1-1-2006

A novel lipase belonging to the hormone-sensitive lipase family induced under starvation to utilize stored triacylglycerol in Mycobacterium tuberculosis

Chirajyoti Deb
University of Central Florida

Jaiyanth Daniel
University of Central Florida

Tatiana D. Sirakova
University of Central Florida

Bassam Abomoelak
University of Central Florida

Vindod S. Dubey
University of Central Florida

See next page for additional authors

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 38, pp. 32979–32988, September 23, 2005

A Novel Platinum Compound Inhibits Constitutive Stat3 Signaling and Induces Cell Cycle Arrest and Apoptosis of Malignant Cells*

Received for publication, March 11, 2005, and in revised form, June 17, 2005 Published, JBC Papers in Press, July 26, 2005, DOI 10.1074/jbc.M502694200

James Turkson[‡§1], Shumin Zhang[‡§], Linda B. Mora[‡§], Audrey Burns[‡¶], Said Sebti[§¶‖], and Richard Jove[‡§¶]**

*From the [‡]Molecular Oncology and [‖]Drug Discovery Programs, H. Lee Moffitt Cancer Center & Research Institute, and the Departments of [§]Oncology, [¶]Biochemistry and Molecular Biology, and **Pathology, University of South Florida College of Medicine, Tampa, Florida 33612*

Previous studies have established constitutive activation of Stat3 protein as one of the molecular changes required for tumorigenesis. To develop novel therapeutics for tumors harboring constitutively active Stat3, compounds from the NCI 2000 diversity set were evaluated for inhibition of Stat3 DNA-binding activity *in vitro*. Of these, a novel platinum (IV) compound, IS3 295, interacted with Stat3 and inhibited its binding to specific DNA-response elements. Further analysis suggested noncompetitive-type kinetics for the inhibition of Stat3 binding to DNA. In human and mouse tumor cell lines with constitutively active Stat3, IS3 295 selectively attenuated Stat3 signaling, thereby inducing cell growth arrest at G_0/G_1 phase and apoptosis. Moreover, in transformed cells, IS3 295 repressed expression of cyclin D_1 and *bcl-*x_L, two of the known Stat3-regulated genes that are overexpressed in malignant cells, suggesting that IS3 295 mediates anti-tumor cell activity in part by blocking Stat3-mediated subversion of cell growth and apoptotic signals. Together, our findings provide evidence for the inhibition of Stat3 activity and biological functions by IS3 295 through interaction with Stat3 protein. This study represents a significant advance in small molecule-based approaches to target Stat3 and suggests potential new applications for platinum (IV) complexes as modulators of the Stat3 pathway for cancer therapy.

Cellular responses to growth factors and cytokines are characterized by the activation of signal transduction pathways, including the signal transducer and activator of transcription (STAT)[2] family of cytoplasmic transcription factors (1–4). Activation of STAT proteins is initiated upon their tyrosine phosphorylation, a key event in the formation of phosphotyrosine-Src homology 2 domain interactions and the dimerization between two STAT monomers. In turn, dimers of STAT proteins translocate to the nucleus and bind to specific DNA-response elements, thereby inducing the expression of genes essential for cellular responses. Normal physiological functions of STAT proteins include regulation of cell proliferation, differentiation, and development and apoptosis (reviewed in Refs. 5–10).

In contrast to the tightly regulated normal STAT signaling, constitutive activation of STAT proteins is frequently observed in human tumors (11, 12) and has been linked to tumor progression. Persistent activation of one STAT family member, Stat3, is detected in breast cancer, prostate cancer, and head and neck squamous cell carcinoma as well as in lymphomas and leukemias (Refs. 13–20; reviewed in Refs. 21–28). In malignant cell lines and tumors that harbor constitutively active Stat3, studies have also revealed overexpression of Stat3-regulated genes encoding the anti-apoptotic proteins Bcl-x_L and Mcl-1, the cell cycle regulators cyclin D_1 and c-Myc, and the angiogenesis factor vascular endothelial growth factor (VEGF) as well as altered expression of immune modulatory factors (15, 16, 18, 20, 29–31). These abnormal gene expression changes contribute to dysregulated cell cycle progression, survival, and angiogenesis and to repressed host immune functions (reviewed in Refs. 27 and 28). Thus, inhibition of abnormal Stat3 signaling is sufficient to repress the induction of these genes, resulting in cell cycle arrest and apoptosis of malignant cells (15, 18, 20, 32), sensitization of tumor cells to chemotherapy-induced apoptosis (33), anti-tumor immune responses (31), and tumor regression (32). Therefore, small molecule inhibitors of Stat3 have the potential to impact tumors that harbor constitutively active Stat3 with significant clinical benefits.

Previous studies have implicated signal transduction pathways in the anti-tumor activity of platinum complexes. Evidence shows that cisplatin might modulate the MAPK family and the phosphatidylinositol 3-kinase/Akt pathway (34–37). We have also reported previously that a novel class of platinum complexes inhibit Stat3 signaling and induce tumor regression (38). Here, we demonstrate that a new platinum (IV) compound, IS3 295, selected from the NCI diversity set (NSC 295558) is another novel Stat3 inhibitor. Analyses of *in vitro* DNA-binding activity and transcriptional regulation indicated that IS3 295 interacted directly with Stat3, thereby inhibiting Stat3 binding to a consensus DNA-response element and Stat3 transcriptional activity. Inhibition of constitutively active Stat3 in malignant cells by IS3 295 suppressed the induction of Stat3-regulated genes, including *bcl-*x_L and cyclin D_1. Studies in v-Src-transformed fibroblasts as well as in human and mouse tumor cell lines that harbor constitutive Stat3 activity revealed G_0/G_1 cell cycle arrest and apoptosis following treatment with IS3 295, which correlated with the inhibition of aberrant Stat3 signaling. Altogether, we provide evidence for direct inhibition of Stat3 and its biological activity by a novel platinum (IV) complex. These findings have wide clinical implications, providing the rationale for further mechanistic studies of these compounds for potential cancer therapy.

EXPERIMENTAL PROCEDURES

Cells and Reagents—The Src-transformed NIH3T3/v-Src, NIH3T3/v-Src/pLucTKS3, NIH3T3/v-Src/pRLSRE, and NIH3T3/hEGFR fibro-

* This work was supported by the Charlotte-Geyer Foundation (to J. T.) and NCI Grant CA55652 from the National Institutes of Health (to R. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: BioMolecular Science Center, Dept. of Molecular Biology and Microbiology, University of Central Florida, 12722 Research Pkwy., Orlando, FL 32826. Tel.: 407-823-4812; E-mail: jturkson@mail.ucf.edu.

[2] The abbreviations used are: STAT, signal transducer and activator of transcription; VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; hSIE, high affinity *sis*-inducible element; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; BrdUrd, bromodeoxyuridine.

blasts and the human breast cancer (MDA-MB-231, MDA-MB-435, MDA-MB-453, and MDA-MB-468), human prostate cancer (DU145), human (U266) and mouse (5TGM1) multiple myeloma, mouse melanoma (B16), and human pancreatic cancer (Panc1) cell lines have all been described previously (15, 19, 32, 39–43). Cells were grown in Dulbecco's modified Eagle's medium containing 5% iron-supplemented bovine calf serum with or without G418 or Zeocin or in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum. Recombinant human epidermal growth factor and interleukin-6 were obtained from R&D Systems (Minneapolis, MN).

Plasmids—The Stat3 reporter (pLucTKS3), the Stat3-dependent VEGF promoter-driven reporter (pGL2-VEGF-Luc), and the Stat3-independent reporter (pLucSRE), all of which drive the expression of firefly luciferase, as well as the Stat3-independent *Renilla* luciferase reporter (pRLSRE) have been described previously (11, 30, 44). The pLucTKS3 plasmid harbors seven copies of a sequence corresponding to the Stat3-specific binding site in the promoter of the human C-reactive protein gene (45). The pRLSRE and pLucSRE plasmids each contain two copies of the serum-response element from the c-*fos* promoter (11, 46) subcloned into the Renilla (pRL-null) or firefly (pGL2) luciferase reporter, respectively (Promega Corp., Madison, WI). The plasmid pNFκB-Luc is firefly luciferase and was obtained from Stratagene (La Jolla, CA). The plasmid pRc/CMV-Stat3-FLAG was a gift from Dr. James Darnell, Jr. (Rockefeller University).

Cytosolic Extract Preparation and Luciferase Assays—Cytosolic extract preparation from fibroblasts and luciferase assays were described previously (11, 44). Briefly, after two washes with phosphate-buffered saline (PBS) and equilibration for 5 min with 0.5 ml of PBS and 0.5 mM EDTA, cells were scraped off the dishes, and the cell pellet was obtained by centrifugation at 4500 × *g* for 2 min at 4 °C. Cells were resuspended in 0.4 ml of low salt HEPES buffer (10 mM HEPES (pH 7.8), 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) for 15 min; lysed by the addition of 20 μl of 10% Nonidet P-40; and centrifuged at 10,000 × *g* for 30 s at 4 °C to obtain the cytosolic supernatant, which was used for luciferase assays (Promega Corp.) and measured with a luminometer. Cytosolic lysates were prepared from recombinant baculovirus-infected Sf9 cells for Stat3 protein as described previously (47). Briefly, cultured dishes of Sf9 cells were washed twice with ice-cold 1× PBS and then PBS containing 1 mM sodium orthovanadate. Cells were lysed in 1% Nonidet P-40 lysis buffer (50 mM HEPES (pH 7.9), 150 mM NaCl, 1% Nonidet P-40, 20 mM NaF, 1 mM sodium orthovanadate, 1 mM tetrasodium pyrophosphate, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM EGTA, 2 mM EDTA, 0.1 μM aprotinin, 1 μM leupeptin, and 1 μM antipain) on ice for 10 min and centrifuged at 13,000 × *g* for 30 s at 4 °C to obtain the lysate.

Nuclear Extract Preparation and Gel Shift Assays—Nuclear extract preparation from NIH3T3 fibroblasts stimulated by recombinant human interleukin-6, NIH3T3/hEGFR fibroblasts stimulated by recombinant human epidermal growth factor, v-Src-transformed NIH3T3/v-Src fibroblasts, and tumor cell lines and electrophoretic mobility shift assay (EMSA) were carried out as described previously (11, 13, 40). The ^{32}P-labeled oligonucleotide probes used were the high affinity *sis*-inducible element (hSIE) from the *c-fos* gene (m67 variant, 5′-AGCTTCATTTCCCGTAAATCCCTA) for Stat1 and Stat3 binding (13, 48) and the mammary gland factor element from the bovine β-casein gene promoter (5′-AGATTTCTAGGAATTCAA) for Stat1 and Stat5 binding (49, 50). Except where indicated, inhibitor compound was preincubated with the nuclear extract for 30 min at room temperature prior to incubation with the radiolabeled probe.

Western Blotting—Whole cell lysates were prepared in boiling SDS sample loading buffer to extract total proteins from the cytoplasm and nucleus. Equivalent amounts of total cellular protein were electrophoresed on an SDS-10% polyacrylamide gel and transferred onto nitrocellulose membranes. Probing of nitrocellulose membranes with primary antibodies and detection of horseradish peroxidase-conjugated secondary antibodies by enhanced chemiluminescence (Amersham Biosciences) were performed as described previously (19, 38, 47). The probes used were anti-cyclin D_1 (Cell Signaling Technologies, Beverly, MA), anti-Bcl-x_L (Cell Signaling Technologies), and anti-β-actin (Sigma) antibodies.

Soft Agar Colony Formation Assays—Colony formation assays were carried out in 6-well dishes as described previously (44). In brief, each well contained 1.5 ml of 1% agarose in Dulbecco's modified Eagle's medium as the bottom layer and 1.5 ml of 0.5% agarose in Dulbecco's modified Eagle's medium containing 4000 or 6000 NIH3T3/v-Src or NIH3T3/v-Ras fibroblasts, respectively, as the top layer. Treatment with IS3 295 was initiated 1 day after seeding cells by adding of 75–100 μl of medium with or without compound and was repeated every 3 days until large colonies were evident. Colonies were quantified by staining with 20 μl of 1 mg/ml iodonitrotetrazolium violet, incubating overnight at 37 °C, and counting the next day.

Cell Proliferation and Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End Labeling (TUNEL) Assays—Proliferating cells were first treated with or without IS3 295 for up to 48 h. A portion of the cells were harvested for bromodeoxyuridine (BrdUrd) (Pharmingen) incorporation following the manufacturer's instructions and analyzed by flow cytometry. Harvested cells were also analyzed for apoptosis via detection by TUNEL assay using a Roche Applied Science apoptosis detection system (fluorescein) according to the manufacturer's instructions.

Oligonucleotide and Plasmid Transfections—The Stat3 antisense (5′-GCTCCAGCATCTGCTGCTTC-3′) and control mismatch (5′-GCTCCAATACCCGTTGCTTC-3′) oligonucleotides were synthesized using phosphorothioate chemistry with 2′-*O*-methoxyethyl modification of the five terminal nucleotides (underlined) (42, 51) to increase stability. Transfections of antisense Stat3 and plasmids were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, cells were seeded at 1–2 × 10^6/10-cm tissue culture plate 18 h before transfection. Immediately before transfection, cells were washed once with PBS. Cells were transfected with luciferase reporters (4 μg) in the presence or absence of v-Src (4 μg) or transfected with Stat3β (4 μg) or pRc/CMV-Stat3-FLAG (4 μg). Antisense Stat3 transfections were carried out Lipofectamine 2000 alone or with Stat3 antisense or mismatch oligonucleotides (final oligonucleotide concentration of 250 nM). After 2–3 h, the transfection medium was aspirated, and cells were washed with PBS before fresh medium containing 10% fetal bovine serum was added. Forty-eight h after transfection, cells were washed, and cytosolic lysates were prepared for luciferase assay as described previously (11, 44) or processed for TUNEL staining.

Immunohistochemistry—The indirect peroxidase/anti-peroxidase test was performed on cytospins prepared from cell lines (control and treated). After inhibition of endogenous peroxidase with 0.3% H_2O_2 and methanol for 30 min, slides were rinsed in PBS (pH 7.4), treated for 30 min with 1.5% normal goat serum, and then incubated for 1 h with primary antibody against Ki67 (Vector Laboratories, Burlingame, CA) at 1:100 dilution. Slides were rinsed in PBS and incubated for 30 min with biotinylated secondary antibody (Vector Laboratories) at 1:200 dilution. Following washing with PBS, the preparations were further incubated in peroxidase-conjugated avidin (Vector Laboratories). Visu-

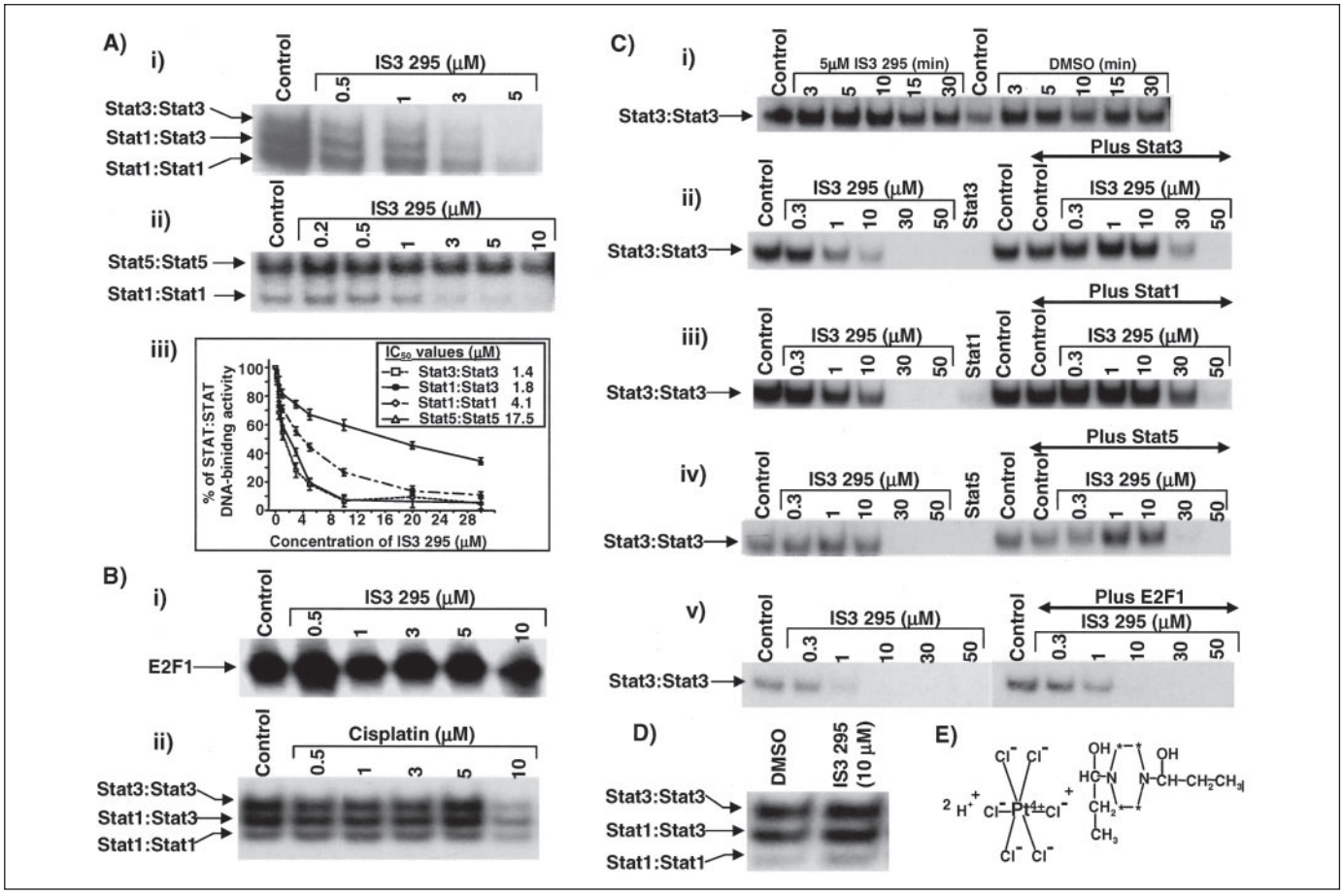

FIGURE 1. **Inhibition of *in vitro* Stat3 DNA-binding activity by a platinum complex.** Nuclear extracts or cell lysates containing activated Stat1, Stat3, or Stat5 or E2F1 prepared from NIH3T3/hEGFR fibroblasts stimulated with epidermal growth factor or from Sf9 insect cells infected with baculovirus expressing Stat1, Stat3, Stat5, or E2F1, respectively, were treated with or without the indicated concentrations of platinum complex (IS3 295) or cisplatin for 30 min at room temperature prior to incubation with radiolabeled oligonucleotide probes and subjected to EMSA analysis. *A*: *panel i*, Stat1 and Stat3 binding to the hSIE probe; *panel ii*, Stat1 and Stat5 binding to the mammary gland factor element probe; *panel iii*, plot of the percentage of STAT/STAT-oligonucleotide probe complexes *versus* the concentration of IS3 295. *Inset*, IC_{50} values for the inhibition of STAT/STAT DNA-binding activity. *B*: *panel i*, E2F1 binding to the dihydrofolate reductase promoter oligonucleotide probe; *panel ii*, Stat1 and Stat3 binding to the hSIE probe. *C*: IS3 295 effect on the *in vitro* DNA-binding activity of Stat3 3–30 min after binding (*panel i*) or in the presence and absence of the inactive Stat3 monomer (*panel ii*), the inactive Stat1 monomer (*panel iii*), the inactive Stat5 monomer (*panel iv*), or E2F1 protein (*panel v*). *D*: Stat1 and Stat3 binding to the IS3 295-treated and untreated (dimethyl sulfoxide (*DMSO*)) radiolabeled hSIE probe. *E*: structural formula of IS3 295. The positions of the STAT/STAT-DNA complexes on the gels are labeled. In *A–C*, the *Control lanes* represent Me_2SO (vehicle) treatment.

alization was carried out with 3,3′-diaminobenzidine (Vector Laboratories) for 2 min. For microscopic evaluation, the preparations were counterstained with hematoxylin and mounted. Negative controls consisted of replacement of the primary antibody with PBS. The presence of Ki67 nuclear staining was calculated as the percentage of positive tumor cells in relation to the total number of cells.

RESULTS

Identification of IS3 295 as an Inhibitor of Stat3 DNA-binding Activity—Compounds from the NCI 2000 diversity set were screened for inhibition of Stat3 signaling in an *in vitro* DNA-binding activity assay based on analysis by EMSA. A platinum (IV) complex, IS3 295 (NSC 295558) (Fig. 1*E*), was identified as a potent inhibitor of Stat3 and was further characterized for its anti-Stat3 properties. In the *in vitro* DNA-binding assay, nuclear extracts of equal total protein containing activated Stat1, Stat3, and Stat5 were preincubated with different concentrations of IS3 295 for 30 min prior to incubation with a ^{32}P-labeled oligonucleotide, the m67 hSIE probe that binds Stat1 and Stat3, or the mammary gland factor element that binds Stat1 and Stat5. Samples were then subjected to EMSA. The results show that the presence of IS3 295 in the nuclear extracts led to a dose-dependent reduction in the levels of the DNA-binding activity of the Stat3/Stat3 homodimer (Fig. 1*A*, *panel i*, *upper band*), Stat1/Stat3 heterodimer (*panel i*, *middle band*), and, to a lesser extent, the Stat1/Stat1 homodimer (*panels i* and *ii*, *lower bands*). In contrast, EMSA analysis showed that the presence of IS3 295 did not significantly affect the level of the DNA-binding activity of the Stat5/Stat5 dimer (Fig. 1*A*, *panel ii*, *upper band*). These results indicate that IS3 295 selectively disrupted Stat3 over Stat1 (IC_{50} = 1.4 and 4.1 μM, respectively) (Fig. 1*A*, *panel iii*), consistent with our previous findings with other platinum(IV) complexes (38).

In control studies, we evaluated the effect of IS3 295 on the DNA-binding activity of the E2F1 protein, which is unrelated to STAT proteins. Analysis by EMSA showed that preincubation with IS3 295 of cell lysates containing E2F1 prior to incubation with a ^{32}P-labeled dihydrofolate reductase promoter sequence as probe had no significant effect on the DNA-binding activity (Fig. 1*B*, *panel i*). In another control study, we similarly evaluated the effect of cisplatin on the DNA-binding activities of Stat1, Stat3, and Stat5 (Fig. 1*B*, *panel ii*) (data not shown) and E2F1 (data not shown) *in vitro* and found no detectable effect. These findings together suggest that the effect of IS3 295 is selective for Stat3 and that it is not a general phenomenon of all platinum compounds to inhibit the activities of transcription factors.

Lack of Effect of IS3 295 on STAT Proteins Pre-bound to DNA—To further characterize the disruption of *in vitro* Stat3 DNA-binding activity by IS3 295, the sequence of the addition of reagents during the DNA-binding assay was changed to determine how it affects the kinetics of IS3 295-mediated inhibition. Nuclear extracts containing activated Stat3

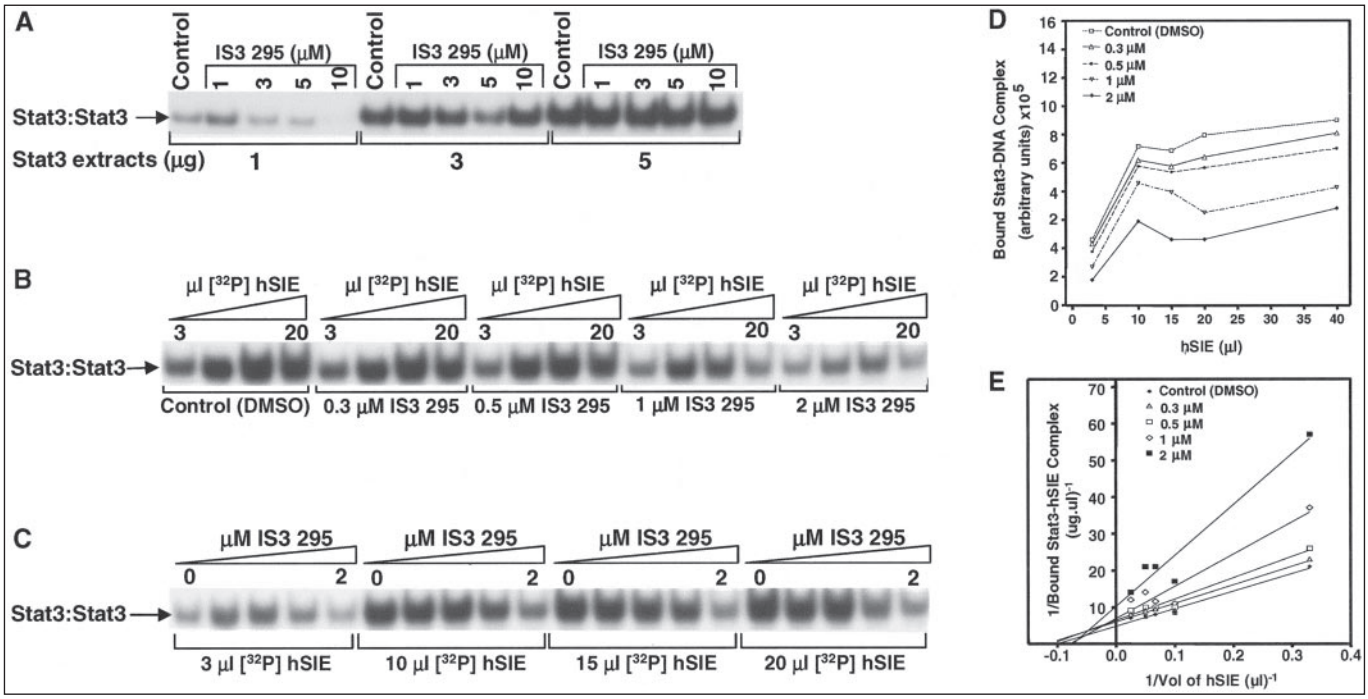

FIGURE 2. **Kinetics of IS3 295-mediated inhibition of *in vitro* Stat3 DNA-binding activity.** Cell lysates containing activated Stat3 were incubated with the radiolabeled hSIE probe for 30 min at room temperature in the presence or absence of IS3 295 and then subjected to EMSA analysis. *A*, DNA-binding activities for different Stat3 protein amounts in the presence of increasing concentrations of IS3 295; *B*, levels of Stat3 binding to increasing amounts of the hSIE oligonucleotide probe in the absence (*Control*) and presence of IS3 295; *C*, levels of Stat3 binding to the hSIE oligonucleotide probe in the presence of increasing concentrations of IS3 295; *D*, plots of the Stat3/Stat3-hSIE complex *versus* the levels of hSIE oligonucleotide probe at different concentrations (0.3–2 μM) of IS3 295; *E*, Lineweaver-Burk analysis (double-reciprocal plot) of the Stat3-hSIE complex *versus* the levels of hSIE at different concentrations (0.3–2 μM) of IS3 295. The positions of the Stat3/Stat3-DNA complexes on the gels are labeled. *DMSO*, dimethyl sulfoxide.

were first incubated with the radiolabeled hSIE probe for 30 min (to allow prior Stat3 binding to the oligonucleotide probe), followed by the addition of IS3 295 for an additional 3–30 min, and then subjected to EMSA analysis. IS3 295 failed to disrupt Stat3 DNA-binding activity when the protein was first bound to the DNA-response element probe (Fig. 1*C*, panel *i*, compare the *first* through *sixth lanes* with the *seventh* through *twelfth lanes* (control)). These findings indicate that DNA-bound Stat3 protein was occluded from inhibition by IS3 295, suggesting that the Stat3 region required for interaction with IS3 295 is already bound to DNA. By contrast, EMSA analysis showed that the simultaneous addition of IS3 295 and the hSIE oligonucleotide probe to nuclear extracts containing activated Stat3 protein resulted in inhibition of Stat3 DNA-binding activity (data not shown), as observed in Fig. 1*A*, suggesting that Stat3 has a higher preference for IS3 295 over hSIE.

Interaction of IS3 295 with STAT Proteins—Because the evidence suggested a possible interaction of activated Stat3 dimers with IS3 295, we wondered whether inactive STAT monomer proteins could do the same. To address this, cell lysates of inactive STAT monomer proteins were added to the cell lysate of activated Stat3, and the mixture was preincubated with IS3 295 for 30 min prior to incubation with the radiolabeled hSIE probe and EMSA analysis. Although inactive STAT monomer proteins did not bind the DNA-response element or alter the binding of activated Stat3 dimer protein to DNA (Fig. 1*C*, *panel ii*, *seventh lane*), if they could interact with IS3 295, we reasoned that they would lower the concentration of IS3 295 and thereby diminish the extent of IS3 295-mediated inhibition of the DNA-binding activity of the activated Stat3 dimer.

Consistent with this possibility, EMSA analysis showed that the presence of the inactive Stat3 monomer significantly diminished the inhibitory effects of IS3 295 on activated Stat3 DNA-binding activity (Fig. 1*C*, *panel ii*, compare the *tenth* through *thirteenth lanes* with the *second* through *fifth lanes*). Indeed, the presence of the inactive Stat3 monomer protein completely restored the DNA-binding activity of activated Stat3 dimers that was hitherto abolished by 1–10 μM IS3 295 (Fig. 1*C*, *panel ii*, *eleventh* and *twelfth lanes versus third* and *fourth lanes*) and resulted in partial recovery of the activated Stat3 DNA-binding activity that was otherwise completely disrupted at high concentrations (30 μM) of IS3 295 (*thirteenth lane versus fifth lane*). However, at the level of protein present in the mixture, the inactive Stat3 monomer was insufficient to impact higher concentrations (50 μM) of IS3 295; and hence, no recovery of activated Stat3 DNA-binding activity was observed (Fig. 1*C*, *panel ii*, *fourteenth lane*). Together, these findings show that the Stat3 monomer interacted with IS3 295 and titrated it out, thereby reducing the levels that were available to inhibit activated Stat3. The interaction of IS3 295 with Stat3 protein is independent of the activation status of the protein.

Similar observations were made when the inactive Stat1 monomer was present in the mixture (Fig. 1*C*, panel *iii*, compare the *eighth* through *twelfth lanes* with the *first* through *sixth lanes*), suggesting that IS3 295 also interacts with Stat1 protein. In contrast, the inactive Stat5 monomer or a non-STAT-related protein, E2F1, failed to significantly influence the effect of IS3 295 on Stat3 DNA-binding activity (Fig. 1*C*, *panel iv*, compare the *eighth* through *fourteenth lanes* with the *first* through *sixth lanes*; and *panel v*, compare the *seventh* through *twelfth lanes* with the *first* through *sixth lanes*).

To investigate the possibility that IS3 295 might alter the integrity of the hSIE oligonucleotide probe that was used in the DNA-binding activity studies and thereby inhibit Stat3 binding, the oligonucleotide was first treated with IS3 295 for 30 min. The pretreated oligonucleotide was then radiolabeled and tested for its ability to bind to activated Stat3 *in vitro*. EMSA analysis revealed that the IS3 295-pretreated radiolabeled hSIE probe bound activated protein similarly to the untreated oligonucleotide probe (Fig. 1*D*, *first lane versus second lane*), indicating that the IS3 295 pretreatment of the oligonucleotide had no observable effect on the ability of the Stat3-responsive DNA sequence to bind to activated Stat3 *in vitro*. Although IS3 295 was in direct contact with the oligonu-

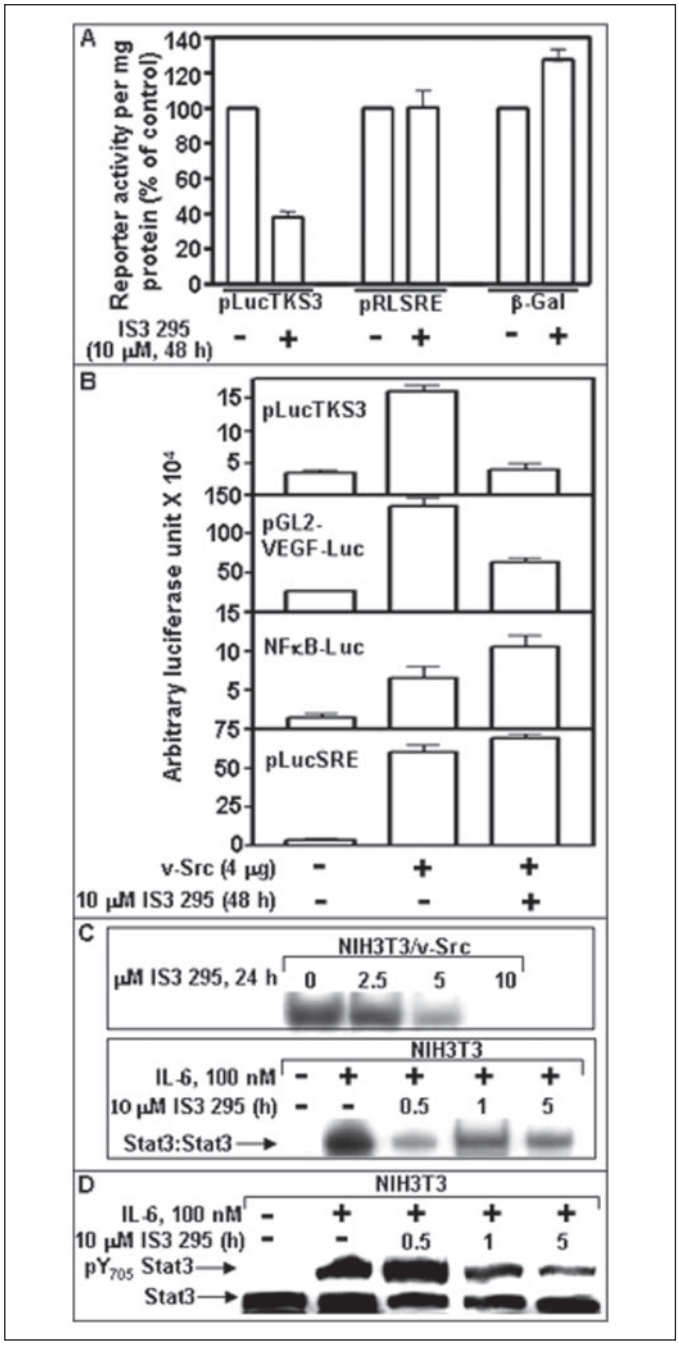

FIGURE 3. **Inhibition of Stat3-mediated gene expression by IS3 295.** *A* and *B*, v-Src-transformed mouse fibroblasts stably expressing Stat3-dependent (NIH3T3/v-Src/pLucTKS3) and Stat3-independent (NIH3T3/v-Src/pRLSRE) luciferase reporters or β-galactosidase (*β-Gal*) and normal mouse fibroblasts (NIH3T3) transiently transfected with pLucTKS3, pGL2-VEGF-Luc, pNFκB-Luc, or pLucSRE with or without v-Src were treated with or without IS3 295 for 48 h. Cytosolic extracts were then prepared from cells for luciferase and β-galactosidase activity measurements. Values are the means ± S.D. of three to five independent assays. *C* and *D*, nuclear extracts or whole cell lysates were prepared from interleukin-6 (*IL-6*)-stimulated normal mouse fibroblasts (NIH3T3) or their v-Src-transformed counterparts (NIH3T3/v-Src) and treated with or without IS3 295 for different times. Samples of equal total proteins were then subjected to *in vitro* DNA-binding assay and EMSA analyses or to SDS-PAGE and Western blot analysis for phosphorylated and total Stat3 proteins. pY_{705}, phospho-Tyr^{705}.

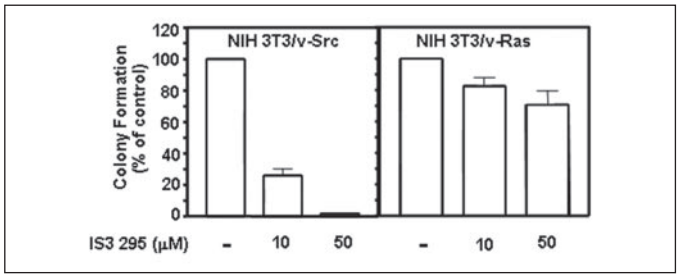

FIGURE 4. **Abrogation of Stat3-dependent v-Src transformation.** v-Src-transformed fibroblasts (NIH3T3/v-Src) and their Ras-transformed counterparts (NIH3T3/v-Ras) were seeded on soft agar, and growing cells were treated every 2–3 days with or without the indicated concentrations of IS3 295 until large colonies were evident. The number of cell colonies on soft agar were counted and are expressed as a percentage of the control (untreated) cells. Values are the means ± S.D. of three independent assays.

cleotide probe during the pretreatment stage and prior to radiolabeling of the probe, it is unlikely to have been retained with the oligonucleotide probe following purification of the probe and therefore would not have come into contact with Stat3 protein at the time of the *in vitro* DNA-binding assay. Thus, under the conditions of the *in vitro* DNA-binding assay, IS3 295 did not directly alter the binding properties of the Stat3-responsive DNA element, suggesting that the DNA element may not directly interact with IS3 295.

Kinetics of IS3 295-mediated Inhibition of Stat3 DNA-binding Activity—To further explore the interaction of IS3 293 with Stat3 protein, the levels of activated Stat3 protein and the radiolabeled hSIE oligonucleotide probe were varied during the *in vitro* DNA-binding assay, and the extent of IS3 295-mediated inhibition was determined. EMSA analysis showed that *in vitro* Stat3 DNA-binding activity increased with increasing protein amounts (Fig. 2*A*, *first*, *sixth*, and *eleventh lanes*). At a low protein amount (1 μg), the presence of IS3 295 caused a strong and dose-dependent decrease in the level of Stat3 DNA-binding activity (Fig. 2*A*, *second* through *fifth lanes*), consistent with Fig. 1*A* (*panel i*). In contrast, there was a diminished effect of IS3 295 on Stat3 DNA-binding activity at higher protein levels (2–3 μg) (Fig. 2*A*, *sixth* through *fifteenth lanes*), suggesting that increasing the Stat3 protein amount overcomes the inhibitory effects of IS3 295 (compare the *eighth* through *tenth* and *thirteenth* through *fifteenth lanes* with the *third* through *fifth lanes*). The apparent restoration of Stat3 DNA-binding activity at higher protein amounts suggests high levels of residual activated Stat3 protein (in excess of the amount that interacted with IS3 295), which in turn bound the probe. Altogether, the findings (Figs. 1 and 2*A*) indicate that IS3 295 interacted with Stat3 protein and thereby inhibited Stat3 DNA-binding activity.

For the same protein amount, the results further show that increasing the level of the radiolabeled hSIE probe resulted in an increased level of *in vitro* Stat3 DNA-binding activity (Fig. 2, *B*; *C*, *first*, *sixth*, *eleventh*, and *sixteenth lanes*; and *D*) until a maximum DNA-binding activity was reached at higher levels of hSIE (a saturation or plateau phase in the plot of the Stat3-DNA complex *versus* the amount of hSIE) (Fig. 2*D*). In the presence of IS3 295, however, the maximum binding levels (plateau phase) attained were diminished, particularly at high concentrations (1–2 μM) of IS3 295 (Fig. 2, *B–D*), suggesting that increasing probe levels fail to overcome the inhibitory effect of higher concentrations of IS3 295. Instead, varying degrees of saturation of Stat3 DNA-binding activity were observed with increasing probe levels under high IS3 295 concentrations (0.3–2 μM) (Fig. 2*D*). Thus, increasing the oligonucleotide probe levels did not restore the maximum DNA-binding activity that was expected for any given amount of protein at different IS3 295 concentrations (1 μM and higher) (Fig. 2*D*). A Lineweaver-Burk (double-reciprocal) plot of the Stat3-DNA complex *versus* the concentration of hSIE suggests that the inhibitory effect of IS3 295 on Stat3 DNA-binding activity displays noncompetitive-type kinetics (Fig. 2*E*), with apparent changes in affinity and maximum binding levels.

IS3 295 Selectively Blocks Intracellular Stat3 Signaling and Stat3-mediated Transformation—To further investigate the activities of IS3 295, we treated malignant cells and measured the effects on Stat3 signaling. In stable cell lines harboring constitutively active Stat3

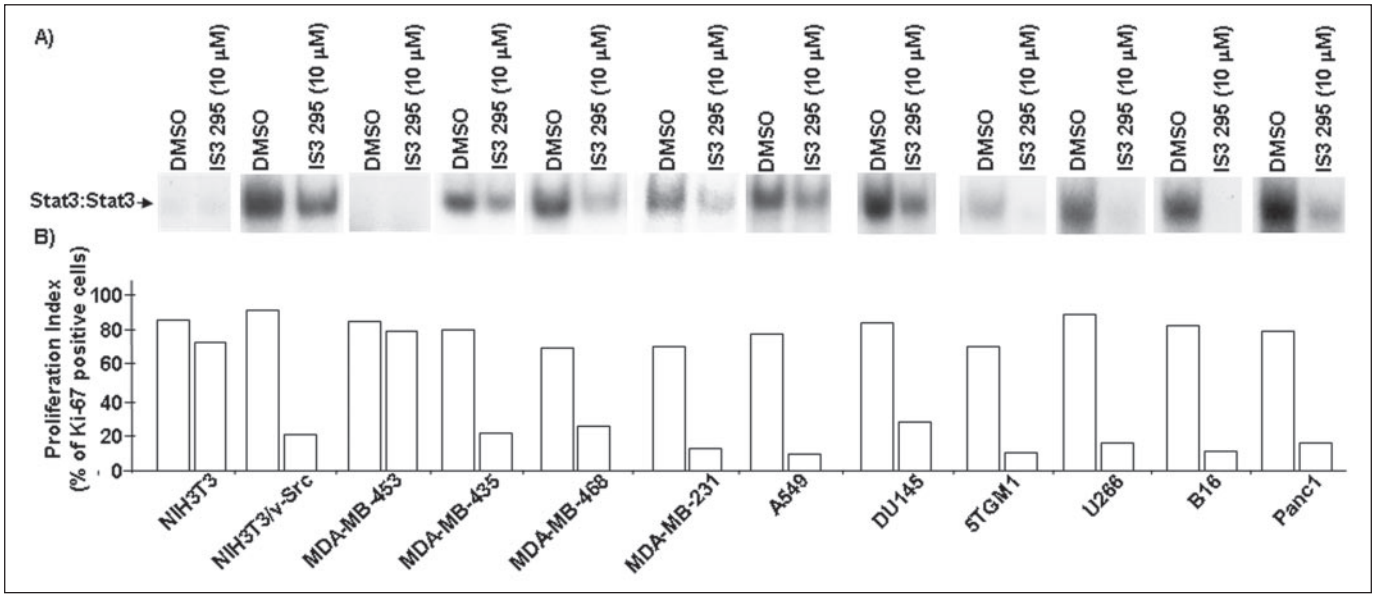

FIGURE 5. **Evaluation of the effects of IS3 295 on cellular constitutive Stat3 activation and cell proliferation.** Normal or malignant cells were treated with or without IS3 295. Nuclear extracts were prepared for Stat3 DNA-binding activity assay with the hSIE probe, or cells were processed for nuclear Ki67 immunohistochemistry. *A*, EMSA analysis of Stat3 DNA-binding activity; *B*, graphical representation of the quantified nuclear staining of the Ki67 proliferation index. The positions of the Stat3/Stat3-DNA complexes on the gels are labeled. The Ki67 proliferation index was calculated as the percentage of positive tumor cells relative to the total number of cells. Ki67 values are representative of three independent assays. *DMSO*, dimethyl sulfoxide.

(NIH3T3/v-Src/pLucTKS3 and NIH3T3/v-Src/pRLSRE) and overexpressing Stat3-dependent firefly (pLucTKS3) and Stat3-independent *Renilla* (pRLSRE) luciferase reporters (41), IS3 295 strongly suppressed the induction of the Stat3-dependent reporter but not the Stat3-independent luciferase reporter or the induction of β-galactosidase in the v-Src-transformed fibroblasts expressing the β-galactosidase vector (Fig. 3). Similar results were obtained in transient transfection studies of mouse NIH3T3 fibroblasts with the Stat3 reporter (pLucTKS3) or the Stat3-dependent VEGF promoter-driven luciferase reporter (pGL2-VEGF-Luc) (30) following activation of Stat3 by v-Src (11) and treatment with IS3 295 (Fig. 3*B*, *upper two panels*). Taken together with the results in Fig. 1, these findings indicate that IS3 295 blocked the binding of activated Stat3 to its responsive elements in the promoters of target genes. Moreover, in a time- and dose-dependent manner, IS3 295 inhibited the constitutive or ligand-induced activation of Stat3 DNA-binding activity (Fig. 3*C*) as well as tyrosine phosphorylation in mouse fibroblasts (Fig. 3*D*) (data not shown). We noted that interleukin-6-induced Stat3 activation was inhibited by IS3 295 as early as 30 min (Fig. 3*C*, *lower panel*), whereas the inhibition of constitutively active Stat3 in v-Src-transformed NIH3T3/v-Src fibroblasts by IS3 295 required >12 h to be significant (data not shown). Changes in Stat3 protein levels were minimum. In contrast, the platinum compound had no inhibitory effects on the induction of Stat3-independent NFκB promoter-driven (pNFκB-Luc) or c-*fos* promoter-driven (pLucSRE) luciferase reporter activity in transient transfection studies in mouse fibroblasts (Fig. 3*B*, *lower two panels*). The results show that, at 5–10 μM IS3 295, which inhibited intracellular Stat3 signaling, there was no observable nonspecific effect on the non-Stat3-related pathways investigated here. These findings together demonstrate that IS3 295 selectively repressed the tyrosine phosphorylation and DNA-binding activity of Stat3 as well as the transcriptional regulation in cells by Stat3.

IS3 295 was next evaluated for effects on Stat3-mediated v-Src transformation (11, 12, 44). The results show that treatment of v-Src-transformed NIH3T3/v-Src fibroblasts grown on soft agar with IS3 295 strongly suppressed growth (Fig. 4, *left panel*). In contrast, similar treatment of v-Ras-transformed NIH3T3/v-Ras fibroblasts grown on soft agar only partially inhibited growth (Fig. 4, *right panel*). These findings are consistent with inhibition of constitutively active Stat3 by IS3 295 and attenuation of growth of v-Src-transformed cells and show that the IS3 295 inhibitory effect was selective against cells harboring constitutively active Stat3.

IS3 295-induced Block of Cell Cycle Progression and Proliferation—We further examined the biological effects of IS3 295 and determined whether any changes might correlate with the inhibition of constitutively active Stat3. Normal NIH3T3 fibroblasts and their v-Src-transformed counterparts (human breast cancer cell lines that harbor constitutively active Stat3 (MDA-MB-435, MDA-MB-231, and MDA-MB-468) and those that do not (MDA-MB-453 and MCF-7) as well as a human non-small cell lung cancer cell line (A549), a human prostate cancer cell line (DU145), and a murine multiple myeloma cell line (5TGM1) all of which harbor constitutively active Stat3) (19, 42, 52) were treated with or without IS3 295 for 24 h. Cells were then harvested for nuclear extract preparation and *in vitro* Stat3 DNA-binding assay with EMSA analysis or processed for cell proliferation and cell cycle analysis by flow cytometry.

EMSA analysis of Stat3 DNA-binding activity in nuclear extracts prepared from malignant cells harboring constitutively active Stat3 and treated with IS3 295 revealed significant inhibition of constitutive activation of Stat3 in those cells (Fig. 5*A*). These observations support the inhibition of Stat3 transcriptional activity (Fig. 3) and together indicate that IS3 295 selectively blocked constitutive activation of Stat3 signaling in diverse cell types. Normal NIH3T3 fibroblasts and the human breast cancer cell line MDA-MB-453 do not harbor constitutively active Stat3 (Fig. 5*A*).

Changes in cell proliferation induced by treatment with IS3 295 were assayed in terms of the Ki67 proliferation index by immunohistochemistry. The presence of Ki67 nuclear staining was calculated as the percentage of positive tumor cells in relation to the total number of cells. In contrast to the lack of effect of IS3 295 on the proliferation of normal NIH3T3 fibroblasts or the human breast cancer cell line MDA-MB-453 (which does not harbor persistent Stat3 activity), treatment with IS3 295 caused significant decreases in Ki67 nuclear staining for the malignant cells harboring constitutively active Stat3 (Fig. 5*B*), which correlated with inhibition of Stat3 activity (Fig. 5*A*).

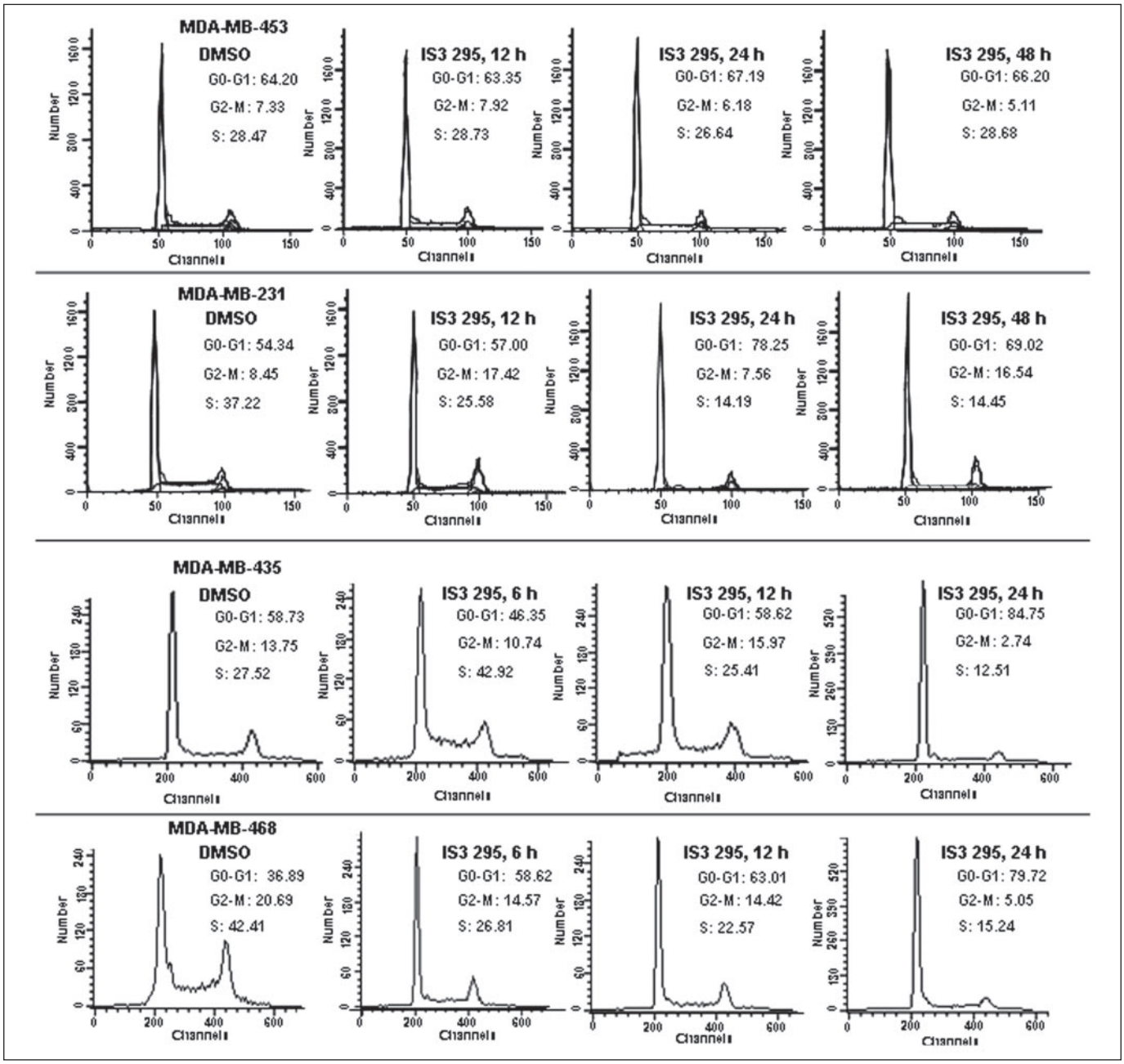

FIGURE 6. **Analysis of relative cellular DNA content by BrdUrd labeling and flow cytometry.** The relative DNA content of human breast cancer cell lines following treatment with or without IS3 295 was analyzed by BrdUrd incorporation and flow cytometry. The population of cells determined from the relative DNA content is shown in each panel for each treatment condition. The results are representative of three independent determinations. *DMSO*, dimethyl sulfoxide.

In flow cytometry analyses for cell cycle changes in IS3 295-treated and untreated (control) cells exposed to BrdUrd, the results show that the breast cancer cell line MDA-MB-231 was arrested at G_0/G_1 phase following IS3 295 treatment (Fig. 6). A significant decrease in S phase cells was also observed, which paralleled the drop in DNA synthesis as judged from the level of incorporation of BrdUrd and persisted for up to 48 h. Similar results were observed after 6 h of IS3 295 treatment of the breast cancer cell line MDA-MB-468 (Fig. 6) (data not shown). In the case of the MDA-MB-435 cell line, similar observations were made following 24 h of treatment (Fig. 6). In contrast, no significant change in the cell cycle profile was observed when the breast cancer cell line MDA-MB-453 (which does not harbor constitutively active Stat3) was treated with IS3 295 (Fig. 6). These findings together show that malignant cells that harbor persistently elevated levels of Stat3 activity were more sensitive to IS3 295 compared with cells that do not, consistent with the ability of IS3 295 to inhibit constitutively active Stat3 and its biological functions.

IS3 295-mediated Apoptosis of Malignant Cells—Malignant cells that harbor persistent Stat3 signaling and those that do not were examined for evidence of apoptosis following treatment with IS3 295. Cells were analyzed for DNA strand breaks by TUNEL staining. Significant TUNEL staining was detected in v-Src-transformed NIH3T3/v-Src fibroblasts; human breast carcinoma cell lines MDA-MB-435, MDA-MB-468, and MDA-MD-231; human non-small cell lung cancer cell line A549; human prostate carcinoma cell line DU145; multiple myeloma cell lines 5TGM1 (mouse) and U266 (human); mouse melanoma cell line B16; and human pancreatic cancer cell line Panc1, all of which harbor constitutively active Stat3, following treatment with IS3 295 (Fig. 7). In contrast, no TUNEL staining was observed in control (Me_2SO-treated) cells or in mouse NIH3T3 fibroblasts and human

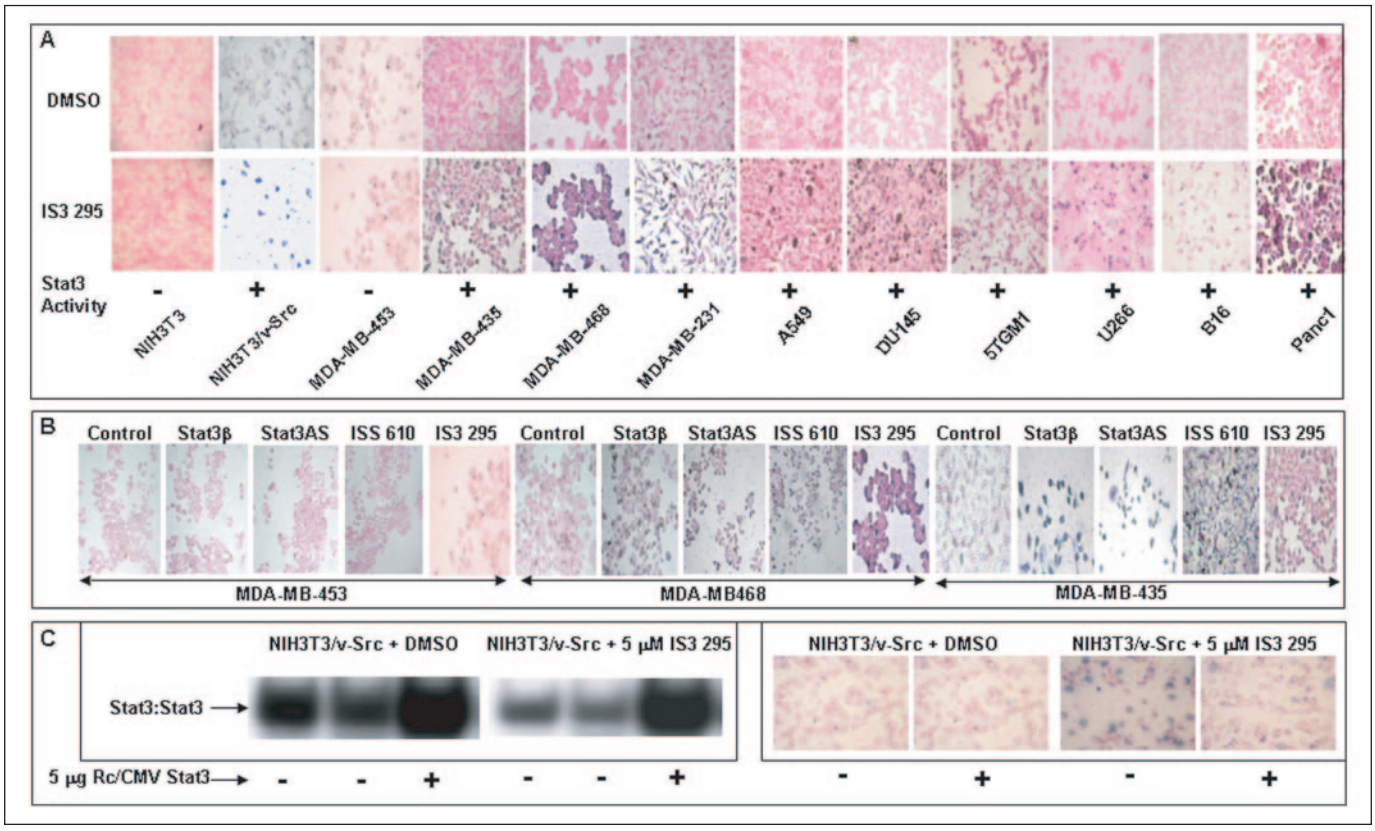

FIGURE 7. **TUNEL analysis of IS3 295-mediated apoptosis.** *A*, normal NIH3T3 fibroblasts and their v-Src-transformed counterparts (NIH3T3/v-Src); human breast carcinoma cell lines MDA-MB-453, MDA-MB-435, MDA-MB-468, and MDA-MB-231; human non-small cell lung cancer cell line A549; human prostate cancer cell line DU145; multiple myeloma cell lines 5TGM1 (mouse) and U266 (human); mouse melanoma cell line B16; and human pancreatic cancer cell line Panc1 were all treated with or without IS3 295 for 48 h and analyzed by TUNEL for DNA damage. For each cell line, the activated Stat3 status is indicated: −, no constitutively active Stat3; +, constitutively active Stat3 (see Fig. 5*A*). Data are representative of three independent determinations. *B*, human breast cancer cell lines MDA-MB-453, MDA-MB-468, and MDA-MB-435 were transfected with or without Stat3β or antisense Stat3 (*Stat3AS*) or were treated with or without the Stat3 peptidomimetic inhibitor ISS 610 (1 mM) or IS3 295 (5 μM). Forty-eight h later, the cells were harvested and processed for TUNEL analysis. *C*, v-Src-transformed NIH3T3/v-Src fibroblasts were transfected with or without wild-type Stat3 (pRc/CMV-Stat3-FLAG (*Rc/CMV Stat3*)) and treated with or without 5 μM IS3 295 for 36 h. Cells were subsequently harvested for nuclear extract preparation and Stat3 DNA-binding assay *in vitro* with EMSA analysis (*left panel*) or were processed for TUNEL analysis (*right panel*). *DMSO*, dimethyl sulfoxide.

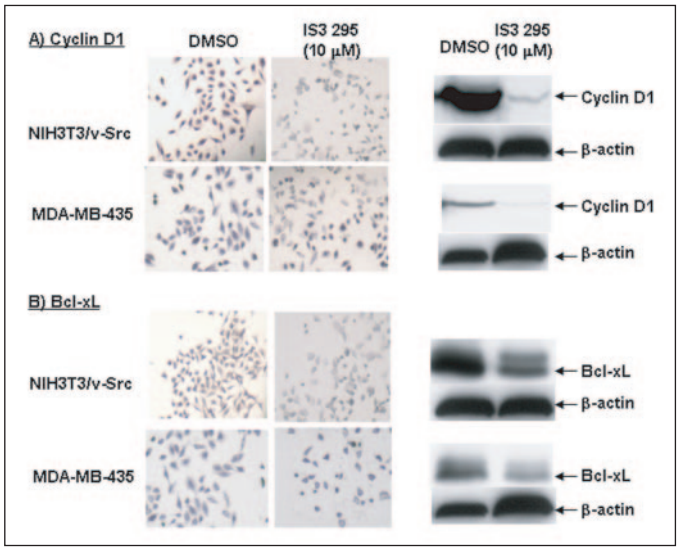

FIGURE 8. **Inhibition of cyclin D_1 and Bcl-x_L induction by IS3 295.** v-Src-transformed NIH3T3/v-Src fibroblasts and human breast cancer cell line MDA-MB-435 (which contains constitutively active Stat3) were treated with or without the platinum complex for 48 h. Cells were processed for immunohistochemistry, or cell lysates were prepared from cells and subjected to 5% PAGE and Western blot analysis as described under "Experimental Procedures." *A*, detection of cyclin D_1; *B*, detection of Bcl-x_L. The positions of the cyclin D_1 and Bcl-x_L proteins are shown. β-Actin levels are shown for normalization of equal total protein. Data are representative of three independent determinations. *DMSO*, dimethyl sulfoxide.

breast cancer cell line MDA-MB-453 (which do not contain aberrant Stat3 activity) following treatment with IS3 295 (Fig. 7). The incidence of apoptosis correlated with the prevalence of constitutively active Stat3 in malignant cells (Fig. 7*A*, *lower panels*). Moreover, the incidence of apoptosis induced by IS3 295 in breast cancer cell lines MDA-MB-468 and MDA-MB-435 (which harbor constitutively active Stat3) compares favorably with that induced by other previously investigated Stat3 inhibitory approaches, including Stat3β (19), antisense Stat3 (42), and ISS 610 (a peptidomimetic inhibitor of Stat3) (53), and contrasts with the absence of apoptosis in breast cancer cell line MDA-MB-453 (which lacks constitutively active Stat3) (Fig. 7*B*). These results indicate differences in the sensitivity of cells to IS3 295 that are dependent on the activation status of Stat3 inside the cells. The susceptible malignant cells are those that harbor constitutively active Stat3; these cells undergo cell cycle arrest and apoptosis in response to IS3 295, consistent with previous reports (15, 18–20, 29, 38, 42).

In Vivo Overexpression of Stat3 Protein Affects IS3 295 Activity and Effect—To further elucidate the interaction between Stat3 and IS3 295, v-Src-transformed mouse fibroblasts were transfected with Stat3 protein and then used to investigate IS3 295 activity. The transfection of wild-type Stat3 into the v-Src-transformed fibroblasts resulted in higher Stat3 DNA-binding activity compared with the parental fibroblasts as measured by *in vitro* DNA-binding activity and EMSA analysis (Fig. 7*C*, *left panel*, *third* and *sixth lanes versus first* and *second lanes*). This was due to the increased expression of Stat3 protein and the consequent

phosphorylation by oncogenic Src tyrosine kinase present in these cells. EMSA analysis of nuclear extracts prepared from parental mock-transfected cells and treated with IS3 295 showed significant reduction in constitutively active Stat3 DNA-binding activity (Fig. 7*C*, *left panel*, *fourth* and *fifth lanes versus first* and *second lanes*), as previously observed (Figs. 3*C*, *upper panel*; and 5*A*, *third* and *fourth lanes*). In contrast, EMSA analysis showed that cells transfected with exogenous wild-type Stat3 and treated with IS3 295 showed no significant reduction in constitutively active Stat3 DNA-binding activity (Fig. 7*C*, *left panel*, *sixth lane versus third lane*). This finding is consistent with data from the *in vitro* DNA-binding assay (Fig. 1*C*, *panel ii*) and indicates that the presence of higher levels of Stat3 protein, either as a monomer or dimer, diminished the relative potency of IS3 295, thus further confirming that IS3 295 interacts with Stat3 protein. Moreover, in diminishing the effect of IS3 295 on Stat3 DNA-binding activity, the results show that the overexpression of Stat3 in v-Src-transformed mouse fibroblasts minimized the extent of IS3 295-mediated apoptosis (Fig. 7*C*, *right panel*, *third* and *fourth images*). Finally, this finding is important in that it suggests that the potency of direct inhibitors of Stat3 activation as therapeutics for tumors harboring constitutively active Stat3 is influenced by the expression levels of Stat3 protein.

IS3 295 Represses Induction of Bcl-x_L and Cyclin D_1—To investigate the molecular changes downstream from Stat3 that may contribute to the biological responses induced by IS3 295, *in situ* detection and Western blot analyses were performed for cell cycle and apoptosis regulators. The results show that cyclin D_1 was significantly diminished in v-Src-transformed mouse NIH3T3/v-Src fibroblasts and human breast cancer cell line MDA-MB-435 in response to IS3 295-induced inhibition of Stat3 activation (Fig. 8). Similar observations were made for the anti-apoptotic protein Bcl-x_L in both malignant cell lines (which harbor constitutively active Stat3) following treatment with IS3 295 (Fig. 8). These findings parallel the cell cycle or proliferation changes and the induction of apoptosis by the IS3 295 treatment (Figs. 5–7) and indicate that inhibition of constitutively active Stat3 by IS3 295 blocked cyclin D_1 and Bcl-x_L induction.

DISCUSSION

Signal transduction pathways and transcription factors have taken on greater significance as molecular targets in cancer drug discovery because of their central roles in carcinogenesis (27, 28, 54). Previously, we reported on novel platinum (IV) complexes that inhibit the Stat3 transcription factor and induce anti-tumor cell activity (38). Here, we present evidence that another novel platinum (IV) complex, IS3 295 (NSC 295558), functions as a Stat3 inhibitor by directly interacting with the protein. The evidence indicates that IS3 295 interacts with Stat3 protein, both the inactive monomer and the activated dimer, and represses Stat3 phosphotyrosine levels, DNA-binding activity, and transcriptional regulation. Differences are evident in the modes of activity and selectivity between IS3 295 and the widely used anti-tumor agent cisplatin (37, 55), which has no effect on Stat3 activity (38). By contrast, IS3 295 blocks the binding of activated Stat3 to a specific DNA-response element. Although the exact site(s) within Stat3 protein that interacts with IS3 295 is not yet known, preliminary evidence implicates cysteine residue(s) (data not shown). This is consistent with previous reports that cisplatin and other platinum complexes interact with cysteine and methionine residues in serum albumin and γ-globulins (36, 56–58), forming thiol conjugates of platinum complexes (57, 59, 60). It is conceivable that such a modification in Stat3 protein by IS3 295 in turn occludes the binding of Stat3 to its consensus DNA-response element.

A noncompetitive-type inhibition by IS3 295 was observed, supported by the reduced maximum DNA-binding activity of Stat3 following IS3 295 treatment. The treatment of activated Stat3 with IS3 295 also induced an apparent change in the binding affinity of the protein for the consensus DNA sequence. Inferring from the known interactions of other platinum complexes with thiol-containing biological molecules (37, 57, 59, 60), the expected reaction of IS3 295 with thiol groups of Stat3 would be irreversible. Those previous studies and ours together suggest that IS3 295 irreversibly modifies Stat3 protein, thereby blocking the DNA-binding activity of the protein and subverting its transcriptional and biological functions. The lack of any IS3 295 effect on Stat3 protein pre-bound to DNA suggests a shielding of the key amino acid(s) within the protein once the latter is first bound to DNA. This raises the possibility that both IS3 295 and the consensus DNA sequence bind to the same region of Stat3 in the DNA-binding domain or that Stat3 protein undergoes conformational changes upon binding to the DNA sequence that restrict access to the key amino acid residue(s) and prevent interaction with IS3 295. Indeed, the crystal structure of the Stat3β dimer bound to DNA (61) shows that the protein is clamped around the DNA double helix, which may be sufficient to impede access by IS3 295 to the DNA-binding domain.

Previous reports have established constitutively active Stat3 as key to the dysregulated growth, survival, angiogenesis, and immune evasion that characterize tumorigenesis (22, 27). The biological effects of IS3 295 are manifest in malignant cells harboring constitutively active Stat3, including inhibition of Stat3-dependent transformation as well as inhibition of cell growth with G_0/G_1 cell cycle arrest and apoptosis of malignant cells (15, 19, 29, 38). Thus, IS3 295 inhibits Stat3-mediated induction of critical genes, including the cell cycle regulator cyclin D_1, anti-apoptotic *bcl-x_L*, and pro-angiogenic VEGF, which are important in tumor processes (15, 30, 62, 63).

In contrast to the DNA denaturation and the formation of platinum-DNA adducts by cisplatin (37, 64), direct modification of DNA is not a key factor in the inhibition of Stat3 signaling and biological functions by IS3 295. Our findings show that the Stat3 binding integrity of the DNA-response element was preserved following treatment of DNA with IS3 295. Moreover, oligonucleotide melting and re-annealing analysis with agarose gel electrophoresis showed a retention of the overall integrity of the IS3 295-treated DNA-response element (data not shown). The effects of IS3 295 on Stat3 observed here also contrast with those of cisplatin and others that modulate the phosphatidylinositol 3-kinase/Akt and MAPK family pathways, which contribute to their biological effects (34–37). We did not observe any effects of IS3 295 on Stat3-independent transcriptional events.

In summary, our results provide proof of concept for a novel platinum compound that interacts with Stat3 and inhibits its biological functions, suggesting that modulation of Stat3 signaling is an important mechanism of anti-tumor cell activity of platinum (IV) compounds. This study represents a significant advance in the development of small molecules that target Stat3 as therapeutics against tumors that harbor constitutively active Stat3 and suggests new applications for platinum (IV) complexes as modulators of Stat3 signal transduction pathways with important clinical implications.

Acknowledgments—We thank colleagues and members of our laboratory for stimulating discussions. We also acknowledge the Molecular Imaging and Flow Cytometry Core Groups of the H. Lee Moffitt Cancer Center & Research Institute for imaging and flow cytometry assistance.

REFERENCES

1. Darnell, J. E., Jr., Kerr, I. M., and Stark, G. R. (1994) *Science* **264,** 1415–1421
2. Schindler, C., and Darnell, J. E., Jr. (1995) *Annu. Rev. Biochem.* **64,** 621–651

3. Darnell, J. E., Jr. (1997) *Science* **277,** 1630–1635
4. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D. (1998) *Annu. Rev. Biochem.* **67,** 227–264
5. Bromberg, J. F., Horvath, C. M., Wen, Z., Schreiber, R. D., and Darnell, J. E., Jr. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 7673–7678
6. Fukada, T., Hibi, M., Yamanaka, Y., Takahashi-Tezuka, M., Fujitani, Y., Yamaguchi, T., Nakajima, K., and Hirano, T. (1996) *Immunity* **5,** 449–460
7. Kotenko, S. V., and Pestka, S. (2000) *Oncogene* **19,** 2557–2565
8. Smithgall, T. E., Briggs, S. D., Schreiner, S., Lerner, E. C., Cheng, H., and Wilson, M. B. (2000) *Oncogene* **19,** 2612–2618
9. Hirano, T., Ishihara, K., and Hibi, M. (2000) *Oncogene* **19,** 2548–2556
10. Akira, S. (2000) *Oncogene* **19,** 2607–2611
11. Turkson, J., Bowman, T., Garcia, R., Caldenhoven, E., de Groot, R. P., and Jove, R. (1998) *Mol. Cell. Biol.* **18,** 2545–2552
12. Bromberg, J. F., Horvath, C. M., Besser, D., Lathem, W. W., and Darnell, J. E., Jr. (1998) *Mol. Cell. Biol.* **18,** 2553–2558
13. Garcia, R., Yu, C. L., Hudnall, A., Catlett, R., Nelson, K. L., Smithgall, T., Fujita, D. J., Ethier, S. P., and Jove, R. (1997) *Cell Growth & Differ.* **8,** 1267–1276
14. Nielsen, M., Kaltoft, K., Nordahl, M., Ropke, C., Geisler, C., Mustelin, T., Dobson, P., Svejgaard, A., and Odum, N. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 6764–6769
15. Catlett-Falcone, R., Landowski, T. H., Oshiro, M. M., Turkson, J., Levitzki, A., Savino, R., Ciliberto, G., Moscinski, L., Fernandez-Luna, J. L., Nuñez, G., Dalton, W. S., and Jove, R. (1999) *Immunity* **10,** 105–115
16. Nielsen, M., Kaestel, C. G., Eriksen, K. W., Woetmann, A., Stokkedal, T., Kaltoft, K., Geisler, C., Ropke, C., and Odum, N. (1999) *Leukemia* (*Basingstoke*) **13,** 735–738
17. Bromberg, J. (2000) *Breast Cancer Res.* **2,** 86–90
18. Grandis, J. R., Drenning, S. D., Zeng, Q., Watkins, S. C., Melhem, M. F., Endo, S., Johnson, D. E., Huang, L., He, Y., and Kim, J. D. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 4227–4232
19. Garcia, R., Bowman, T. L., Niu, G., Yu, H., Minton, S., Muro-Cacho, C. A., Cox, C. E., Falcone, R., Fairclough, R., Parson, S., Laudano, A., Gazit, A., Levitzki, A., Kraker, A., and Jove, R. (2001) *Oncogene* **20,** 2499–2513
20. Epling-Burnette, P. K., Lui, J. H., Catlette-Falcone, R., Turkson, J., Oshiro, M., Kothapalli, R., Li, Y., Wang, J.-M., Yang-Yen, H.-F., Karras, J., Jove, R., and Loughran, T. P., Jr. (2001) *J. Clin. Investig.* **107,** 351–362
21. Bowman, T., Garcia, R., Turkson, J., and Jove, R. (2000) *Oncogene* **19,** 2474–2488
22. Turkson, J., and Jove, R. (2000) *Oncogene* **19,** 6613–6626
23. Song, J. I., and Grandis, J. R. (2000) *Oncogene* **19,** 2489–2495
24. Coffer, P. J., Koenderman, L., and de Groot, R. P. (2000) *Oncogene* **19,** 2511–2522
25. Lin, T. S., Mahajan, S., and Frank, D. A. (2000) *Oncogene* **19,** 2496–2504
26. Buettner, R., Mora, L. B., and Jove, R. (2002) *Clin. Cancer Res.* **8,** 945–954
27. Yu, H., and Jove, R. (2004) *Nat. Rev. Cancer* **4,** 97–105
28. Turkson, J. (2004) *Expert Opin. Ther. Targets* **8,** 409–422
29. Bowman, T., Broome, M., Sinibaldi, N., Wharton, W., Pledger, W. J., Sedivy, J., Irby, R., Yeatman, T., Courneidge, S. A., and Jove, R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 7319–7324
30. Niu, G., Wright, K. L., Huang, M., Song, L., Haura, E., Turkson, J., Zhang, S., Wang, T., Sinibaldi, D., Coppola, D., Heller, R., Ellis, L. M., Karras, J., Bromberg, J., Pardoll, D., Jove, R., and Yu, H. (2002) *Oncogene* **21,** 2000–2008
31. Wang, T., Niu, G., Kortylewski, M., Burdelya, L., Shain, K., Zhang, S., Bhattacharya, R., Gabrilovich, D., Heller, R., Coppola, D., Dalton, W., Jove, R., Pardoll, D., and Yu, H. (2004) *Nat. Med.* **10,** 48–54
32. Niu, G., Heller, R., Catlett-Falcone, R., Coppola, D., Jaroszeski, M., Dalton, W., Jove, R., and Yu, H. (1999) *Cancer Res.* **59,** 5059–5063
33. Oshiro, M. M., Landowski, T. H., Catlett-Falcone, R., Hazlehurst, L. A., Huang, M., Jove, R., and Dalton, W. S. (2001) *Clin. Cancer Res.* **7,** 4262–4271
34. Sanchez-Perez, I., Murguia, J. R., and Perona, R. (1998) *Oncogene* **16,** 533–540
35. Persons, D. L., Yazlovitskaya, E. M., Cui, W., and Pelling, J. C. (1999) *Clin. Cancer Res.* **5,** 1007–1014
36. Bose, R. N. (2002) *Mini. Rev. Med. Chem.* **2,** 103–111
37. Siddik, Z. H. (2003) *Oncogene* **22,** 7265–7279
38. Turkson, J., Zhang, S., Palmer, J., Kay, H., Stanko, J., Mora, L. B., Sebti, S., Yu, H., and Jove, R. (2004) *Mol. Cancer Ther.* **3,** 1533–1542
39. Johnson, P. J., Coussens, P. M., Danko, A. V., and Shalloway, D. (1985) *Mol. Cell. Biol.* **5,** 1073–1083
40. Yu, C. L., Meyer, D. J., Campbell, G. S., Larner, A. C., Carter-Su, C., Schwartz, J., and Jove, R. (1995) *Science* **269,** 81–83
41. Turkson, J., Ryan, D., Kim, J. S., Zhang, Y., Chen, Z., Haura, E., Laudano, A., Sebti, S., Hamilton, A. D., and Jove, R. (2001) *J. Biol. Chem.* **276,** 45443–45455
42. Mora, L. B., Buettner, R., Seigne, J., Diaz, J., Ahmad, N., Garcia, R., Bowman, T., Falcone, R., Fairclough, R., Cantor, A., Muro-Cacho, C., Livingston, S., Karras, J., Pow-Sang, J., and Jove, R. (2002) *Cancer Res.* **62,** 6659–6666
43. Oyajobi, B. O., Franchin, G., Williams, P. J., Pulkrabek, D., Gupta, A., Munoz, S., Grubbs, B., Zhao, M., Chen, D., Sherry, B., and Mundy, G. R. (2003) *Blood* **102,** 311–319
44. Turkson, J., Bowman, T., Adnane, J., Zhang, Y., Djeu, J. Y., Sekharam, M., Frank, D. A., Holzman, L. B., Wu, J., Sebti, S., and Jove, R. (1999) *Mol. Cell. Biol.* **19,** 7519–7528
45. Zhang, D., Sun, M., Samols, D., and Kushner, I. (1996) *J. Biol. Chem.* **271,** 9503–9509
46. Yamauchi, K., Holt, K., and Pessin, J. E. (1993) *J. Biol. Chem.* **268,** 14597–14600
47. Zhang, Y., Turkson, J., Carter-Su, C., Smithgall, T., Levitzki, A., Kraker, A., Krolewski, J. J., Medveczky, P., and Jove, R. (2000) *J. Biol. Chem.* **275,** 24935–24944
48. Wagner, B. J., Hayes, T. E., Hoban, C. J., and Cochran, B. H. (1990) *EMBO J.* **9,** 4477–4484
49. Gouilleux, F., Moritz, D., Humar, M., Moriggl, R., Berchtold, S., and Groner, B. (1995) *Endocrinology* **136,** 5700–5708
50. Seidel, H. M., Milocco, L. H., Lamb, P., Darnell, J. E., Jr., Stein, R. B., and Rosen, J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 3041–3045
51. Karras, J., McKay, R., Lu, T., Pych, J., Frank, D., Rothstein, T., and Monia, B. (2000) *Cell. Immunol.* **202,** 124–135
52. Song, L., Turkson, J., Karras, J. G., Jove, R., and Haura, E. B. (2003) *Oncogene* **22,** 4150–4165
53. Turkson, J., Kim, J. S., Zhang, S., Yuan, J., Huang, M., Glenn, M., Haura, E., Sebti, S., Hamilton, A. D., and Jove, R. (2004) *Mol. Cancer Ther.* **3,** 261–269
54. Darnell, J. E., Jr. (2002) *Nat. Rev. Cancer* **2,** 740–749
55. Wang, G., Reed, E., and Li, Q. Q. (2004) *Oncol. Rep.* **12,** 955–965
56. Trynda-Lemiesz, L., Kozlowski, H., and Keppler, B. K. (1999) *J. Inorg. Biochem.* **77,** 141–146
57. Allain, P., Heudi, O., Cailleux, A., Le Bouil, A., Larra, F., Boisdron-Celle, M., and Gamelin, E. (2000) *Drug Metab. Dispos.* **28,** 1379–1384
58. Trynda-Lemiesz, L., and Luczkowski, M. (2004) *J. Inorg. Biochem.* **98,** 1851–1856
59. Heudi, O., Mercier-Jobard, S., Cailleux, A., and Allain, P. (1999) *Biopharm. Drug Dispos.* **20,** 107–116
60. Heudi, O., Brisset, H., Cailleux, A., and Allain, P. (2001) *Int. J. Clin. Pharmacol. Ther.* **39,** 344–349
61. Becker, S., Groner, B., and Muller, C. W. (1998) *Nature* **394,** 145–151
62. Bromberg, J. F., Wrzeszczynska, M. H., Devgan, G., Zhao, Y., Pestell, R. G., Albanese, C., and Darnell, J. E., Jr. (1999) *Cell* **98,** 295–303
63. Sinibaldi, N., Wharton, W., Turkson, J., Bowman, T., Pledger, W. J., and Jove, R. (2000) *Oncogene* **19,** 5419–5427
64. Perez, J. M., Kelland, L. R., Montero, E. I., Boxall, F. E., Fuertes, M. A., Alonso, C., and Navarro-Ranninger, C. (2003) *Mol. Pharmacol.* **63,** 933–944

A Novel Platinum Compound Inhibits Constitutive Stat3 Signaling and Induces Cell Cycle Arrest and Apoptosis of Malignant Cells

James Turkson, Shumin Zhang, Linda B. Mora, Audrey Burns, Said Sebti and Richard Jove

J. Biol. Chem. 2005, 280:32979-32988.
doi: 10.1074/jbc.M502694200 originally published online July 26, 2005
