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 auto-phosphorylation. Exposure of various cell lines to SD-1008 decreases levels of the STAT3-dependent proteins, Bcl-XL 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 exploitation 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). This pathway 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 pathway activation is associated with high-grade tumors, drug resistance, and induction of antiapoptotic proteins such as survivin and Bcl-XL (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-
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 Srfc 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-6Rα, 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-XL, 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; Pedranzini 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 pathways, 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, Bel-2, 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 μg/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 cell line BHK-21, human osteosarcoma U-2OS cells, or human ovarian cell line 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 μM 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 × 71 fluorescence microscope (Olympus, Tokyo, Japan), and the data were captured as digital images using IPLab Software from Scionalytics (Rockville, MD). AG490 (20 μM) 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 NuPage 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 μl 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 autophosphorylation 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 cells 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 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 μM paclitaxel, 30 μM AG490, 5 or 10 μM 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 μM), or AG490 (20 μM) 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.](image-url)
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 μM) 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 μM in both BHK-21 and U2-OS 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 representing 1 S.D. from the mean cytoplasmic/nuclear ratio. 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 OVCAR8TR 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 μM AG490 and by >8-fold after exposure to 50 μM SD-1008. Similar inhibition was also observed in human osteosarcoma cell line U-2OSTR 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-XL, MCL-1, and survivin. Incubation in SD-1008 for 24 h significantly down-regulated Bcl-XL and survivin expression in both OVCAR8TR and MDA-MB-468 cells (Fig. 5). After 24 h incubation, the expression of MCL-1 protein expression also decreased in OVCAR8TR 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 OVCAR8TR and MDA-MB-468 cell lines in vitro demonstrated the suppression of levels
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_T or MDA-MB-468 cells with 10 μM SD-1008. A dose-response analysis revealed the appearance of PARP cleavage products with a 1 μM 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 μM SD-1008 for 24 h, paclitaxel, or the combination of paclitaxel and SD-1008. Apoptosis was scored using the M30-Apoptose ELISA assay. SD-1008 treatment resulted in a marked 20-fold induction of apoptosis in the OVCAR8_T 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 μM) 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_T cells and MDA-MB-468 cells were treated with 10 μM SD-1008 in a time-dependent manner or with SD-1008 for 24 h in a dose-response manner. For Western blot analysis, 25 μg 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 expression, 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-XL, 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-XL and survivin is significantly decreased in cancer cells treated with SD-1008 and is consistent with a hypothesis that the decrease in Bcl-XL and survivin expression contributes to the induction

Fig. 4. SD-1008 inhibits Stat3-dependent luciferase activity in OVCAR8TR cells. OVCAR8TR 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.

Fig. 5. SD-1008 down-regulated Stat3-targeted antiapoptotic proteins Bcl-XL and survivin. OVCAR8TR 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-XL, 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<sub>L</sub> (STAT3 target protein) inhibitor, ABT-737, significantly enhanced activities of paclitaxel in lung cancer cells (Oltersdorf et al., 2005).

We identified previously SD-1029, a halogenated xanthene
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 μM AG490 and 5 μM SD-1029 compared with 10 μM 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 distinguishes 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 strategies 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.

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Fig. 8. SD-1008 inhibits cell growth and induces apoptosis in OVCAR3R ovarian cancer cells. OVCAR3R 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.
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