# Antitumor activity of a novel small molecule STAT3 inhibitor against a human lymphoma cell line with high STAT3 activation

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**Abstract.** Signal transducer and activator of transcription (STAT)3, a member of a family of DNA-binding molecules mediating numerous physiological and oncogenic signaling pathways, is a novel target in cancer cells which show high phosphorylation of STAT3. Recently, we identified a novel small-molecule inhibitor of STAT3 dimerization, STX-0119, as a cancer therapeutic. We investigated the mechanisms responsible for the antitumor activity *in vitro* and *in vivo* through numerous biochemical and biological assays. Specifically, the effects of STX-0119 on target genes (c-myc, cyclin D1, survivin) and apoptosis induction were analyzed in tumors treated with STX-0119 *in vivo*. STX-0119 showed strong growth-inhibitory activity against a broad range of hematological cancer cell lines, particularly lymphomas. STX-0119 suppressed the growth of SCC3 cells, a human lymphoma cell line with highly activated STAT3, through apoptosis and down-regulation of STAT3 targets such as c-myc, cyclin D1, survivin and Bcl-xL. Notably, Tyr-705-phosphorylated STAT3 up-regulation was not significantly suppressed by STX-0119, as opposed to other STAT3 inhibitors. STX-0119 demonstrated potent antitumor effects *in vivo* in SCC3-bearing nude mice by way of the down-regulation of STAT3 target genes and induction of apoptosis in the tumors. Thus, STX-0119 may be a new type of STAT3 inhibitor exhibiting strong antitumor activity.

## Introduction

The signal transducer and activator of transcription (STAT) protein family is a group of transcription factors that play an important role in relaying signals from growth factors and cytokines (1-3). STAT3 is reported to be involved in oncogenesis by up-regulating the transcription of several genes that control tumor cell survival, resistance to apoptosis, cell cycle progression and angiogenesis. Targets of STAT3 include Bcl-2 (4), Bcl-xL (5), c-myc (6), cyclin D1 (7), vascular endothelial growth factor (8) and human telomerase reverse transcriptase (9).

Several STATs have been associated with the proliferation and survival of cancer cells; however STAT3 is also widely expressed in normal cells. Generally, STAT3 is temporarily activated in a manner strictly regulated by the protein inhibitor of STATs (PIAS), Src homology (SH)2-containing tyrosine phosphatases (SHP1 and SHP2) (10), and suppressor of cytokine signaling/extracellular signal-regulated kinase (SOCS/ERK) (11) to protect cells from excess inflammatory signaling.

Moreover, persistent activation of STAT3 occurs in many hematological malignancies (12,13) and solid cancers. Constitutive STAT3 activation up-regulates the expression of anti-apoptotic factors including Bcl-xL, Bcl-2 and Mcl-1 and promotes the survival of tumor cells, thus contributing to resistance to FAS- or chemotherapy-induced apoptosis (14). Blocking of STAT3 signaling demonstrated significant growth inhibition of cancer cells by down-regulation of the expression of anti-apoptotic genes, leading to apoptosis.

The activation of STAT3 occurs when it is phosphorylated at the Tyr-705 residue. This leads to dimerization and a subsequent transfer to the nucleus where further activation promotes the binding of DNA and the translation of target genes. We are currently designing inhibitors of STAT3

dimerization by simulating docking *in silico* and by screening for active compounds through biochemical methods and with *in vitro* as well as *in vivo* systems (15).

In the present study we demonstrated the antitumor activity of a novel STAT3-SH2 antagonist, STX-0119, against a constitutively STAT3-activated human lymphoma cell line and investigated the mechanism responsible for the efficacy of STX-0119.

## Materials and methods

*Cell lines and reagents.* All hematological cancer cell lines including IM-9, U937, Jurkat, KG-1, MOLT-3, HL-60, THP-1, RPMI8226, HUT78, HH, K562, BC-3, U266B1, NCI-H929 and TF-1 were purchased from American Type Culture Collection (Manassas, VA, USA). SCC3 was supplied by Health Science Research Resources Bank (Osaka, Japan). The CMK cell line was kindly provided by Dr T. Sato of Chiba University School of Medicine. All cell lines were cultured in RPMI1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 U/ml) and 10% (v/v) fetal bovine serum (FBS; Gibco, Paisley, UK). Various antibodies against STAT3, phospho-specific STAT3 (Tyr-705), c-myc, cyclin D1, survivin, Bcl-xL and β-actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA) and Becton-Dickinson (BD) Biosciences (Franklin Lakes, NJ) for Western blotting (WB). Anti-STAT3 and anti-phospho-specific STAT3 polyclonal antibodies (Cell Signaling Technology), anti-Ki76 antibody (clone MIB-1; Dako, Glostrup, Denmark) and anti-c-myc antibody (Abcam, Cambridge, MA) were purchased for immunohistochemistry (IHC). Phycoerythin (PE)-labeled mouse anti-phosphorylated STAT3 monoclonal antibody (mAb) was purchased from BD Biosciences for FACS analysis.

*Chemicals.* STX-0119 and the JAK-specific inhibitor AG490 were synthesized and supplied by the Center for Drug Discovery, University of Shizuoka, Shizuoka, Japan and by Dr Okawara, Kumamoto Health Science University, Kumamoto, Japan, respectively. Cytarabine (Ara-C) was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. These compounds were dissolved and diluted in a sterile 0.5% (w/v) methylcellulose 400 cp solution (Wako, Tokyo, Japan) or sterile 0.9% NaCl solution (Ohtsuka Pharmaceutical, Tokyo, Japan) just prior to their use. The chemical structure of STX-0119 is shown in Fig. 1.

*Cell proliferation assay.* Cell proliferation was determined using the WST-1 assay (Dojin Kagaku Corp., Japan). A 100 $\mu$l volume of floating cells ($3 \times 10^4$ to $1 \times 10^5$/ml) was seeded into each well of a 96-well microculture plate (Corning, NY), and 100 $\mu$l of various doses of compounds sequentially diluted with DMSO (100-0.1 $\mu$M) was added. After 72 h at 37°C, 10 $\mu$l of combined WST-1 substrate was added to each well and incubated for 3 h at 37°C. The optical density (OD) was measured at 450 and 620 nm using an immunoreader (Immuno Mini NJ-2300; Nalge Nunc International, Roskilde, Denmark). The $IC_{50}$ value was defined as the dose needed for a 50% reduction in OD calculated from the survival curve. Percent survival was calculated as follows: (Mean OD of the
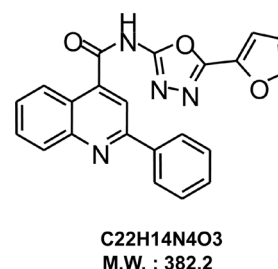


**C22H14N4O3**
**M.W. : 382.2**

Figure 1. Chemical structure of STX-0119. Formula, $C_{22}H_{14}N_4O_3$. Molecular weight, 382.2.

test wells - Mean OD of the background wells)/(Mean OD of the control wells - Mean OD of the background wells).

*STAT3 phosphorylation assay.* Hematological cancer cell lines were treated with dimethyl sulfoxide (DMSO; Sigma-Aldrich), STX-0119 or AG490 overnight in RPMI1640 medium containing 10% FBS. After being washed, cells were incubated in serum-free RPMI1640 medium for 1-2 h and stimulated with 50 ng/ml of human interleukin (IL)-6 (Peprotech Biotech, Rocky Hill, NJ). Cells were fixed with fixation buffer (BD Bioscience), permealized with Perm buffer (BD Bioscience) and washed with staining buffer (BD Bioscience). Treated cells were stained with a PE-labeled anti-phospho-specific STAT3 antibody or isotype control antibody. Stained cells were fixed with 0.5% paraformaldehyde (Sigma-Aldrich) and analyzed on a flow cytometer (FACSCanto, BD).

*Apoptosis assay.* Cancer cell lines were double-stained with Annexin V and 7-amino-actinomycin D (7-AAD) using an Annexin V-PE apoptosis detection kit (BD Biosciences) according to the manufacturer's instructions. Briefly, after incubation with STX-0119 or AG490 for 24 h, cells were re-suspended in 1X binding buffer at a concentration of $1 \times 10^6$ cells/ml and incubated with Annexin V-PE and 7-AAD solutions at room temperature (RT) for 15 min. The percentage of apoptotic cells was analyzed on a flow cytometer (FACSCanto). Early apoptotic cells were defined as Annexin $V^+/7\text{-}AAD^-$.

*Western blotting.* SCC3 cells were treated with STX-0119, AG490 or DMSO for 24 h in RPMI1640 medium containing 10% FBS. Cells were lysed using RIPA buffer (Thermo Fisher Scientific Inc., Rockford, IL) containing protease inhibitors and phosphatase inhibitors. RIPA buffer consisted of 50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1.5 mM $MgCl_2$, 100 mM NaF, 10 mM $Na_4P_2O_7 \cdot 10\ H_2O$, 1 mM $NaVO_3$, 10% glycerol and 1% NP-40. The cell lysate was subjected to SDS-PAGE using a 7.5% polyacrylamide separating gel, and was subsequently transferred to PVDF membranes. The membranes were blocked with 5% skim milk in TBS containing 0.1% Tween-20 (TBS-T) at RT for 60 min or 4°C overnight, and incubated at 4°C overnight with the primary antibody against STAT3, phospho-specific STAT3, cleaved caspase-3, c-myc, cyclin D1, Bcl-xL, survivin and β-actin (1:200-1:2000) in blocking solution.

After washing in TBS-T, the membranes were incubated for 1 h with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:5000). Membranes were treated with

ECL plus reagent (GE Healthcare) and analyzed on a chemiluminescence scanner (LAS-3000; FujiFilm, Tokyo). Subcellular fractionation analysis of phosphorylated STAT3 in the STX-0119-treated SCC3 cells was also performed using a Subcellular Proteome Extraction kit (Merck kGaA, Darmstadt, Germany). Each subcellular fraction derived from the SCC3 cells was analyzed by WB. In some experiments, SCC3 tumors transplanted into nude mice and treated with STX-0119 were resected on day 3, 7, 14 and 21 after the start of STX-0119 administration and used for WB. WB signals obtained by a chemiluminescence scanner were transferred to a computer-associated image analyzer (WinRoof, version 6; Mitani Corporation, Fukui, Japan). The entire area of each signal was measured and utilized for quantitative analysis.

*Cell cycle analysis.* Cancer cell lines were incubated with STX-0119 for 24 h, washed with phosphate-buffered saline (PBS) and fixed with ice-cold 70% (vol/vol) ethanol. After incubation in PBS containing 0.25 mg/ml of RNase A (type 1-A; Sigma-Aldrich) and 0.1% (vol/vol) Nonidet P-40 (Nacalai Tesque, Kyoto, Japan) for 30 min at 37˚C, the cells were stained with 50 $\mu$g/ml of propidium iodide (PI; Sigma-Aldrich) for at least 20 min on ice. Finally, the DNA content was analyzed with a flow cytometer (FACSCalibur, BD) using ModFit LT™ 3.1 (Verity Software House, Inc., Topsham, ME).

*Animal experiments.* Male nude mice (BALB/cA-nu/nu, 5-6 weeks old) were obtained from Nippon Clea (Tokyo, Japan), housed in a separate experimental room, and given sterilized food and water *ad libitum*. All animals were cared for and used humanely according to Guidelines for the Welfare and Use of Animals in Cancer Research (Committee of the National Cancer Research Institute, 2010) and procedures approved by the Animal Care and Use Committee of Shizuoka Cancer Center Research Institute.

SCC3 cells re-suspended in RPMI1640 medium (100 $\mu$l) containing Matrigel (BD Biosciences) at $1x10^7$/ml, were inoculated into the flank of BALB/cA-nu/nu mice. To evaluate the antitumor activity against subcutaneously (s.c.) inoculated tumors, tumor volume was calculated based on the National Cancer Institute formula as follows: Tumor volume $(mm^3)$ = Length (mm) x [Width $(mm)]^2$ x 1/2.

The efficacy of the compounds against the human tumors inoculated into the nude mice was expressed as the mean $V/V_0$ value, where V is the tumor volume on the day of evaluation and $V_0$ is that on the day of treatment. The tumor/control (T/C) value was calculated as the mean $V/V_0$ value of the treated group versus that of the untreated group.

In another experiment, SCC3 tumors treated with vehicle or STX-0119 were resected on day 3, 7 14 and 21 after the start of STX-0119 administration.

*Immunohistochemistry (IHC).* SCC3 tumors treated with vehicle or STX-0119 were resected on day 3, 7, 14 and 21 after the start of STX-0119 administration and fixed with formalin solution. Hematoxylin and eosin staining was performed according to the manufacturer's instructions. For immunostaining, antibodies against STAT3, phospho-specific STAT3, c-myc and Ki67 as primary antibody and goat or rabbit anti-mouse IgG antibody as secondary antibody were used.

Table I. Cell proliferation assay using hematological cancer cell lines.

| Cell line | IC$_{50}$ | |
| --- | --- | --- |
| | STX-0119 ($\mu$M) | AG490 ($\mu$M) |
| IM-9 | 5.1 | 2.2 |
| U937 | 7.7 | 31.5 |
| Jurkat | 5.3 | 4.2 |
| KG-1 | 6.1 | 15.6 |
| MOLT-3 | 4.6 | 3.2 |
| HL-60 | 17.2 | 86.3 |
| THP-1 | 13.5 | 37.7 |
| RPMI8226 | 8.8 | 19.7 |
| SCC3 | 5.5 | 16.4 |
| HUT78 | 8.2 | 3.5 |
| HH | 1.4 | 4.9 |
| K562 | 18.0 | 50.9 |
| CMK | 7.4 | 7.4 |
| BC-3 | 18.3 | 23.1 |
| U266B1 | 73.0 | >100 |
| NCI-H929 | 16.3 | 14.9 |
| TF-1 | 6.4 | 33.6 |

HRP and hydrogen peroxide were utilized for producing color according to the manufacturer's instructions (Histofine mousestain kit; Nichirei Biosciences Inc., Tokyo).

*Statistical analysis of tumor models.* Statistical analysis was performed with corrected p-values for comparison with the untreated control using the Steel multiple plus Kruskal-Wallis method and Mann-Whitney's rank-sum test.

## Results

*Cell proliferation assay.* STX-0119 exhibited a potent antiproliferative effect, with an IC$_{50}$ of 5-10 $\mu$M, on hematological cancer cells, particularly acute lymphocytic leukemia (ALL), T-cell lymphoma and myeloma cell lines (Table I). Acute myelocytic leukemia (AML) and chronic myelocytic leukemia (CML) cell lines were not very sensitive. Notably, the T-cell lymphoma cell line HH was the most sensitive (IC$_{50}$, 1.4 $\mu$M) among the 17 hematological cancer cell lines. Moreover, the JAK2 kinase inhibitor, AG490, was not effective against T-cell lymphoma cell lines such as U937, SCC3 and HH when compared with STX-0119.

*STAT3 phosphorylation assay.* The activation (phosphorylation) of STAT3 was investigated in 10 hematological cell lines with or without IL-6 treatment (Fig. 2A). The phosphorylation was promoted in UP37, KG-1, RPMI8226, THP-1 and CMK cells by IL-6 or leukemia inhibitory factor (LIF). In contrast, SCC3 which had the strongest STAT3 activation, was demonstrated to be constitutively STAT3-autophosphorylated.

*Effect of STX-0119 on phosphorylated STAT3 expression.* The effect of STX-0119 on phosphorylated STAT3 expression in the SCC3 and U937 lymphoma cell lines was investigated
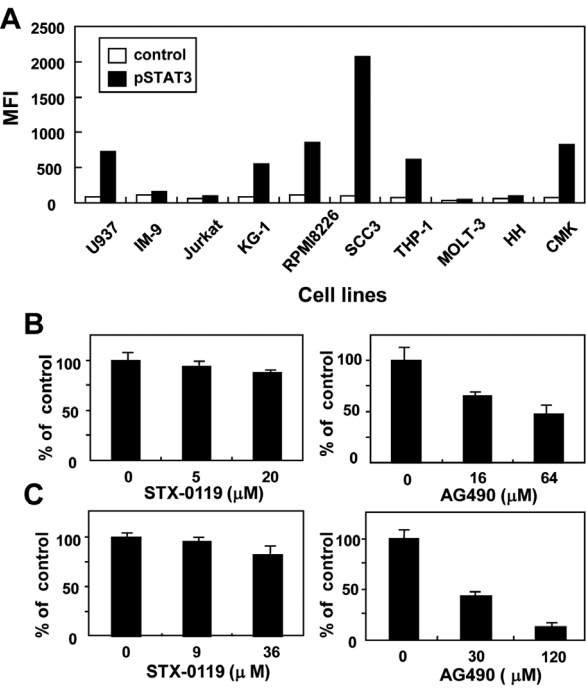
Figure 2. Effect of STX-0119 on STAT3 phosphorylation. (A) STAT3 activation status in hematological cancer cell lines. STAT3 was activated by IL-6 in U937, KG-1, RPMI8226, THP-1 and by LIF in CMK cells. SCC3 cells showed high constitutive STAT3 activation without IL-6 stimulation. After SCC3 (B) or U937 (C) cells were treated with STX-0119 or AG490 overnight, they were stimulated with IL-6 and subsequently stained with anti-phospho-specific STAT3 Ab.
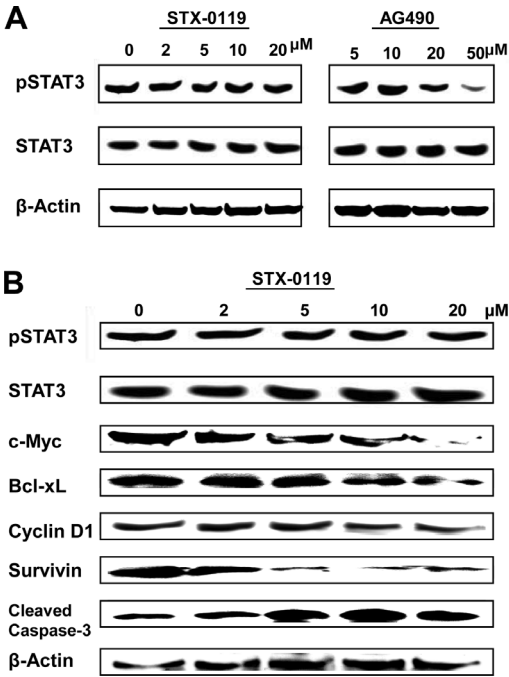


Figure 4. Down-regulation by STX-0119 of the expression of genes targeted by STAT3 in the SCC3 cells. The lysate of SCC3 cells treated with STX-0119, AG490 or DMSO for 24 h was subjected to WB analysis of target gene expression. Downstream target genes including Bcl-Xl, c-myc, cyclin D1 and survivin were significantly down-regulated in their expression by STX-0119. In contrast, an increase in cleaved caspase-3 expression was noted. (A) STAT3 and phosphorylated STAT3 expression, (B) STAT3 downstream target gene expression.
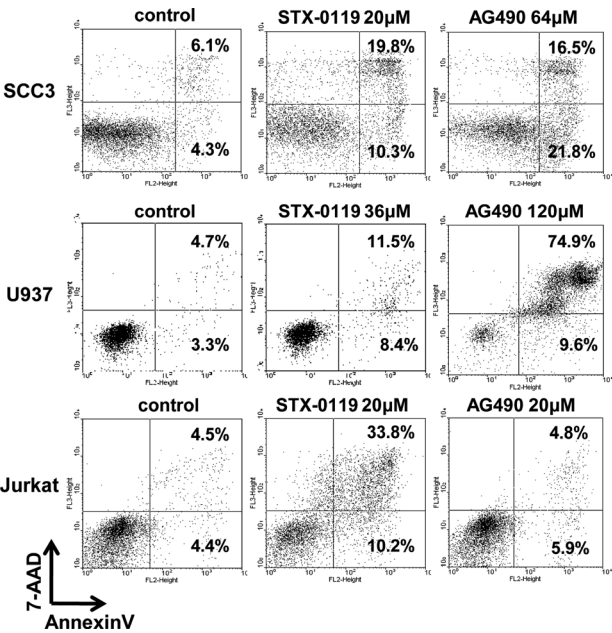


Figure 3. Apoptosis-inducing activity of STX-0119. After incubation with STX-0119 or AG490 for 24 h, SCC3, U937 and Jurkat cells were stained with Annexin V-PE and 7-AAD solutions. STX-0119 showed a weak to moderate apoptosis-inducing activity in the SCC3 and U937 cells while AG490 exhibited a stronger effect than STX-0119.

using flow cytometry. The cells were treated with the $IC_{50}$ and 4-fold the $IC_{50}$ of STX-0119 or AG490 overnight and stained with an anti-phospho-specific STAT3 Ab. No significant effect of STX-0119 at any dose was noted (Fig. 2B and C) in either the SCC3 or U937 cells. By contrast, AG490 suppressed phosphorylated STAT3 levels in both cell lines in a dose-dependent manner.

*Induction of apoptosis by STX-0119 in hematological cancer cell lines.* The cell lines SCC3 (STAT3 constitutively activated), U937 (STAT3 activated by IL-6) and Jurkat (STAT3 unactivated) were used for the apoptosis assays. STX-0119 showed weak to moderate apoptosis-inducing activity in the SCC3 and U937 cells; however AG490 showed a stronger effect on Annexin+ apoptotic cells, particularly U937 cells (Fig. 3). Notably, STX-0119 exhibited far stronger apoptosis-inducing activity in Jurkat cells compared to AG490.

*Regulation of STAT3 target gene expression by STX-0119 in SCC3 cells.* Downstream targets including Bcl-xL, c-myc, cyclin D1 and survivin were significantly down-regulated by STX-0119 at ~10 $\mu$M (Fig. 4). Meanwhile, an increase in cleaved caspase-3 was noted, consistent with the evidence of apoptosis shown previously. In contrast, the expression of STAT3 and phosphorylated STAT3 was not significantly affected by STX-0119, although AG490 strongly suppressed STAT3 phosphorylation in the SCC3 cells.

*Effect of STX-0119 on the amount of phosphorylated STAT3 in subcellular fractions.* To localize Tyr-705-phosphorylated STAT3 in SCC3 cells treated with STX-0119, subcellular extraction was performed using a Subcellular Proteome Extraction kit. Extracted fractions consisted of cytosolic,
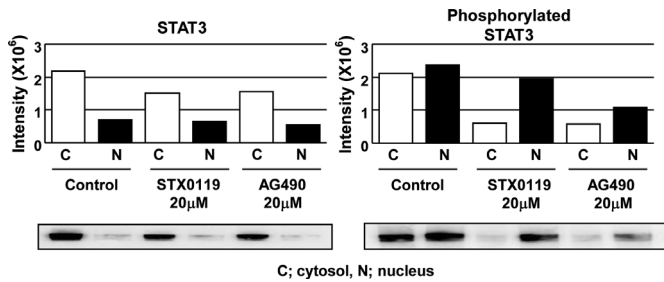
Figure 5. Subcellular localization of phosphorylated STAT3 in STX-0119-treated SCC3 cells. SCC3 cells treated with DMSO (control), STX-0119 (20 $\mu$M) and AG490 (20 $\mu$M) were subjected to subcellular fractionation and WB. C, cytosol; N, nucleus. Membrane and cytoskeletal fractions are not shown. STAT3 was not detected in these fractions.

membrane, nuclear and cytoskeletal fractions. Total STAT3 was located mainly in the cytosol and membrane fractions. In contrast, phosphorylated STAT3 accumulated in the nuclear fraction. STX-0119 decreased the amount of phosphorylated STAT3 in the cytosol, but not in the nuclear fraction. However, phosphorylated STAT3 levels in the whole lysate appeared to be similar even after STX-0119 treatment. In contrast, AG490 significantly decreased the amount of phosphorylated STAT3 in both the cytosol and nuclear fractions (Fig. 5).

*Effect of STX-0119 on the cell cycle.* After incubation with 20 $\mu$M STX-0119 or 20 $\mu$M AG490, SCC3 cells were stained with PI, and the DNA content was analyzed with a flow cytometer. STX-0119 had no significant effect on the cell cycle of SCC3 cells compared to the untreated cells (data not

shown). Similarly, AG490 showed no obvious effect on the cells at concentrations up to 20 $\mu$M.

*STX-0119 inhibits tumor growth in a subcutaneous model of lymphoma.* To determine whether STX-0119 inhibits the growth of human tumors, nude mice were transplanted with SCC3 human lymphoma cells. STX-0119 at doses of 40 and 160 mg/kg suspended with methylcellulose was orally administered to SCC3-bearing nude mice for five consecutive days. Cytarabine (Ara-C) at 13 mg/kg as a reference was intraperitoneally administered for 5 consecutive days. In some experiments, AG490 at 25 mg/kg was administered intra-peritoneally to compare its activity to STX-0119 at 20 mg/ kg. STX-0119 at more than 80 mg/kg caused a more than 50% inhibition of SCC3 tumor growth on day 4 (early phase) similar to Ara-C (Fig. 6). STX-0119 at 160 mg/kg alone maintained the growth inhibition at ~50% as the T/C ratio even after day 21 (late phase), although rebound growth was noted in other groups. In the *in vivo* experiments using SCC3-bearing nude mice, AG490 at 25 mg/kg revealed a less potent antitumor activity than STX-0119 at 20 mg/kg (Fig. 6D).

Additionally, no significant side effects including weight loss were noted in the STX-0119-treated mice (Fig. 6C). A brief toxicity test with 160 mg of STX-0119 administered orally for 5 consecutive days showed no changes in hemato-logical or biochemical parameters (data not shown).

*Regulation of STAT3 target gene expression by STX-0119 in the SCC3 tumors.* The SCC3 tumors treated with vehicle, 80 or 160 mg/kg of STX-0119 were resected on day 3, 7, 14 and 21 and subjected to WB analysis for STAT3 target gene expression. The
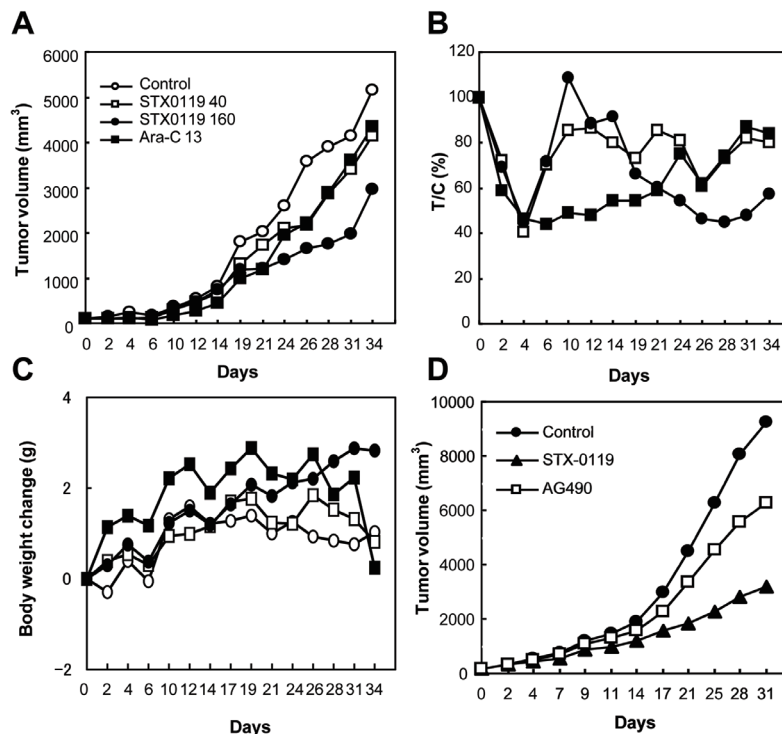


Figure 6. STX-0119 inhibits tumor growth in a subcutaneous model of human lymphoma. Nude mice were transplanted with SCC3 human lymphoma cells. STX-0119 and Ara-C were administered and (A) tumor volume, (B) tumor/control (T/C) ratio and (C) body weight change were assessed. ○, control; □, STX-0119 (40 mg/kg); ●, STX-0119 (160 mg/kg); ■, Ara-C (13 mg/kg). STX-0119 (20 mg/kg) and AG490 (25 mg/kg) were administered and (D) tumor volume was assessed. ●, control; ▲, STX-0119 (20 mg/kg); □, AG490 (25 mg/kg).
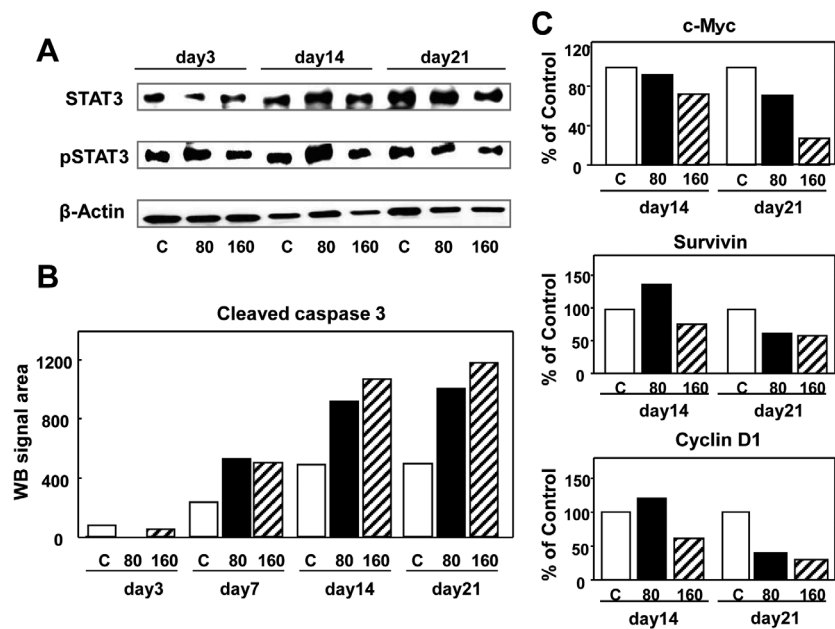
Figure 7. Regulation of STAT3 target gene expression by STX-0119 in SCC3 *in vivo* tumors. SCC3 tumors treated with 80 and 160 mg/kg of STX-0119 were resected on day 3, 7, 14 and 21 after the start of administration and were subjected to WB analysis for target gene expression. Expression of (A) STAT3 and phosphorylated STAT3; (B) cleaved caspase 3; (C) c-Myc, survivin and cyclin D1. C (white column), control; 80 (black column), treated with 80 mg/kg of STX-0119; 160 (hatched column), treated with 160 mg/kg of STX-0119.
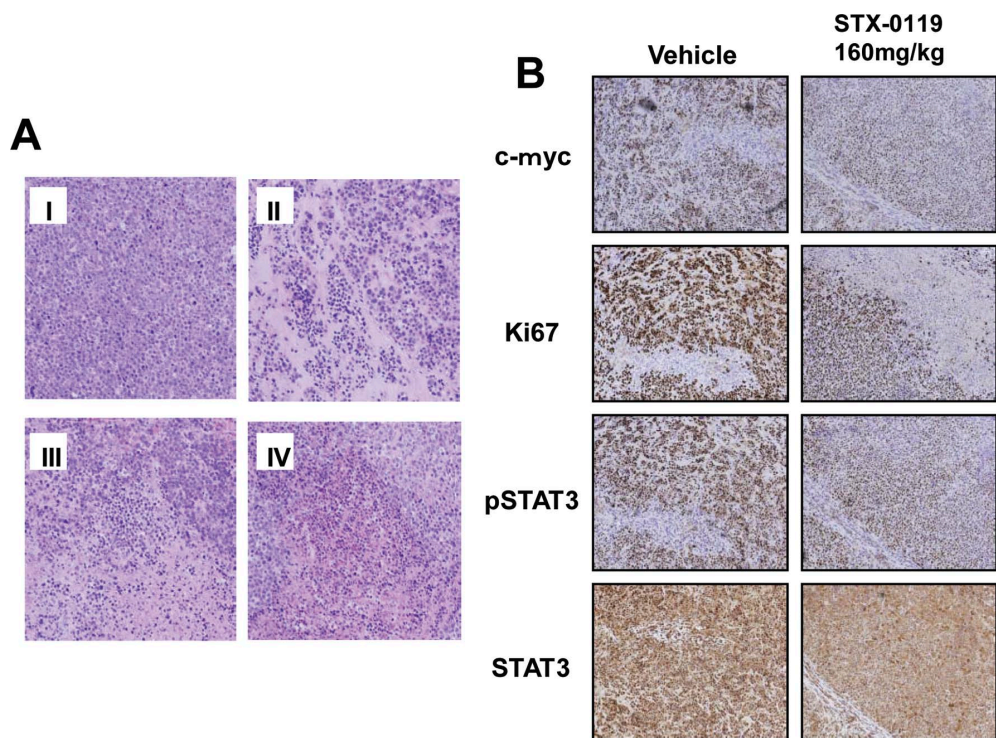


Figure 8. IHC analysis of SCC3 tumors treated with STX-0119. (A) H&E staining of tumors treated with 160 mg/kg STX-0119. I, control (untreated); II, 7 days after STX-0119 administration; III, 14 days; IV, 21 days. (B) Down-regulation of c-myc and Ki67 proteins in STX-0119-treated tumors. SCC3 tumors were resected on day 21 after the start of STX-0119 administration (160 mg/kg). Immunostaining using antibodies against STAT3, phosphorylated STAT3, c-myc and Ki67 was performed. Magnification, x100.

expression of STAT3 and phosphorylated STAT3 was maintained before and after the treatment. Downstream target genes like c-myc, cyclin D1 and survivin apeared to be down-regulated in the late phase, particularly in the tumors treated with 160 mg/kg of STX-0119 (Fig. 7). Similar to the *in vitro* experiments, the

up-regulation of cleaved caspase-3 expression was noted in the tumors in the late phase (from day 14) of STX-0119 treatment.

*IHC analysis of SCC-3 tumors treated with STX-0119*. Seven days after the start of STX-0119 administration, morpho-

logical change was noted suggesting a cytotoxic effect. From day 13, cell infiltration with necrosis occurred (Fig. 8A). In viable tumors, most cells were positive for STAT3; however Ki67- and c-myc-positive tumor cells appeared to decrease in number compared to the control (Fig. 8B).

## Discussion

STAT3 is a member of a family of DNA-binding molecules, which regulate numerous physiological and oncogenic signaling pathways leading to the induction of target gene expression through STAT3-SH2 dimerization and phosphorylation, and to the promotion of cell proliferation and induction of anti-apoptotic activity and angiogenesis.

Recent studies have showed that STAT3 is constitutively activated in various types of cancers including hematological and solid cancers. Evidence has demonstrated that persistent STAT3 activation may impair immune responses by enhancing immunological tolerance and inducing immunological evasion in the tumor site. Additionally, aberrant STAT3 activation plays an important role in establishing loops of autocrine and/ or paracrine cytokines, particularly IL-6, which may partly contribute to tumorigenesis in some types of cancers (16,17).

Experimental approaches to blocking STAT3 signaling using small interfering RNA (siRNA), small hairpin RNA (shRNA) and STAT3 antisense have been successful in inhibiting cell proliferation in vitro (18) and tumor growth in vivo (19). However, STAT3 silencing has been reported to have no toxic effect on normal cells (20). These observations suggest that STAT3 may be a potential target in cancer therapy.

Previously, we identified a novel small-molecule inhibitor of STAT3 dimerization, STX-0119, using biochemical and virtual screening methods (STAT3-dependent luciferase reporter gene assay and STAT3 dimerization assay using FRET) for antagonizing the activity of STAT3-SH2 binding (15). The crystal structure of a STAT3-based computer model (in silico) obtained with a docking simulation algorithm named Consensus-Dock revealed that STX-0119 binds directly to the SH2 region of the STAT3 monomer and antagonizes the dimerization.

In the present study, we investigated the antitumor activity of STX-0119 in vitro and in vivo and investigated the mechanism involved through biochemical and biological assays. Our study revealed that STX-0119 had a potent growth-inhibitory effect on the human lymphoma cell line SCC3 (STAT3-activated), and effectively down-regulated downstream target genes of the STAT3 signaling pathway including c-myc, Bcl-xL, cyclin D1 and survivin without significantly affecting STAT3 phosphorylation. Matsuno et al (15) reported similar results from experiments in vitro using human breast cancer MDA-MB-468 cells. More impressively, STX-0119 exhibited stronger growth inhibition against SCC3 tumors in vivo than did AG490.

A notable observation in vivo was the two-phased effect of STX-0119 against SCC3 tumors: rapid inhibition at approximately day 4 (early) and sustained inhibition after day 21 (late). Biochemical analysis using resected tumors treated with STX-0119 demonstrated extensive apoptosis of the tumor in the late phase; however there may be some inhibitory effect on cancer stem cells leading to late-phase inhibition.

Recently, Sherry et al (21) demonstrated STAT3-dependent proliferation and maintenance in glioblastoma stem cells, which can be inhibited by a STAT3 signaling inhibitor. These results suggest cancer stem cells to be a novel target for small-molecule STAT3 signaling inhibitors.

Recently, many JAK-STAT3 inhibitors, which down-regulate STAT3 phosphorylation (Tyr-705) and induce apoptosis in cancer cells by inhibiting STAT3 signaling, have been developed, including STAT3 DNA decoy (22), LLL-3 (23), indirubin derivative E804 (24), AG490 (25), WP1066 (26), FLLL31 and 32 (27), cucurbitacin analogue (28), SD1029 (29), JSI-124 (30) and the JAK2 inhibitor AZD1480 (31). Only a few compounds showed antitumor activity in vivo; however AZD1480 exhibited very strong antitumor activity against solid tumors like breast and prostate cancer in vitro (IC$_{50}$ <0.1 $\mu$M) and in vivo compared to other compounds. By contrast, only a few inhibitors of STAT3-SH2 dimerization have been reported, namely peptide mimetics (32), STA-21 (33), Stattic (34), S3I-201 (NSC74859) (35) and 5,15-DPP (36). Based on these findings, STX-0119 showed the strongest antitumor activity, particularly in vivo, among the STAT3 dimerization inhibitors. Notably, STAT3 phosphorylation was suppressed by Static and S3I-201, but not STA-21 and STX-0119. As STX-0119 was demonstrated to regulate STAT3 transcriptional activity and induce apoptosis in cancer cells, there may be different mechanisms affecting the phosphorylation or translocation of STAT3 into the nucleus. In the present study, we obtained evidence that STX-0119 inhibited phosphorylated STAT3 in the cytosol, but not STAT3 in the nucleus, using a subcellular fractionation kit and WB (Fig. 5). These results suggest mainly two possibilities: i) the inhibition of STX-0119 activity against dimerization and translocation of STAT3 leading to the breakdown of phosphorylated STAT3 in the cytosol, or ii) no significant effect of STX-0119 on transported phosphorylated STAT3 in the nucleus. In contrast, AG490 significantly suppressed STAT3 phosphorylation in both the cytosol and nucleus. Seemingly, there may be a different mechanism of activity between the dimerization inhibitor, STX-0119, and the kinase inhibitor, AG490. To investigate the precise mechanism of STX-0119 activity, a time-course study of phosphorylated STAT3 localization is warranted.

Finally, one important issue which requires investigation is the fact that the antitumor efficacy in nude mice was weak compared to that of STAT3 signaling inhibitors such as AZD1480. Considering the high concentration of STX-0119 in mouse plasma after an oral administration in a pharmacokinetic study reported previously (15), there may be a discrepancy between the plasma concentration and antitumor effect. Poor uptake of STX-0119 in the tumor site and a weak immunological antitumor effect in nude mice triggered by overcoming the STAT3-mediated immunosuppression induced in the tumor microenvironment (37) may be responsible for this discrepancy. In the future, intratumoral pharmacokinetics and immunocompetent mice should be utilized.

In conclusion, we identified a novel small-molecule inhibitor of STAT3 dimerization using biochemical and virtual screening methods and demonstrated that it shows a potent antitumor effect in vitro and in vivo against constitutively

STAT3-activated cancer cells. These observations suggest STX-0119 to be a promising candidate for STAT3-signaling therapeutics in STAT3-activated hematological malignancies.

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