA-MB-468 cells with 10 μ M SD-1008. A dose-response analysis revealed the appearance of PARP cleavage products with a 1 μ M SD-1008 concentration when cells were allowed to incubate for 24 h (Fig. 7).

SD-1008 Enhanced 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 hypothesis, several pSTAT3-activated cell

lines were treated with either 5 or 10 μ M SD-1008 for 24 h, paclitaxel, or the combination of paclitaxel and SD-1008. Apoptosis was scored using the M30-Apoptosense ELISA assay. SD-1008 treatment resulted in a marked 20-fold induction of apoptosis in the OVCAR8_{TR} cells that express constitutively activated STAT3 (Fig. 8). In addition, the combination of the paclitaxel with SD-1008 resulted in significantly greater cell death compared with paclitaxel or SD-1008 alone.

Discussion

Nuclear translocation of activated STAT3 is required for its function as a transcription factor (Schindler and Strehlow, 2000; Liu et al., 2005). Compounds capable of inhibiting STAT3 phosphorylation and translocation represent a desirable approach for the inhibition of the STAT3 signaling pathway in human cancer. In this study, we report that SD-1008 is a novel JAK/STAT3 signaling pathway inhibitor whose mechanism of action includes the inhibition of STAT3 phosphorylation and nuclear translocation. SD-1008 is the second inhibitor of STAT3 signaling identified through a cell-based assay of STAT3 nuclear translocation. The inhibition of JAK2 and Src phosphorylation by SD-1008 in cell culture and inhibition of pJAK2 in cell-free autophosphorylation in vitro

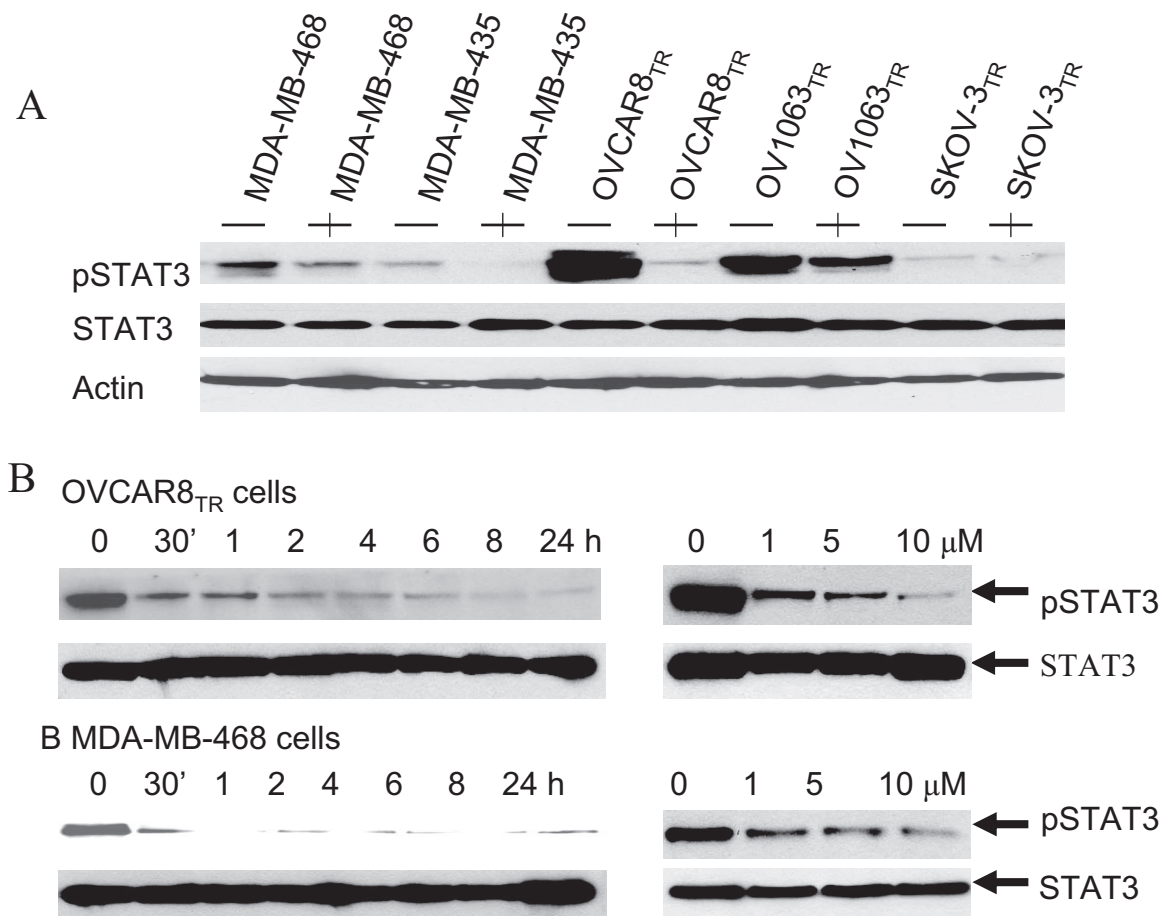


Fig. 3. SD-1008 inhibits phosphorylation of Stat3 levels in different cell lines. A, SD-1008 reduces pStat3 levels in different human ovarian and breast cancer cell lines. The cells were treated for 24 h with SD-1008 (10 μ M) and then harvested and processed for pStat3 Western blotting as described under *Materials and Methods*. B, dose- and time-dependent inhibition of Stat3 phosphorylation by SD-1008. OVCAR8_{TR} cells and MDA-MB-468 cells were treated with 10 μ M SD-1008 in a time-dependent manner or with SD-1008 for 24 h in a dose-response manner. For Western blot analysis, 25 μ g of cellular proteins was subjected to immunoblotting with specific antibodies to pStat3 and Stat3 as described under *Materials and Methods*.

assays suggests that the principal mechanism of SD-1008 activity is kinase inhibition. In particular, incubation with SD-1008 down-regulates STAT3-dependent protein expres-

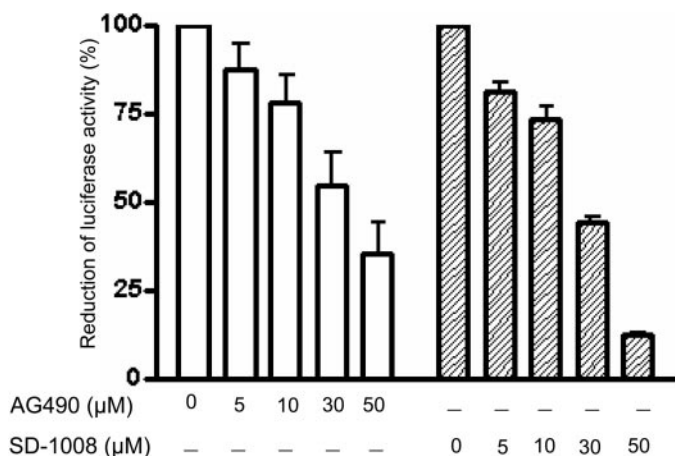
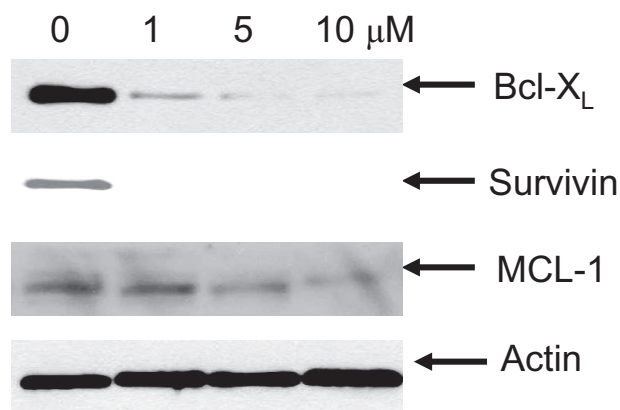
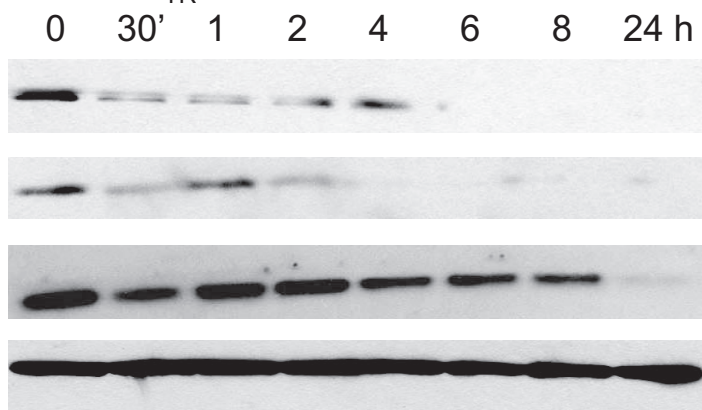


Fig. 4. SD-1008 inhibits Stat3-dependent luciferase activity in OVCAR8_{TR} cells. OVCAR8_{TR} cells stably cotransfected with pLucTKS3 Stat3-dependent luciferase reporter vector and pIRESneo vector. The cells were treated with 0, 5, 10, 30, or 50 μM concentration of SD-1008 or AG490 for 24 h and with quantitation of luciferase activity as described under *Materials and Methods*. The results were based on the means and S.D. from three experiments performed in triplicate.

sion, 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. Potentially, these effects were through decreased expression of STAT3 downstream antiapoptotic genes such as Bcl-X_L, survivin, and MCL-1. Many studies have found these growth- and survival-promoting genes are highly expressed in human cancer, especially in high-grade tumors (Silver et al., 2004; Gritsko et al., 2006). For example, we demonstrated recently that STAT3 and more notably pSTAT3 expression levels are increased in recurrent tumors collected after chemotherapy compared with matched primary tumors collected before chemotherapy (Duan et al., 2006b). The Src-induced STAT3 activation also increases survival of fibrosarcoma cells in response to doxorubicin (Vigneron et al., 2005). Constitutive activation of STAT3 could be a predictive marker of drug resistance (Barré et al., 2007). The finding that SD-1008 induces apoptosis in cells that express constitutively activated STAT3, both as a single agent and when combined with paclitaxel, suggests that this agent or other agents that target the STAT3 pathway may be useful in the clinic. The expression of antiapoptotic proteins Bcl-X_L and survivin is significantly decreased in cancer cells treated with SD-1008 and is consistent with a hypothesis that the decrease in Bcl-X_L and survivin expression contributes to the induction

OVCAR8_{TR} cells



MDA-MB-468 cells

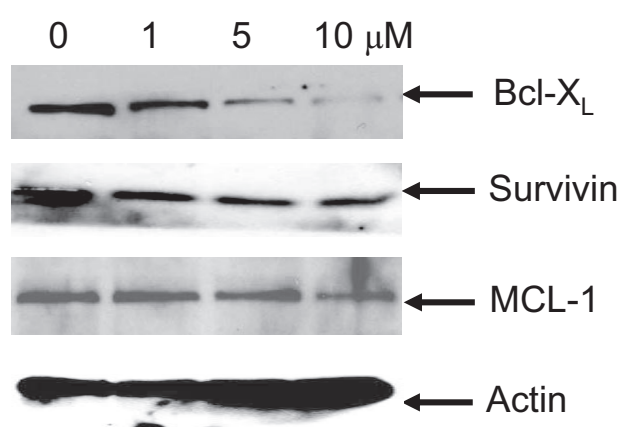
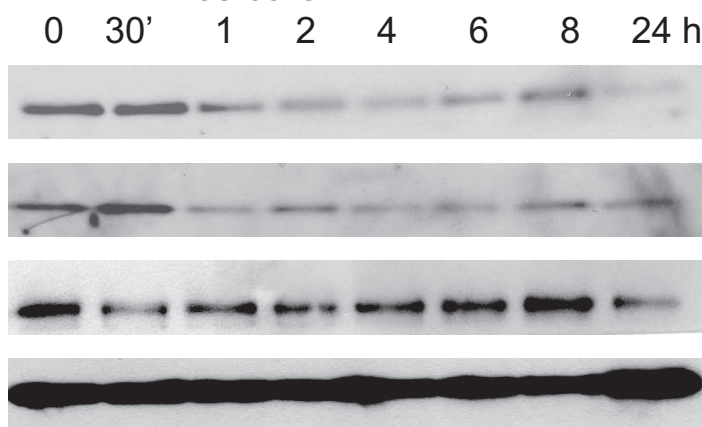


Fig. 5. SD-1008 down-regulated Stat3-targeted antiapoptotic proteins Bcl-X_L and survivin. OVCAR8_{TR} or MDA-MB-468 cells were treated with SD-1008 in a time- or dose-dependent manner. For Western blot analysis, 25 μg of total cellular proteins was subjected to immunoblotting with specific antibodies to Bcl-X_L, MCL-1, survivin or β-actin. The results were detected by a chemiluminescence detection system as described under *Materials and Methods*.

of apoptosis (Diaz et al., 2006; Gritsko et al., 2006). This hypothesis is also supported by a study indicating that a novel Bcl-X_L (STAT3 target protein) inhibitor, ABT-737, sig-

nificantly enhanced activities of paclitaxel in lung cancer cells (Oltersdorf et al., 2005).

We identified previously SD-1029, a halogenated xanthene

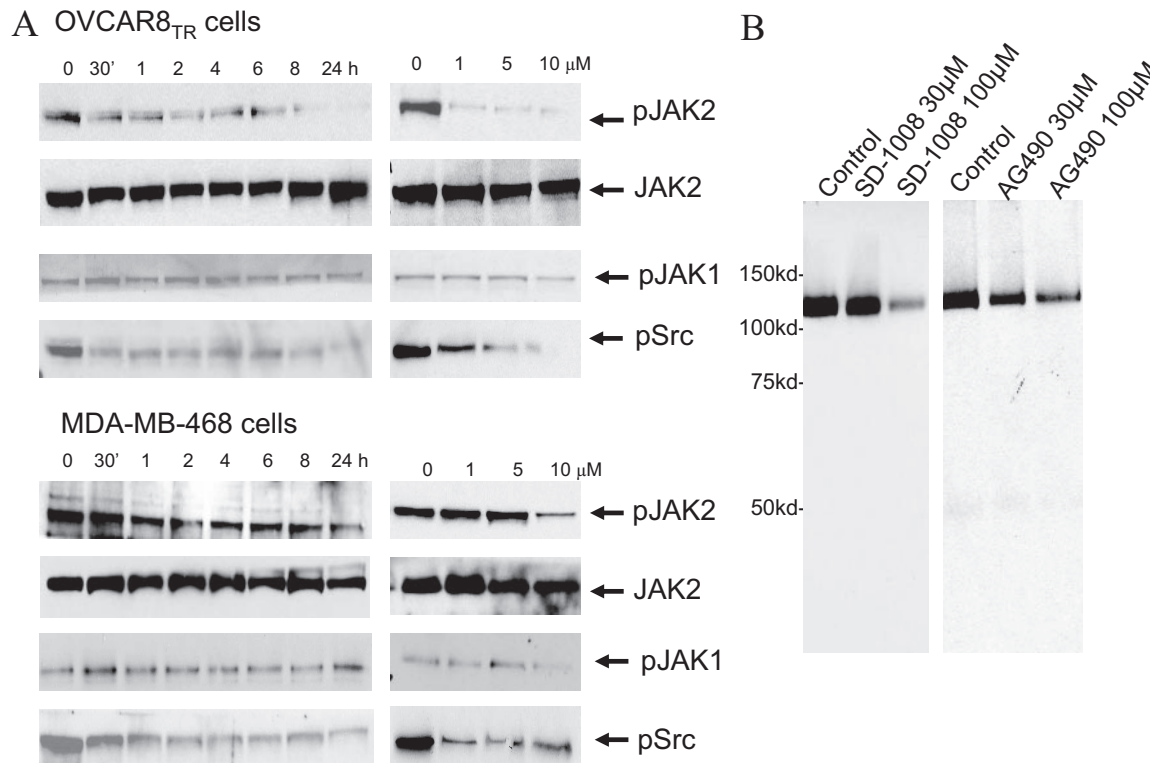


Fig. 6. SD-1008 blocks Jak2 and Src tyrosine phosphorylation. A, SD-1008 inhibits phosphorylation of Jak2 and Src in cell lines. OVCAR8_{TR} cells and MDA-MB-468 cells were treated with 10 μ M SD-1008 in a time-dependent manner or with SD-1008 for 24 h in a dose-response manner. For Western blot analysis, 25 μ g of cell-free extracts was subjected to immunoblotting with specific antibodies to pJAK1, pJAK2, and pSrc as described under *Materials and Methods*. B, SD-1008 directly inhibits Jak2 kinase autophosphorylation in vitro. Equal amounts of Jak2 recombinant protein were divided and preincubated in the presence or absence of various concentration of SD-1008, AG490, or DMSO control for 1 h. After the addition of 1 mM ATP and an additional 60 min of incubation at room temperature, the reaction was halted using stop buffer. Jak2 autophosphorylation was visualized as described under *Materials and Methods*.

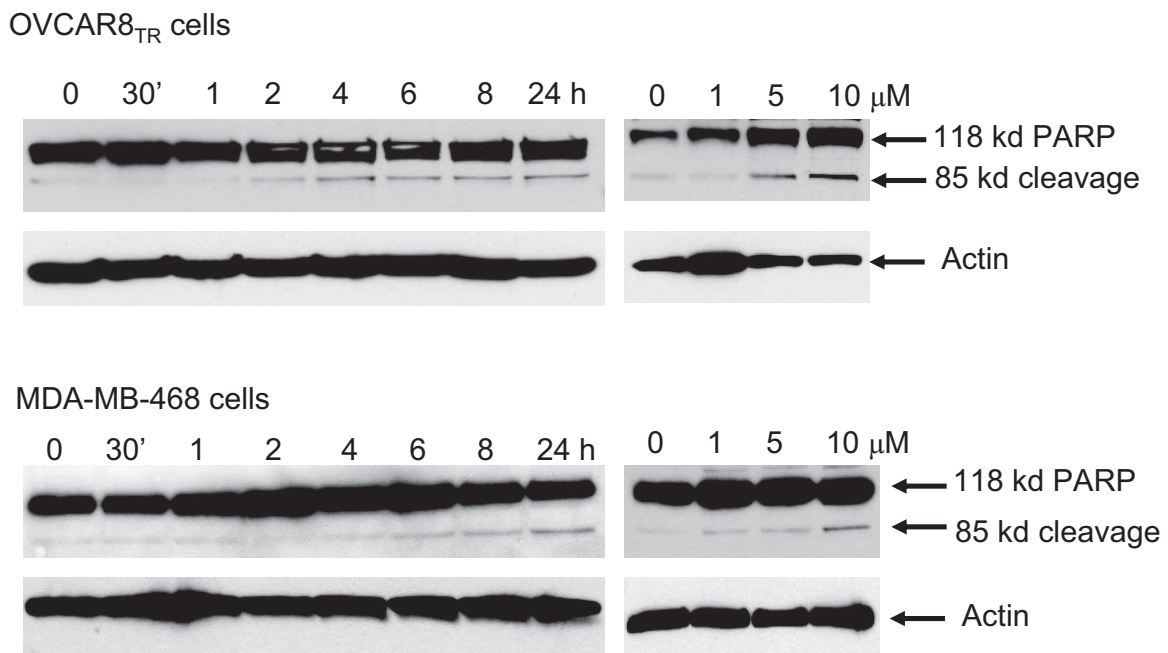


Fig. 7. SD-1008 induces apoptosis in cancer cells. OVCAR8_{TR} or MDA-MB-468 cells were treated with SD-1008 in a time- or dose-dependent manner. Total cellular proteins were subjected to immunoblotting with specific antibodies to PARP and β -actin as described under *Materials and Methods*.

compound, as the first inhibitor of STAT3 signaling selected by a cell-based assay of STAT3 nuclear translocation (Duan et al., 2006a). Maximum inhibition of nuclear translocation was seen at a concentration of 20 μ M AG490 and 5 μ M SD-1029 compared with 10 μ M SD-1008. Although the inhibition of nuclear transport could be explained by inhibition of JAK2, it is notable that SD-1008 significantly inhibits the level of phosphorylation of Src, which distinguish it from SD-1029 and may provide an alternate mechanism for STAT3 inhibition. In addition, SD-1008 is structurally distinct from SD-1029 and AG490. This is important because it could define a new scaffold for JAK inhibitors or suggest a non-nucleoside pocket binding motif.

This study provides evidence that phenotypic screening using a cell-based assay is an effective approach for identifying inhibitors of the JAK-STAT pathway. Similar to SD-1029, SD-1008 distinguishes itself from other inhibitors of the JAK-Stat pathway because it was identified through an ability to inhibit nuclear translocation. More importantly, this biologically relevant cell-based biomarker of pathway activation is compatible with miniaturization and antibody-independent high-content, high-throughput screening. Further screening studies are underway using both biased and large, diverse chemical libraries.

In conclusion, we have identified SD-1008 as a novel inhibitor of STAT3 activation. Biochemical and cellular assays support the hypothesis that this molecule inhibits JAK and Src activity with resultant inhibition of Stat phosphorylation, nuclear transport, and a decrease in STAT3-dependent transcription, leading to apoptosis and enhanced chemosensitivity (Alas and Bonavida, 2003; George et al., 2005). It is noteworthy that the superior potency of SD-1008 compared with AG490 enables experimentation in animal models of human malignancies in which the JAK-STAT pathway contributes to the malignant phenotype. Furthermore, a wealth of evidence supporting JAK2 inhibition has a treatment potential in a variety of myeloproliferative disorders (Levine et al., 2005b; Tefferi and Gilliland, 2005). These results add

further evidence that the JAK-STAT pathway is a potential therapeutic pathway in a number of human malignancies.

Acknowledgments

We thank Drs. Doug Smallwood and Robert J. Schultz from the NCI Developmental Therapeutics Program for providing the Diversity Set. We thank the National Cancer Institute and the Initiative for Chemical Genetics, who provided support for this publication, and the Chemical Biology Platform of the Broad Institute of Harvard and Massachusetts Institute of Technology for their assistance in this work.

References

- Alas S and Bonavida B (2003) Inhibition of constitutive STAT3 activity sensitizes resistant non-Hodgkin's lymphoma and multiple myeloma to chemotherapeutic drug-mediated apoptosis. *Clin Cancer Res* 9:316–326.
- Alvarez JV, Febbo PG, Ramaswamy S, Loda M, Richardson A, and Frank DA (2005) Identification of a genetic signature of activated signal transducer and activator of transcription 3 in human tumors. *Cancer Res* 65:5054–5062.
- Barré B, Vigneron A, Perkins N, Roninson IB, Gamelin E, and Coqueret O (2007) The STAT3 oncogene as a predictive marker of drug resistance. *Trends Mol Med* 13:4–11.
- Bellone S, Watts K, Cane S, Palmieri M, Cannon MJ, Burnett A, Roman JJ, Pecorelli S, and Santin AD (2005) High serum levels of interleukin-6 in endometrial carcinoma are associated with uterine serous papillary histology, a highly aggressive and chemotherapy-resistant variant of endometrial cancer. *Gynecol Oncol* 98:92–98.
- Bromberg J (2002) Stat proteins and oncogenesis. *J Clin Invest* 109:1139–1142.
- Bromberg J and Darnell JE Jr (2000) The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* 19:2468–2473.
- Bromberg JF, Horvath CM, Besser D, Lathem WW, and Darnell JE Jr. (1998) Stat3 activation is required for cellular transformation by v-src. *Mol Cell Biol* 18:2553–2558.
- Cao X, Tay A, Guy GR, and Tan YH (1996) Activation and association of Stat3 with Src in v-Src-transformed cell lines. *Mol Cell Biol* 16:1595–1603.
- Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R, Ciliberto G, Moscinski L, Fernandez-Luna JL, Nunez G, et al. (1999) Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. *Immunity* 10:105–115.
- Darnell JE Jr (1997) STATs and gene regulation. *Science* 277:1630–1635.
- Diaz N, Minton S, Cox C, Bowman T, Gritsko T, Garcia R, Eweis I, Wloch M, Livingston S, Seijo E, et al. (2006) Activation of stat3 in primary tumors from high-risk breast cancer patients is associated with elevated levels of activated SRC and survivin expression. *Clin Cancer Res* 12:20–28.
- Duan Z, Bradner JE, Greenberg E, Levine R, Foster R, Mahoney J, and Seiden MV (2006a) SD-1029 inhibits signal transducer and activator of transcription 3 nuclear translocation. *Clin Cancer Res* 12:6844–6852.
- Duan Z, Feller AJ, Penson RT, Chabner BA, and Seiden MV (1999) Discovery of differentially expressed genes associated with paclitaxel resistance using cDNA array technology: analysis of interleukin (IL) 6, IL-8, and monocyte chemoattractant protein 1 in the paclitaxel-resistant phenotype. *Clin Cancer Res* 5:3445–3453.
- Duan Z, Foster R, Bell DA, Mahoney J, Wolak K, Vaidya A, Hampel C, Lee H, and Seiden MV (2006b) Signal transducers and activators of transcription 3 pathway activation in drug-resistant ovarian cancer. *Clin Cancer Res* 12:5055–5063.
- Duan Z, Lamendola DE, Duan Y, Yusuf RZ, and Seiden MV (2005) Description of paclitaxel resistance-associated genes in ovarian and breast cancer cell lines. *Cancer Chemother Pharmacol* 55:277–285.
- Faderl S, Ferrajoli A, Harris D, Van Q, Priebe W, and Estrov Z (2005) WP-1034, a novel JAK-STAT inhibitor, with proapoptotic and antileukemic activity in acute myeloid leukemia (AML). *Anticancer Res* 25:1841–1850.
- Gamero AM, Young HA, and Wiltrott RH (2004) Inactivation of Stat3 in tumor cells: releasing a brake on immune responses against cancer? *Cancer Cell* 5:111–112.
- George JA, Chen T, and Taylor CC (2005) SRC tyrosine kinase and multidrug resistance protein-1 inhibitions act independently but cooperatively to restore paclitaxel sensitivity to paclitaxel-resistant ovarian cancer cells. *Cancer Res* 65:10381–10388.
- Gritsko T, Williams A, Turkson J, Kaneko S, Bowman T, Huang M, Nam S, Eweis I, Diaz N, Sullivan D, et al. (2006) Persistent activation of stat3 signaling induces survivin gene expression and confers resistance to apoptosis in human breast cancer cells. *Clin Cancer Res* 12:11–19.
- Huang M, Page C, Reynolds RK, and Lin J (2000) Constitutive activation of stat 3 oncogene product in human ovarian carcinoma cells. *Gynecol Oncol* 79:67–73.
- Ikuta K, Takemura K, Kihara M, Nishimura M, Ueda N, Naito S, Lee E, Shimizu E, and Yamauchi A (2005) Overexpression of constitutive signal transducer and activator of transcription 3 mRNA in cisplatin-resistant human non-small cell lung cancer cells. *Oncol Rep* 13:217–222.
- Lamendola DE, Duan Z, Yusuf RZ, and Seiden MV (2003) Molecular description of evolving paclitaxel resistance in the SKOV-3 human ovarian carcinoma cell line. *Cancer Res* 63:2200–2205.
- Levine RL, Loriaux M, Huntly BJ, Loh ML, Beran M, Stoffregen E, Berger R, Clark JJ, Willis SG, Nguyen KT, et al. (2005a) The JAK2V617F activating mutation occurs in chronic myelomonocytic leukemia and acute myeloid leukemia, but not in acute lymphoblastic leukemia or chronic lymphocytic leukemia. *Blood* 106:3377–3379.
- Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, Boggon TJ,

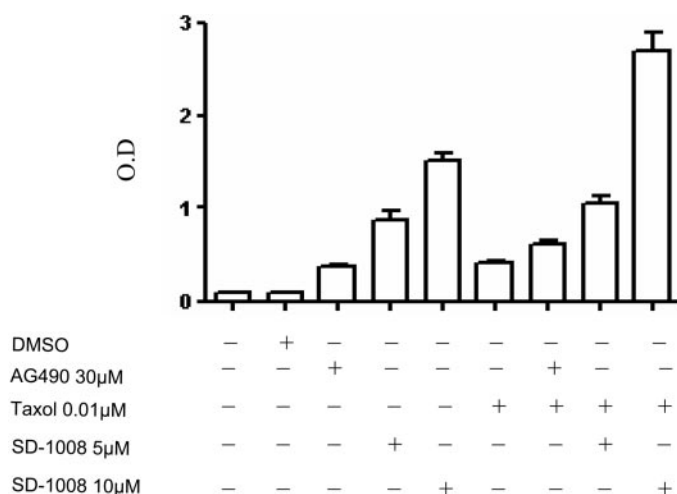


Fig. 8. SD-1008 inhibits cell growth and induces apoptosis in OVCAR₈ ovarian cancer cells. OVCAR₈ cells were seeded at a density of 8000 cells/well in a 96-well plate for 24 h. Cells were then treated with different drugs for 24 h. The cells were lysed with 10% Nonidet P-40, and the M30-Apoptosense ELISA assay was performed as described under *Materials and Methods*.

- Wlodarska I, Clark JJ, Moore S, et al. (2005b) Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell* **7**:387–397.
- Liu L, McBride KM, and Reich NC (2005) STAT3 nuclear import is independent of tyrosine phosphorylation and mediated by importin- α . *Proc Natl Acad Sci U S A* **102**:8150–8155.
- Mora LB, Buettner R, Seigne J, Diaz J, Ahmad N, Garcia R, Bowman T, Falcone R, Fairclough R, Cantor A, et al. (2002) Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. *Cancer Res* **62**:6659–6666.
- Nam S, Buettner R, Turkson J, Kim D, Cheng JQ, Muehlbeyer S, Hippe F, Vatter S, Merz KH, Eisenbrand G, et al. (2005) Indirubin derivatives inhibit Stat3 signaling and induce apoptosis in human cancer cells. *Proc Natl Acad Sci U S A* **102**:5998–6003.
- Nilsson MB, Langley RR, and Fidler IJ (2005) Interleukin-6, secreted by human ovarian carcinoma cells, is a potent proangiogenic cytokine. *Cancer Res* **65**:10794–10800.
- Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, Bruncko M, Deckwerth TL, Dinges J, Hajduk PJ, et al. (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* **435**:677–681.
- Pedranzini L, Dechow T, Berishaj M, Comenzo R, Zhou P, Azare J, Bornmann W, and Bromberg J (2006) Pyridone 6, a pan-janus-activated kinase inhibitor, induces growth inhibition of multiple myeloma cells. *Cancer Res* **66**:9714–9721.
- Schindler C and Strehlow I (2000) Cytokines and STAT signaling. *Adv Pharmacol* **47**:113–174.
- Schust J, Sperl B, Hollis A, Mayer TU, and Berg T (2006) Stattic: a small-molecule inhibitor of STAT3 activation and dimerization. *Chem Biol* **13**:1235–1242.
- Silver DL, Naora H, Liu J, Cheng W, and Montell DJ (2004) Activated signal transducer and activator of transcription (STAT) 3: localization in focal adhesions and function in ovarian cancer cell motility. *Cancer Res* **64**:3550–3558.
- Song H, Wang R, Wang S, and Lin J (2005) A low-molecular-weight compound discovered through virtual database screening inhibits Stat3 function in breast cancer cells. *Proc Natl Acad Sci U S A* **102**:4700–4705.
- Syed V, Ulinski G, Mok SC, and Ho SM (2002) Reproductive hormone-induced, STAT3-mediated interleukin 6 action in normal and malignant human ovarian surface epithelial cells. *J Natl Cancer Inst* **94**:617–629.
- Tefferi A and Gilliland DG (2005) The JAK2V617F tyrosine kinase mutation in myeloproliferative disorders: status report and immediate implications for disease classification and diagnosis. *Mayo Clin Proc* **80**:947–958.
- Turkson J, Bowman T, Garcia R, Caldenhoven E, De Groot RP, and Jove R (1998) Stat3 activation by Src induces specific gene regulation and is required for cell transformation. *Mol Cell Biol* **18**:2545–2552.
- Turkson J and Jove R (2000) STAT proteins: novel molecular targets for cancer drug discovery. *Oncogene* **19**:6613–6626.
- Turkson J, Zhang S, Mora LB, Burns A, Sehti S, and Jove R (2005) A novel platinum compound inhibits constitutive Stat3 signaling and induces cell cycle arrest and apoptosis of malignant cells. *J Biol Chem* **280**:32979–32988.
- Vigneron A, Roninson IB, Gamelin E, and Coqueret O (2005) Src inhibits Adriamycin-induced senescence and G2 checkpoint arrest by blocking the induction of p21waf1. *Cancer Res* **65**:8927–8935.
- Yu H and Jove R (2004) The STATs of cancer—new molecular targets come of age. *Nat Rev Cancer* **4**:97–105.

Address correspondence to: Dr. Zhenfeng Duan, Massachusetts General Hospital, Department of Hematology/Oncology, 100 Blossom St. Jackson 1008, Boston, MA 02114. E-mail: zduan@partners.org
