

Diallyl Trisulfide Inhibits Leptin-induced Oncogenic Signaling in Human Breast Cancer Cells but Fails to Prevent Chemically-induced Luminal-type Cancer in Rats

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Previous studies have demonstrated inhibitory effect of garlic component diallyl trisulfide (DATS) on growth of breast cancer cells in vitro and in vivo. This study investigated the effect of DATS on oncogenic signaling regulated by leptin, which plays an important role in breast carcinogenesis. Leptin-induced phosphorylation and nuclear translocation of STAT3 was inhibited significantly in the presence of DATS in MCF-7 (a luminal-type human breast cancer cell line) and MDA-MB-231 (a basal-like human breast cancer cell line). Leptin-stimulated cell proliferation, clonogenic cell survival, and migration and/or invasion ability in MCF-7 and/or MDA-MB-231 cells were also suppressed by DATS treatment. DATS exposure resulted in inhibition of leptin-stimulated expression of protein and/or mRNA levels of Bcl-2, Bcl-xL, Cyclin D1, vascular endothelial growth factor, and matrix metalloproteinase-2. Western blotting revealed a decrease in protein levels of phosphorylated STAT3 in breast cancer xenografts from DATS-treated mice when compared to controls in vivo. However, the incidence of *N*-methyl-*N*-nitrosourea-induced luminal-type breast cancer development in rats was not affected by oral administration of 5 mg/kg or 25 mg/kg DATS. The present study reveals that oncogenic signaling induced by leptin is inhibited in the presence of DATS but higher doses of this phytochemical may be required to achieve chemopreventive activity.

Key Words Breast cancer, Leptin, STAT3, Diallyl trisulfide, Chemoprevention

INTRODUCTION

Epidemiological studies have implied an inverse relationship between dietary intake of *Allium* vegetables (e.g., garlic and onions) and the risk of breast cancer, which continues to be a major cause of cancer-associated mortality in women worldwide [1-3]. For example, breast cancer risk was inversely associated with increasing intake of garlic and onions in a French case-control study of 345 pathologically confirmed cases and the same number of controls matched for age and social and economic status [3]. The bioactive phytochemicals with anticancer activity in *Allium* vegetables include water-soluble (e.g., *S*-allyl cysteine) and fat-soluble sulfur compounds such as diallyl trisulfide (DATS) but the later class exhibits greater anticancer potency compared with the water-soluble phytochemicals at least in cellular models of cancer [4,5]. The γ -glutamyl-*S*-alk(en)yl-L-cysteine that is hydrolyzed and oxidized to produce alliin is the odorless

precursor of bioactive sulfur compounds in *Allium* vegetables [4]. Cutting or chewing of *Allium* vegetables releases a vacuolar enzyme (allinase) that acts upon alliin to give rise to alliin and other alkyl alkane-thiosulfinate compounds [4]. Eventually, various sulfur compounds including DATS are generated due to decomposition of alliin [4].

Growth inhibitory effects and associated mechanisms of DATS have been studied using human breast cancer cell lines and athymic xenograft models [6-9]. DATS treatment resulted in suppression of MCF-7 (a human breast cancer cell line with molecular characteristics of luminal-subtype) cell viability that was accompanied by a decrease in percent of cells in G₂ phase and induction of apoptotic cell death [6]. These effects of DATS in MCF-7 cell line were associated with increased expression of FAS, Bax, and p53 and downregulation of Akt and Bcl-2 proteins [6]. Another study implicated pro-apoptotic protein Bim in cytotoxicity of DATS in the MDA-MB-231 cell line [7]. Specifically, DATS

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treatment caused oxidative stress leading to dissociation of glutaredoxin from apoptosis signal-regulating kinase 1 that resulted in activation of downstream signal transduction of c-Jun N-terminal kinase-Bim pathway [7]. A role for reactive oxygen species-mediated activation of c-Jun N-terminal kinase and activator protein 1 was demonstrated in DATS-induced apoptosis in MCF-7 cells [8]. The *in vivo* growth of MCF-7 xenografts was inhibited significantly by oral administration of DATS [8]. We showed that DATS-induced apoptosis in MDA-MB-231 and MCF-7 cells was significantly abrogated by ectopic expression of superoxide dismutase due to attenuation of Bak activation [9]. Furthermore, the DATS-mediated inhibition of breast cancer cell migration was also significantly attenuated by superoxide dismutase overexpression [9]. Cellular effects in a non-tumorigenic MCF-10A cell line induced by an environmental carcinogen (benzo[a]pyrene) were reversible following DATS exposure [10]. These studies indicate that DATS is a promising agent potentially useful for treatment and/or prevention of breast cancer. Therefore, elucidation of the mechanism(s) underlying anticancer effect of DATS is/are important for its clinical development.

In this study, we investigated the effect of DATS treatment on leptin-induced signaling in human breast cancer cells *in vitro* and *in vivo*. We also tested the possibility of cancer prevention by DATS using a chemically-induced rat model of breast cancer. Impetus for the present study was based on following observations: (a) signal transducer and activator of transcription 3 (STAT3) is a known downstream target of leptin, and DATS treatment inhibits STAT3 activation in cancer cells [11,12], and (b) leptin is known to regulate breast cancer stem-like cells and DATS suppresses self-renewal of this population in breast cancer cells [13,14]. Leptin is a 16 kDa cytokine that exercises its action through binding to its specific receptors on the cell membrane of a variety of tissues [11]. Leptin has been implicated in initiation and progression of breast cancer [11]. Moreover, biological effects of leptin are regulated in cooperation with multiple oncogenes (e.g., STAT3, mitogen-activated protein kinases, phosphoinositide 3-kinase/Akt etc.), cytokines (interleukin-1 and interleukin-8), and growth factors like insulin-like growth factor-1/epidermal growth factor receptor [11]. Leptin is also known to stimulate production of pro-inflammatory cytokines as well as promote T-helper 1 responses [11].

MATERIALS AND METHODS

Ethics statement

The use of rats for determining the effect of DATS administration on incidence of *N*-methyl-*N*-nitrosourea (MNU)-induced luminal type breast cancer was approved by the University of Pittsburgh Animal Care and Use Committee (protocol # 18114222).

Reagents

DATS was purchased from LKT Laboratories (St. Paul, MN, USA). Cell culture media were purchased from Cellgro (CORNING, Corning, NY, USA). Leptin was purchased from R&D Systems (Minneapolis, MN, USA). Matrigel was from BD Biosciences (San Jose, CA, USA) and Transwell permeable support was from Costar (CORNING). Antibodies against phospho-STAT3 (Tyr705), phospho-STAT3 (Ser727), and total STAT3 were from Cell Signaling (Danvers, MA, USA). Antibodies against Bcl-xL, Cyclin D1 and phospho-STAT3 (Tyr705) for immunofluorescence microscopy were from Santa Cruz Biotechnology (Dallas, TX, USA). Anti- β -actin antibody was from Sigma (Sigma-Aldrich, St. Louis, MO, USA) and anti-Bcl-2 antibody was from DakoCytomation (Glostrup, Denmark). SYTOX Green reagent and Alexa Fluor 568 goat anti-rabbit antibody were from Invitrogen (Waltham, MA, USA).

Cell lines and treatment

The MCF-7 and MDA-MB-231 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained as suggested by the supplier. Both these cell lines were last authenticated by us in March of 2017. Stock solution of DATS was prepared in dimethyl sulfoxide (DMSO), and an equal volume of DMSO (0.1%) was added to controls.

For treatment, cells were seeded and then serum starved for 16 hours. The cells were treated with or without DATS (40 μ mol/L) for 2 hours followed by exposure to leptin (100 ng/mL) for specified time for each experiment.

Western blotting

Whole lysates from cells treated with DMSO (control) or DATS and/or leptin were prepared as described by us previously [15]. Lysate proteins were resolved by polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane. After blocking with 5% non-fat dry milk, the membrane was incubated with the desired primary antibody for overnight at 4°C. The membrane was then treated with secondary antibody and the bands were visualized by enhanced chemiluminescence method.

Immunocytochemistry for nuclear localization of P-STAT3

Cells (1×10^5) were plated on coverslips and allowed to attach by overnight incubation. The following day, cells were serum-starved for 16 hours and pretreated with DATS for 2 hours prior to exposure to leptin for 3 hours. The cells were fixed with 2% paraformaldehyde for 1 hour at room temperature and permeabilized with 0.05% Triton X-100 for 10 minutes. The cells were then incubated with PBS containing 0.5% bovine serum albumin and 0.15% glycine for 1 hour followed by overnight incubation with anti-phospho (P)-STAT3 antibody at 4°C. The cells were then treated with 2 μ g/mL of Alexa Fluor 568-conjugated secondary antibody

for 1 hour at room temperature. After washing with PBS, the cells were counterstained with 0.5 $\mu\text{mol/L}$ SYTOX Green for 5 minutes at room temperature. Subsequently, the cells were mounted and observed under a Leica DC300F fluorescence microscope at 100 \times objective magnification.

Cell viability and clonogenic assays

Cells were seeded in 12-well plates at a density of 10^5 cells/well. After 16 hours of serum starvation, the cells were treated with or without DATS (40 $\mu\text{mol/L}$) for 2 hours and then treated with leptin (100 ng/mL) for 22 hours in the absence or presence of DATS. The effects of leptin and/or DATS treatments on cell viability was determined by trypan blue dye exclusion assay as described by us previously [5]. For clonogenic assay, the cells were plated in 6-well plates at a density of 500 cells/well and treated with leptin and/or DATS as described for the cell viability assay. Medium containing DATS and/or leptin was replaced every 3 days. After 12 days, the medium was removed and colonies were stained with 0.5% crystal violet. The colonies were counted under a microscope.

Reverse transcription-PCR

Total RNA from MCF-7 and MDA-MB-231 cells treated with DMSO, leptin and/or DATS was extracted with the use of the RNeasy kit (Qiagen, Hilden, Germany). The cDNA was synthesized with the use of 1 μg of total RNA using SuperScript III reverse transcriptase with oligo(dT)₂₀ primer. Specific primers and amplification conditions were as follow: *Bcl-2* (forward) 5'-TGCACCTGACGCCCTTACAC-3' and (reverse) 5'-TAGCTGATTGACGTTTTGCCTGA-3'; *Bcl-xL* (forward) 5'-CCCAGAAAGGATACAGCTGG-3' and (reverse) 5'-GCGATCCGACTACCAATAC-3'; *Cyclin D1* (forward) 5'-TGTTTGCAAGCAGGACTTTG-3' and (reverse) 5'-ACGTCAGCCTCCACACTCTT-3'; *matrix metalloproteinase (MMP)-2* (forward) 5'-AGATCTTCTTCTCAAGGACCGTT-3' and (reverse) 5'-GGCTGGTCAGTGGCTTGGGGTA-3'; *MMP-9* (forward) 5'-GCGGAGATTGGAACCAAGCTGTA-3' and (reverse) 5'-GACGCGCCTGTGTACACCCACA-3'; *VEGF* (forward) 5'-CCATGAACTTCTGCTGTCTTGG-3' and (reverse) 5'-CTCACCGCCTCGGCTTGTACAC-3'; *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* (forward) 5'-GGACCTGACCTGCCGTCTAGAA-3' and (reverse) 5'-GGTGTGCTGTTGAAGTCAGAG-3'. Amplification conditions were: *Bcl-2*: 94°C/15 seconds, 56°C/30 seconds, 72°C/30 seconds, 28 cycles; *Bcl-xL*: 94°C/15 seconds, 68°C/1 minute, 72°C/1 minute, 28 cycles; *Cyclin D1*: 94°C/40 seconds, 59°C/40 seconds, 72°C/1 minute, 32 cycles; *MMP-2*: 94°C/45 seconds, 60°C/1 minute, 72°C/1 minute, 30 cycles; *MMP-9*: 94°C/30 seconds, 68°C/1 minute, 72°C/30 seconds, 34 cycles; *VEGF*: 94°C/45 seconds, 60°C/1 minute, 72°C/1 minute, 34 cycles; *GAPDH*: 94°C/30 seconds, 60°C/30 seconds, 72°C/30 seconds, 30 cycles.

Determination of cell migration and invasion

For cell migration assay, we used 24-well plate inserts with 8.0 μm pore size polycarbonate filters (Costar; CORNING). For cell invasion assay, filters were pre-coated with 25 μL diluted Matrigel in serum-free medium (1 : 2) for 5 hours at 37°C. Serum starved MDA-MB-231 cells were seeded in upper chamber in 150 μL of serum-free media, while media supplemented with 10% FBS (600 μL) was placed in the lower chamber. The following procedures were the same for both cell migration and cell invasion assays. Twenty-four hour following treatment with DMSO, DATS and/or leptin, the cells remaining in the upper chamber were gently removed with a cotton swap. Cells that migrated or invaded through the Matrigel to the lower chamber were fixed in methanol, stained with hematoxylin and eosin and photographed. The migrated or invaded cells from each filter were counted under a microscope at 100 \times magnification. The experiments were performed in triplicate and repeated to ensure reproducibility of the results.

RNA interference

The MDA-MB-231 (5×10^4) cells were seeded in 6-well plates and transfected at ~50% confluency with 100 nmol/L of control siRNA or STAT3-targeted siRNA using Oligofectamine. Twenty-four hours after transfection, the cells were serum starved for 16 hours and then treated with DMSO or DATS/and or leptin for 24 hours. After treatment, the cells were processed for immunoblotting and invasion assay.

Analysis of the RNA-Seq data from The Cancer Genome Atlas

Association of *STAT3* expression with that of *Bcl-2*, *Bcl-xL* (*BCL2L1*), *Cyclin D1* (*CCND1*), *VEGF*, *MMP-2*, and *MMP-9* was determined from the breast cancer RNA-Seq data from The Cancer Genome Atlas (TCGA) with the help of University of California Santa Cruz Xena Browser (<http://xena.ucsc.edu/public-hubs/>).

Chemoprevention study in rats

To determine the in vivo efficacy of DATS for chemoprevention of luminal type breast cancer, a Sprague-Dawley rat model of breast cancer induced by MNU was used. MNU was freshly prepared in 0.9% sodium chloride solution (Sigma) in the dark prior to injection. Female rats (21-day old) were injected intraperitoneally with MNU (50 mg/kg body weight). One week after MNU injection, the rats were randomly divided into 3 groups. The control group of rats ($n = 21$) were orally administered with 100 μL of corn oil (vehicle for DATS) 5 times/wk. The second and third groups of rats were orally treated with 100 μL of corn oil containing 5 mg DATS/kg body weight ($n = 20$) or 25 mg DATS/kg body weight ($n = 20$) 5 times/wk. The body weights of rats of each group were recorded at weekly intervals. Each rat of every

group was palpated daily for the presence of mammary tumor. Two rats from the control group and one rat from the 25 mg DATS/kg body weight group were sacrificed early (at 7-9 weeks post-MNU injection) due to tumor burden. Rest of the rats were sacrificed at 11 weeks after MNU injection.

Statistical analysis

GraphPad Prism (ver. 7.02; <https://www.graphpad.com/scientific-software/prism/>) was used for statistical analyses. ANOVA followed by Bonferroni's test was used for multiple group comparisons. Student's *t*-test was used for comparison of two groups. Fisher's exact test was used for statistical comparison of tumor incidence in the rat study.

RESULTS

DATS inhibited leptin-stimulated activation of STAT3 in breast cancer cells

We used 40 μmol/L DATS concentration for in vitro studies based on its pharmacokinetics in rats after administration of a single 10 mg dose [16]. The maximum blood concentration

of DATS was found to be ~31 μmol/L [16]. Leptin treatment alone resulted in an increase in phosphorylation of STAT3, a measure of its activation, in MCF-7 and MDA-MB-231 cells that was not due to induction of total STAT3 protein (Fig. 1A). Constitutive phosphorylation of STAT3 was markedly suppressed following DATS treatment in both cell lines (Fig. 1A). Furthermore, leptin-stimulated activation of STAT3 was also decreased in the presence of DATS (Fig. 1A).

Extracellular signal-regulated kinase (ERK) is another downstream target of leptin in cancer cells [17]. We therefore examined the effects of leptin and/or DATS treatment on protein levels of phosphorylated-ERK and total ERK in MCF-7 and MDA-MB-231 cells by western blotting. However, the results were not consistent (data not shown). As the effects of leptin and/or DATS were consistent for STAT3 in both cell lines, we focused on this pathway for further investigation.

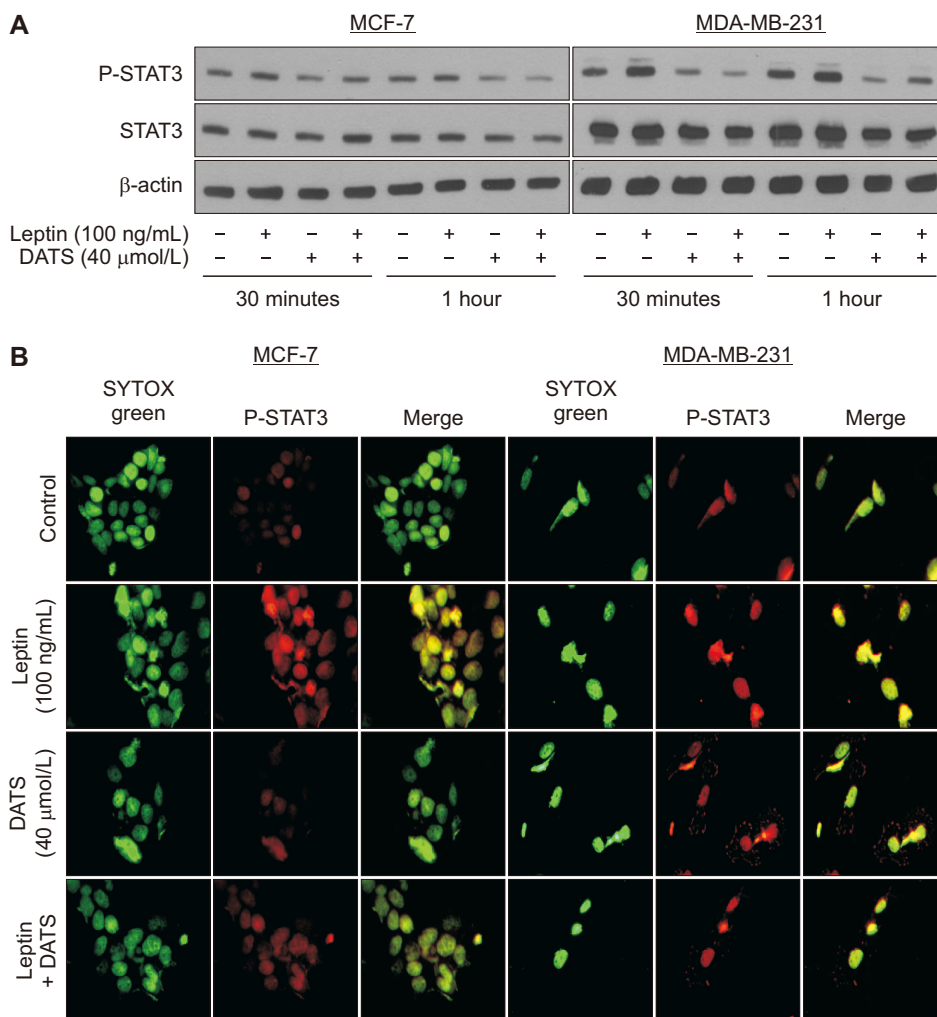


Figure 1. Diallyl trisulfide (DATS) treatment inhibits constitutive as well as leptin-induced STAT3 phosphorylation at Tyr-705 in human breast cancer cells. (A) Immunoblotting for phospho (P)-STAT3, STAT3, and β-actin using lysates from 16-hour serum starved MCF-7 (left panel) and MDA-MB-231 (right panel) cells. Cells were first serum starved for 16 hours and then exposed to dimethyl sulfoxide (DMSO; control) or 40 μmol/L DATS for 2 hours and then further treated for 30 minutes or 1 hour after adding 100 ng/mL leptin. Experiment was repeated twice. (B) Immunocytochemistry for P-STAT3 (red fluorescence) in MCF-7 (left panel) and MDA-MB-231 (right panel) cells. Cells were serum starved for 16 hours and then treated with DMSO (control) or 40 μmol/L DATS for 2 hours and further incubated for 3 hours after adding 100 ng/mL leptin. SYTOX Green was used to stain nucleus. Images were captured at 100 × objective magnification. Experiment was repeated twice with comparable results.

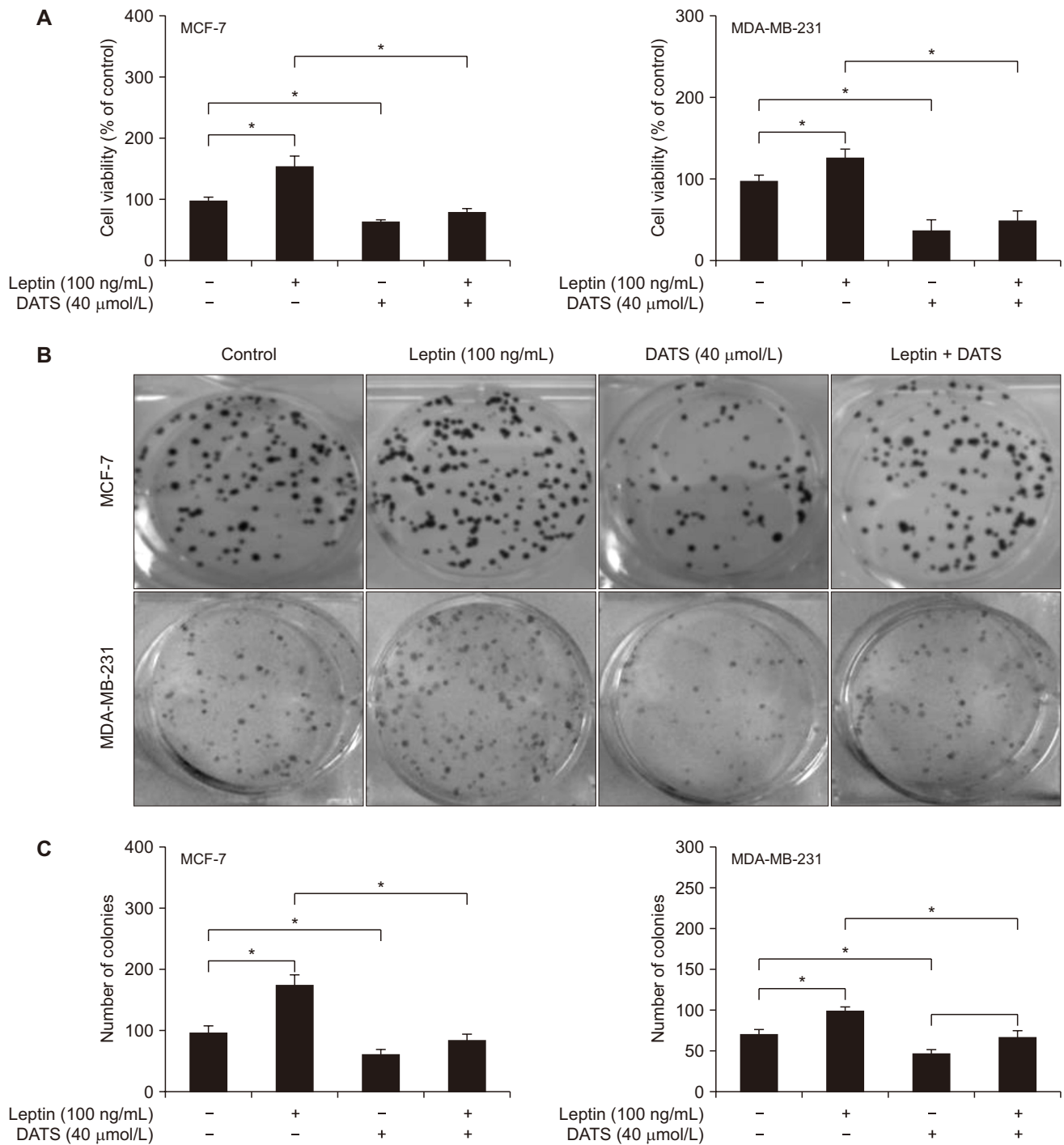


Figure 2. Diallyl trisulfide (DATS) treatment inhibits basal as well as leptin-induced cell growth in human breast cancer cells. (A) Quantitation of cell viability of 16-hour serum-starved MCF-7 (left panel) and MDA-MB-231 (right panel) cells treated with dimethyl sulfoxide (DMSO; control) or 40 μmol/L DATS for 2 hours and then further incubated for 22 hours after adding 100 ng/mL leptin. Results shown are mean ± SD (n = 3) and statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparisons test (**P* < 0.05). Experiment was repeated twice with consistent results. (B) Representative images of colonies from 16-hour serum-starved MCF-7 (upper panel) and MDA-MB-231 (lower panel) cells treated with DMSO (control) or 40 μmol/L DATS for 2 hours and then further incubated for 12 days after adding 100 ng/mL leptin. Media containing agents was changed every third day. (C) Quantitation of colony formation from data shown in panel B. Results shown are mean ± SD (n = 3) and statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparisons test (**P* < 0.05). Experiment was repeated twice with consistent results.

DATS inhibited leptin-stimulated nuclear translocation of STAT3 in breast cancer cells

STAT3 is a transcription factor and hence its nuclear translocation is essential for its activity. Figure 1B depicts results of immunocytochemistry for P-STAT3 in DMSO-treated control MCF-7 and MDA-MB-231 cells and those treated with leptin and/or DATS. Consistent with Western blotting results, leptin treatment alone caused an increase

in nuclear staining for phosphorylated STAT3 in MCF-7 and MDA-MB-231 cells reflected by merging of red fluorescence (P-STAT3) and SYTOX Green fluorescence (a nuclear stain) (Fig. 1B). The leptin-stimulated nuclear localization of P-STAT3 was decreased by DATS treatment (Fig. 1B). DATS alone was also effective in reducing the nuclear level of P-STAT3 (Fig. 1B). Collectively, these results indicated inhibition of leptin-stimulated activation of STAT3 following

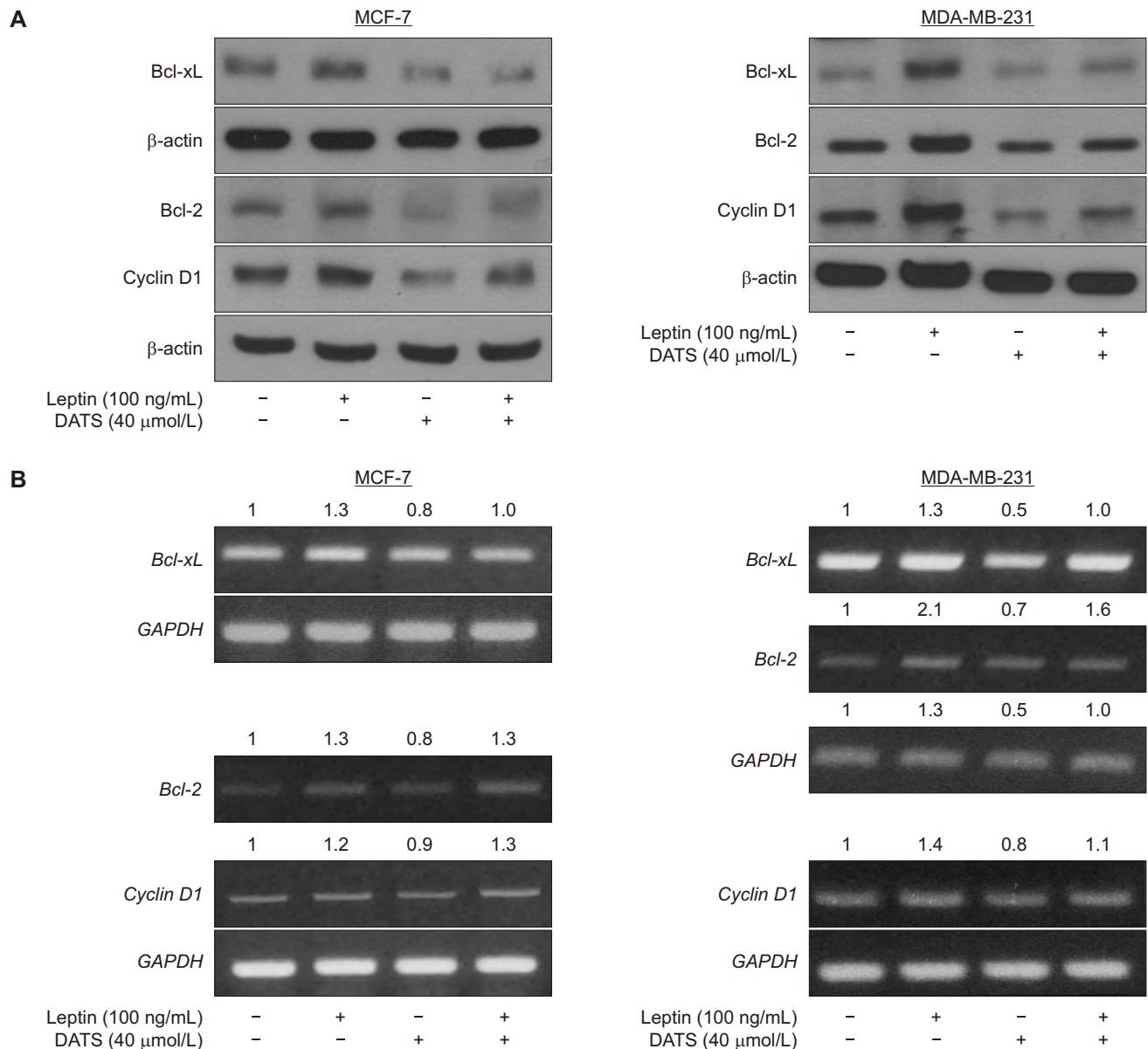


Figure 3. Diallyl trisulfide (DATS) treatment inhibits basal as well as leptin-stimulated expression of pro-survival proteins regulated by STAT3. (A) Immunoblotting for Bcl-2, Bcl-xL, Cyclin D1, and β-actin using lysates from 16-hour serum starved MCF-7 (left panel) and MDA-MB-231 (right panel) cells treated with dimethyl sulfoxide (DMSO; control) or 40 μmol/L DATS for 2 hours and then further incubated for 22 hours after adding 100 ng/mL leptin. Experiment was repeated at least twice. (B) Reverse transcription-PCR analysis for *Bcl-2*, *Bcl-xL*, *Cyclin D1*, and *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* using 16-hour serum starved MCF-7 (left panel) and MDA-MB-231 (right panel) cells treated with DMSO (control) or 40 μmol/L DATS for 2 hours and then further incubated for 22 hours after adding 100 ng/mL leptin. Numbers above bands indicate change in expression relative to solvent control treated cells. Experiment was repeated at least twice.

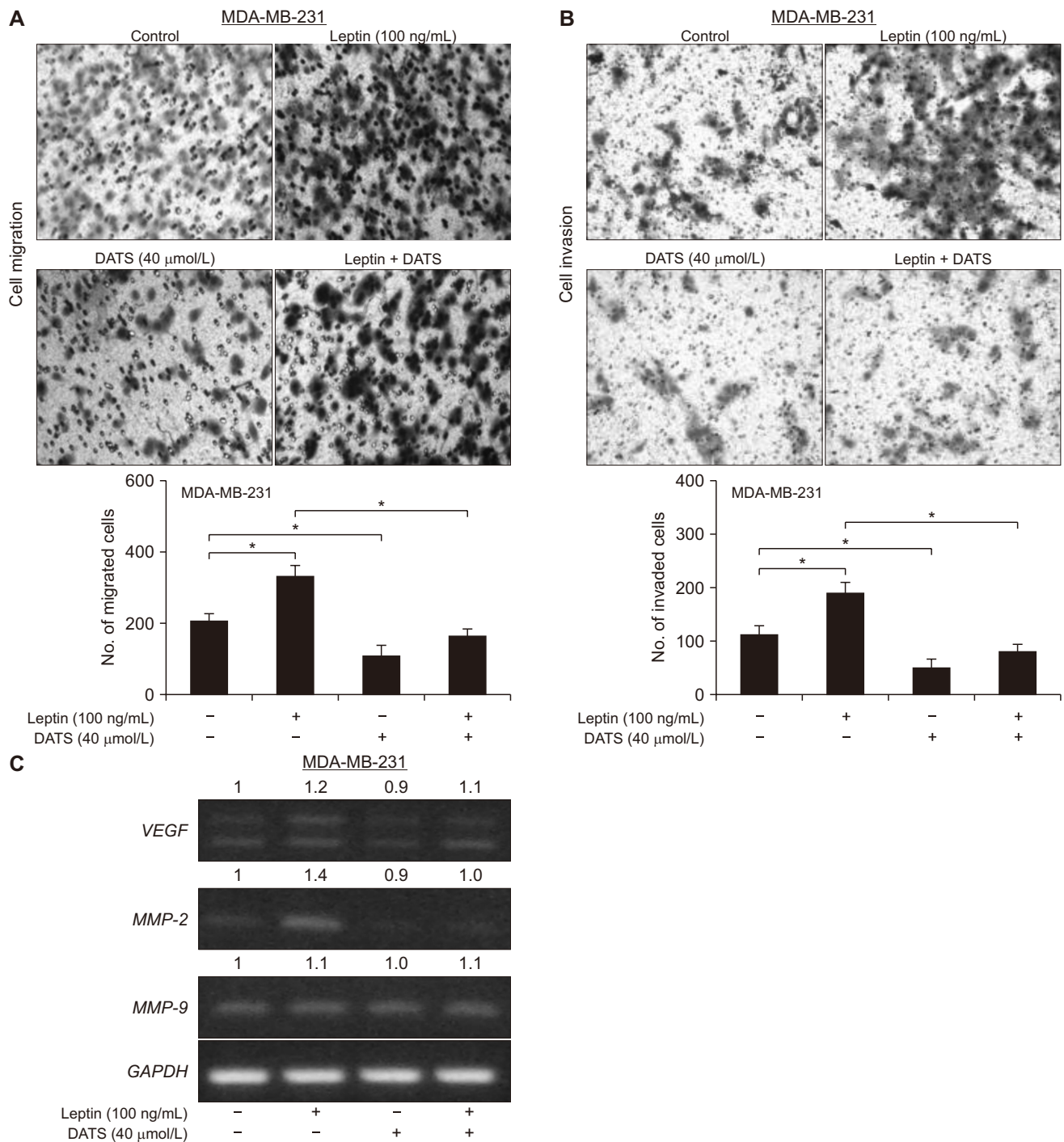


Figure 4. Diallyl trisulfide (DATS) treatment inhibits leptin-stimulated migration and invasion of MDA-MB-231 cells. (A) Representative H & E-stained images ($\times 100$ magnification) showing cell migration and its quantification. Cells were serum starved for 16 hours and then treated with dimethyl sulfoxide (DMSO; control) or $40 \mu\text{mol/L}$ DATS for 24 hours in the absence or presence of 100 ng/mL leptin. Results are shown as mean \pm SD ($n = 3$) and statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparisons test ($*P < 0.05$). Experiment was repeated thrice with consistent results. (B) Representative H & E-stained images ($\times 100$ magnification) showing cell invasion and its quantification. Cells were serum starved for 16 hours and then treated with DMSO (control) or $40 \mu\text{mol/L}$ DATS for 24 hours in the absence or presence of 100 ng/mL leptin. Results are shown as mean \pm SD ($n = 2-3$) and statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparisons test ($*P < 0.05$). Experiment was repeated thrice with consistent results. (C) Reverse transcription-PCR analysis for *VEGF*, *matrix metalloproteinase MMP-2*, *MMP-9*, and *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* using lysates from 16-hour serum starved MDA-MB-231 cells treated with DMSO (control) or $40 \mu\text{mol/L}$ DATS for 2 hours and then further incubated for 22 hours after adding 100 ng/mL leptin. Numbers above bands indicate change in expression relative to solvent control treated cells. Experiment was repeated thrice with consistent results.

DATS treatment in a luminal-type (MCF-7) and a basal-like (MDA-MB-231) human breast cancer cell line.

DATS inhibited leptin-stimulated survival of breast cancer cells

The viability of MCF-7 and MDA-MB-231 cells was increased significantly following leptin stimulation (Fig. 2A). DATS treatment alone resulted in inhibition of cell viability (Fig. 2A). The leptin-stimulated survival of MCF-7 and MDA-MB-231 cells was also decreased in the presence of DATS (Fig. 2A). Figure 2B shows images of colonies of MCF-7 and MDA-MB-231 cells following 12 days of treatment with leptin and/or DATS or solvent control. Consistent with cell viability data (Fig. 2A), the number of colonies was increased in the presence of leptin (Fig. 2C). The leptin-stimulated clonogenic survival of MCF-7 and MDA-MB-231 cells was decreased significantly by DATS treatment (Fig. 2C).

DATS inhibited leptin-stimulated expression of pro-survival proteins in breast cancer cells

The expression of pro-survival proteins, including Bcl-2, Bcl-xL, and Cyclin D1 was increased after leptin treatment in MCF-7 and MDA-MB-231 cells (Fig. 3A). Leptin-stimulated induction of these proteins was decreased by DATS (Fig. 3A). DATS treatment alone also caused a decrease in the levels of these proteins in both cells (Fig. 3A). The results of reverse transcription-PCR for *Bcl-2*, *Bcl-xL*, and *Cyclin D1* mRNA expression in leptin and/or DATS treated cells (Fig. 3B) were generally consistent with the results of Western blotting data (Fig. 3A). These results may explain DATS-mediated inhibition of leptin-stimulated survival of MCF-7 and MDA-MB-231 cells.

Next, we analyzed the association of *STAT3* expression with that of *Bcl-2*, *Bcl-xL* (*BCL2L1*), *Cyclin D1* (*CCND1*), *VEGF*, *MMP-2*, and *MMP-9* from the breast cancer RNA-Seq data from TCGA. Statistically significant positive association was discernible between *STAT3* and *Bcl-2* or *MMP-2*. On the other hand, *STAT3* expression was negatively associated with that of *Bcl-xL*, *VEGFB*, and *MMP-9* (Figure S1).

DATS inhibited leptin-stimulated migration and invasion ability of breast cancer cells

As can be seen in Figure 4A, migration of MDA-MB-231 cells was increased significantly by leptin exposure that was sensitive to inhibition by DATS treatment. Similarly, leptin-mediated increase in invasion of MDA-MB-231 cells was inhibited significantly in the presence of DATS (Fig. 4B). DATS treatment alone also inhibited migration and invasion of MDA-MB-231 cells. Induction of pro-metastasis genes, including *VEGF* and *MMP-2* resulting from leptin treatment was suppressed in the presence of DATS (Fig. 4C). These results provided mechanistic insights into the DATS-mediated inhibition of leptin-stimulated migration and invasion of MDA-MB-231 cells. However, *MMP-9* may not be involved in

DATS-mediated inhibition of leptin-stimulated cell invasion. Finally, these experiments were not done with MCF-7 cells as they migrate and invade poorly as compared to MDA-MB-231 cells.

Effect of RNA interference of STAT3 on invasion of MDA-MB-231 cells

Figure 5A shows knockdown of *STAT3* protein after transfection of MDA-MB-231 cells with a *STAT3*-targeted siRNA when compared to cells transfected with a control non-specific siRNA. RNA interference of *STAT3* decreased

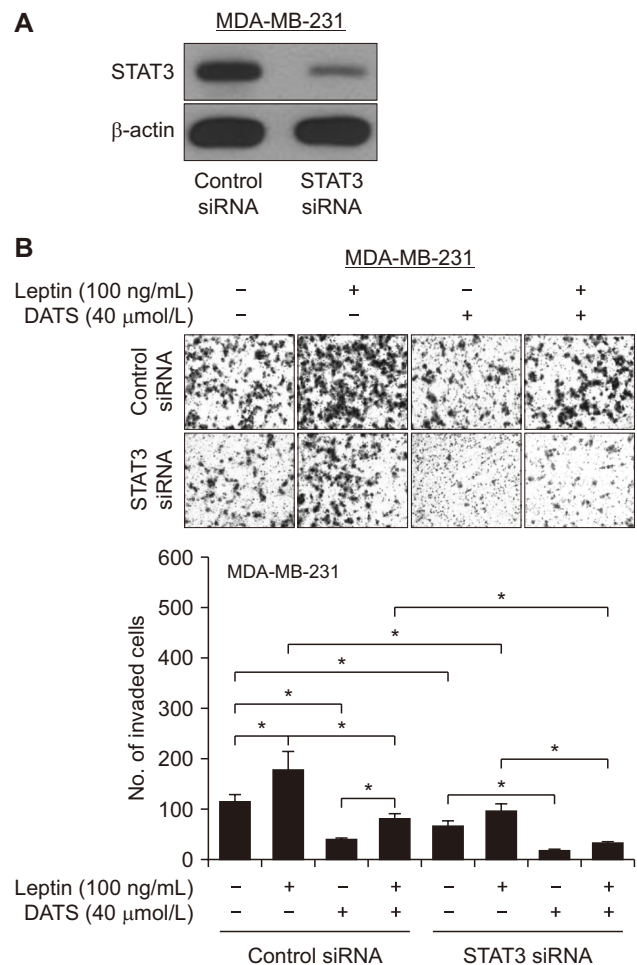


Figure 5. *STAT3* knockdown augments diallyl trisulfide (DATS)-mediated inhibition of leptin-stimulated cell invasion in MDA-MB-231 cells. (A) Immunoblotting for *STAT3* and β -actin in MDA-MB-231 cells transfected with control or *STAT3*-targeted siRNA. (B) Representative H & E-stained images ($\times 100$ magnification) and quantification of cell invasion in 16-hour serum-starved transiently transfected MDA-MB-231 cells with control or *STAT3* siRNA and treated for 24 hours with dimethyl sulfoxide (control) or 40 μ mol/L DATS in the absence or presence of 100 ng/mL leptin. Results are shown as mean \pm SD ($n = 3$) and statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparisons test ($*P < 0.05$). Experiment was repeated twice with consistent results.

the ability of leptin to increase cell invasion (Fig. 5B). On the other hand, DATS-mediated inhibition of leptin-stimulated invasion of MDA-MB-231 cells was augmented by knockdown of STAT3 protein (Fig. 5B). As DATS still inhibits the invasiveness of the cells even in the absence of STAT3, these findings suggest that DATS exerts antiproliferative and anti-migrative effects, at least in part, independently of targeting STAT3.

DATS treatment differentially inhibited phosphorylation of STAT3 in vivo

We have shown previously that oral treatment with 2 mg DATS (in 100 μ L of PBS beginning on the day of tumor cell implantation) 3 times/wk decreases tumor take (incidence) of a basal-like SUM159 xenografts by 50% when compared

to control mice [14]. The average wet weight of the SUM159 xenografts was also lower in DATS-treated mice compared with control mice [14]. Supernatants from SUM159 xenografts from this study were used to check the levels of phosphorylated and total STAT3. The level of P-STAT (S727), but not P-STAT (Y705), was significantly lower (Fig. 6A and 6B).

N-methyl-N-nitrosourea-induced breast cancer formation in rats was not prevented by oral administration of DATS

We next proceeded to determine whether DATS administration could prevent cancer development using a well-established rat model of chemically-induced breast cancer. DATS administration did not cause weight loss (Fig.

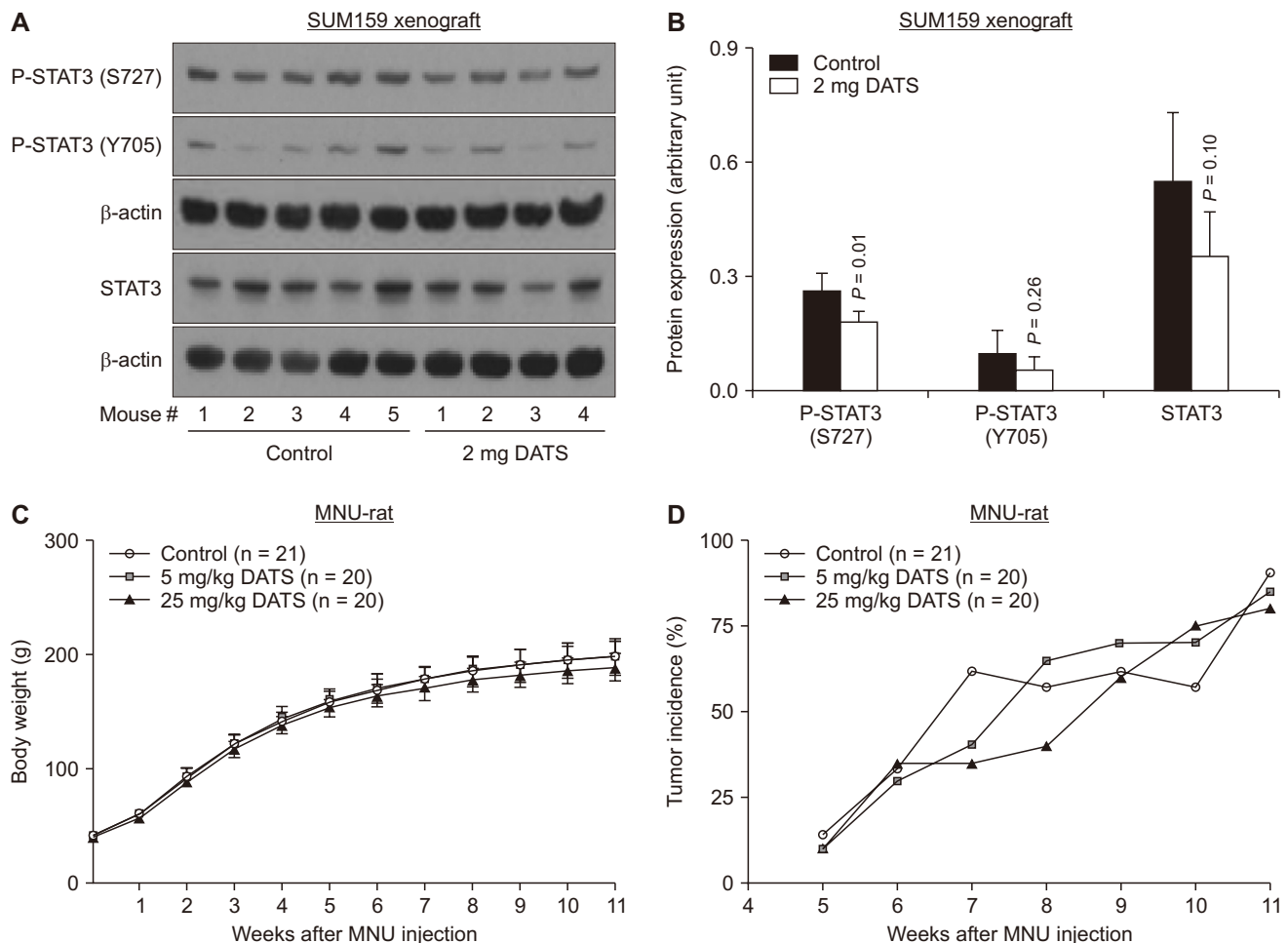


Figure 6. Diallyl trisulfide (DATS) administration inhibits phosphorylation of STAT3 at Ser-727 in SUM159 xenografted mice and shows no signs of toxicity in N-methyl-N-nitrosourea (MNU)-induced Sprague–Dawley rats. (A) Immunoblots showing protein levels of P-STA3 (S727), P-STAT3 (Y705), and total STAT3 using tumor lysates from SUM159 xenografts. (B) The bar graph shows quantitation of immunoreactive bands for P-STAT3 (S727), P-STAT3 (Y705), and total STAT3. Results shown are mean \pm SD (n=5 for control group and n=4 for DATS treatment group). Statistical analysis was performed by unpaired Student's *t*-test. (C) Body weight over time in MNU-injected rats fed vehicle (control) or DATS (5 or 25 mg/kg body weight). Results shown are mean \pm SD (n = 21 for control, n = 20 for 5 mg/kg DATS, and n = 20 for 25 mg/kg DATS). Statistical analysis was performed by one-way ANOVA followed by Dunnett's test. (D) Palpable mammary tumor incidence over time in MNU-injected rats fed vehicle or DATS (5 or 25 mg/kg body weight). Statistical analysis was performed by Fisher's exact test.

6C) or any other obvious side effects like water and food withdrawal or gastrointestinal issues such as diarrhea or bleeding. Despite an early promising effect (e.g., 7 weeks post-MNU injection), the overall incidence of breast cancer was comparable in control and DATS-treated rats (Fig. 6D). The tumor volume also did not differ significantly between control and DATS-treated rats (data not shown). These results indicated that DATS was ineffective in preventing MNU-induced luminal-type tumors in a rat model at least at the tested doses. Because the mammary cancer incidence was not affected, expression of STAT3 or its target proteins was not determined from rat tumor tissues.

DISCUSSION

Leptin is an adipokine that signals by binding to its receptor Ob-R and regulates energy balance in normal subjects [18]. Leptin has an oncogenic role in breast cancer and is one of the mechanistic links explaining obesity-breast cancer connection [19]. A study using 76 invasive ductal carcinomas and 32 samples of corresponding normal mammary gland showed weak expression of Ob-R in normal mammary cells but its overexpression in 83% of ductal carcinoma cells [20]. Moreover, overexpression of leptin was also reported in 92% of ductal carcinoma specimens [20]. Leptin not only promotes growth and invasion of breast cancer cells but also stimulates epithelial-mesenchymal transition [21-23]. The present study demonstrates, for the first time that oncogenic effects of leptin are counteracted by a safe phytochemical from garlic that is a key ingredient of human diet for many cultures worldwide.

STAT3 is one of the oncogenes that is activated by leptin in breast and other cancers [11,17,24]. STAT3 is overexpressed in breast cancer and it is a highly investigated therapeutic target for this malignancy [25]. In this study, we show that DATS treatment inhibits activation of STAT3 in breast cancer cells in vitro as well as in SUM159 xenografts in vivo. The leptin-mediated activation of STAT3 is also suppressed in the presence of DATS. Inhibition of STAT3 signaling following DATS treatment has been reported in other cell types including mouse colitis and prostate cancer [12,26]. Activation of STAT3 is mediated by Janus activated kinase (JAK) [27]. It is possible that DATS treatment inhibits JAK to suppress phosphorylation of STAT3 in breast cancer cells. Even though further work is necessary to explore this mechanistic possibility, we have shown previously that DATS treatment decreases interleukin 6-stimulated phosphorylation of JAK2 in prostate cancer cells [12].

STAT3 is known to regulate expression of many cancer relevant genes [28-30]. STAT3 occupancy at the promoter of *Bcl-2* was shown in HeLa cells by chromatin immunoprecipitation assay [28]. Breast cancer TCGA data also shows excellent positive association between expression of *STAT3* and *Bcl-2*. In agreement with these results, we also found that leptin-mediated activation of STAT3 was

accompanied by induction of *Bcl-2*. Likewise, the positive association for expression of *STAT3* with that of *MMP-2* in breast cancer TCGA is consistent with the published literature [29]. A role for *STAT3* in regulation of *MMP-2* expression was established from an electrophoretic mobility shift assay [30]. Surprisingly, analysis of the breast cancer TCGA did not reveal statistically significant positive association for *STAT3* expression with that of *Bcl-xL*, *Cyclin D1*, *VEGF*, and *MMP-9* even though literature suggests regulatory function of *STAT3* for these genes [11,30-33]. The reasons for these discrepancies are not clear but may be explained by the fact that leptin can activate other signaling pathways and transcription factors [18,19,21-24]. Nevertheless, the present study reveals that DATS inhibits constitutive as well as leptin-inducible expression of many pro-survival and pro-metastatic proteins leading to inhibition of cell proliferation, migration, and invasion.

It was surprising to us that DATS administration did not prevent development of MNU-induced breast cancer in rats because this agent is highly effective for prevention or therapy of breast and other cancers [8,34,35]. Moreover, DATS is known to inhibit estrogen receptor α expression and activity in MCF-7 and T47D cells [36] and MNU-induced mammary tumors in rats are estrogen receptor α positive with similarities to luminal-type disease [37-39]. For example, 17 β -estradiol-stimulated growth of MCF-7 cells implanted on the left or right thoracic area in female Balb/c nude mice was inhibited by >80% by oral administration of 5 μ mol DATS/kg body weight only twice per week for 1 month [8]. Noticeably, 50% of the DATS-treated mice were alive after 12 weeks but none of the control mice survived at this time point [8]. Likewise, lung and liver metastasis induced by orthotopic injection of MDA-MB-231 cells into the mammary fat pad of Balb/c nude mice was inhibited by daily oral administration of DATS at 25 mg/kg and 50 mg/kg body weight although the growth of primary tumor was not affected [34]. Interestingly, the DATS-mediated inhibition of pulmonary metastasis in the MDA-MB-231 model was accompanied by a statistically significant inhibition of *MMP-2* and *MMP-9* protein level at the 50 mg/kg dose [34]. DATS treatment (5-20 μ M for 24 hours) inhibited dissemination and metastasis of MDA-MB-231 cells in a zebrafish model that was associated with suppression of *MMP-2* and *MMP-9* activity [35]. In another study, the incidence as well as burden of poorly differentiated prostate adenocarcinoma and pulmonary metastasis multiplicity were suppressed significantly following oral administration of 1 mg (about 40 mg DATS/kg body weight for a 25 g mouse) and 2 mg DATS (about 80 mg DATS/kg body weight for a 25 g mouse) [40]. It is possible that DATS concentrations higher than 25 mg/kg body weight might be required for prevention of MNU-induced mammary cancer in rats.

In conclusion, the present study reveals that DATS is a potent inhibitor of leptin-stimulated activation of *STAT3* in vitro in estrogen-responsive and estrogen-independent

human breast cancer cells. Further work is necessary to determine the *in vivo* efficacy of DATS for prevention of MNU-induced breast cancer in rats at doses above 25 mg/kg. In future studies, it would also be worthwhile investigating the promotion of experimentally induced mammary carcinogenesis by leptin and its possible modulation by DATS.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via <https://doi.org/10.15430/JCP.2020.25.1.1>

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