Impact of STAT3 Inhibition on Survival of Osteosarcoma Cell Lines

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Abstract. *Background/Aim: Osteosarcoma is often a fatal malignancy. Constitutive STAT3 activation is associated with various human cancers and commonly suggests poor prognosis. We aimed to investigate the effect and potential molecular mechanisms of STAT3 inhibition on osteosarcoma. Materials and Methods: STAT3 inhibitor S3I-201 was investigated in six osteosarcoma cell lines. Crystal violet colorimetric, clonogenic, cleaved caspase-3 assays and western blot were performed to measure the effect and mechanisms of STAT3 inhibition. Results: All osteosarcoma cell lines expressed phosphorylated STAT3. Anti-proliferative effects of S3I-201 were dose- and time-dependent. S3I-201 also inhibited colony-formation and induced apoptosis through the caspase cleavage pathway. Finally, molecular mechanism studies suggested that down-regulation of STAT3 phosphorylation and downstream STAT3-target genes such as cyclin D1 and survivin may contribute to S3I-201-mediated anti-proliferation and apoptosis. Conclusion: Inhibition of STAT3 signalling suppressed osteosarcoma cell growth and induced apoptosis, and indicated that STAT3 targeted-therapy may have therapeutic potential in osteosarcoma.*

Osteosarcoma, the most frequently occurring primary bone malignancy, occurs mostly in children and adolescents aged 0 to 24 years, and there is a second incidence peak in the elderly (ages over 60 years) (1). The current treatment approach incorporates surgery, radiation therapy and multi-drug chemotherapy, which has significantly improved patient outcomes from 1970s (2). Currently, the overall survival for localized osteosarcoma is approximately 70% (3). However, the prognosis for metastatic disease remains poor with an overall survival of less than 20% (4). Patients diagnosed with primary osteosarcoma over the age of 40 years or with a non-extremity tumor have a significantly inferior outcome (22% and 16%, respectively) (5). The overall survival in osteosarcoma has stagnated in the past several decades (6). The lack of significant improvement for osteosarcoma highlights the need for further research into innovative drugs and approaches.

A better understanding of the tumor microenvironment and molecular pathogenesis of osteosarcoma leads to the development of new strategies involving the identification of new potential targets. The receptor tyrosine kinases including insulin-like growth factor receptor (IGF-R), platelet-derived growth factor receptor (PDGFR), human epidermal growth factor receptor 2 (HER2) and vascular endothelial growth factor (VEGF) have been found abundantly expressed in osteosarcoma cells and correlated with tumor metastases and overall survival, suggesting that inhibition of these receptors or their pathways may be effective in osteosarcoma (6-8). For a number of these receptors, antagonists, including monoclonal antibodies and small-molecule inhibitors, have been developed and have been or are currently being introduced in clinical trials for patients with osteosarcoma. However, they have not yet been successful in improving current survival rates for osteosarcoma patients (6). Identification of additional active agents is needed. Further testing of these agents is required in order to fully-assess their role in osteosarcoma treatment.

Signal transducer and activator of transcription 3 (STAT3), one of seven members of a family of transcription factors, is a latent cytoplasm transcription factor that relays signals from the cell membrane to the nucleus to regulate gene expression of proliferation, survival, angiogenesis, migration and invasion (9). Its activation is regulated through epidermal growth factor receptor (EGFR) kinase, Janus-activated

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kinases (JAK) and non-receptor protein tyrosine kinase Src in response to external stimulation by cytokines (interleukin-6 (IL-6)) and growth factors (epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and transforming growth factor (TGF)) (10). STAT3 phosphorylation at tyrosine 705 leads to its dimerization, nuclear translocation, DNA binding and gene transcription. STAT3 regulates the expression of genes that mediate survival (survivin), apoptosis (caspase 3) and proliferation (cyclin D1) (11-13). Constitutive activation of STAT3 has been reported at high frequency in a wide range of tumors and commonly suggests poor prognosis. Recently, IL-6/JAK/STAT3 signaling was identified as a resistance pathway against pan-HER inhibition (14), suggesting a role for inhibition of phosphorylated STAT3 as a therapeutic target.

Our previous data showed that constitutive activation of STAT3 was detected in a broad range of human soft-tissue sarcoma (STS) cell lines. In addition, we demonstrated that a small molecular STAT3 inhibitor, S3I-201, induced anti-proliferative effects on a majority of STS cell lines harboring aberrant STAT3 by blocking STAT3 phosphorylation (15). The S3I-201 (NSC74859) was identified from the National Cancer Institute chemical libraries and inhibited STAT3 dimerization and STAT3 DNA-binding and transcriptional activates (16). To our knowledge, S3I-201 has not yet been evaluated on osteosarcoma cell lines. This study aimed to characterize the expression and activation of STAT3, as well as investigate the effect and mechanisms of S3I-201 therapy on a panel of six osteosarcoma cell lines.

Materials and Methods

Cell lines and culture. Three human osteosarcoma cell lines (MG63, U2OS and Saos-2) were purchased from the American Type Culture Collection (Manassas, VA, USA). Another three osteosarcoma cell lines (HOS, 143B and SJSA) were kindly provided by Professor David Thomas (Peter MacCallum Cancer Centre, Australia) and Dr. Florence Pedeatour (Nice University Hospital, France). All cells were grown in Roswell Park Memorial Institute (RPMI-1640) media supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and 50U/ml penicillin-streptomycin. All cells were incubated in a humidified 5% CO_2 environment at 37˚C and fed every 3-4 days. The cell lines were all tested mycoplasma-free as well as had an identification test using short tandem repeat (STR) profiling by CellBank Australia and shown to be consistent with their stated cell lineage.

Crystal violet colorimetric assay. 24 h after cells were seeded in 96-well plates, STAT3 inhibitor S3I-201 (also named NSC 74859; Merck, Darmstadt, Germany) was added into relevant wells at a concentration of 0 to 100μM. After required time period (24, 48 and 72 hours), cells were washed with Dulbecco's phosphate-buffered saline (DPBS; Invitrogen, Mulgrave, VIC, Australia), stained with 0.5% crystal violet and incubated with Elution Solution (0.1 M Sodium citrate (pH 4.2) +100% ethanol; Sigma, Castle Hill, NSW, Australia) for 30 minutes, followed by light absorbance at 540 nm on SpectraMax M3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA, USA). Relative cell proliferation percentages were derived by comparing mean OD values of drug treatment groups to those of control.

Clonogenic survival assay. Single-cell suspensions of 143B and HOS were plated in duplicate into six-well plates. The number of cells plated per well was adjusted according to the plating efficiency of the cell lines. After 24 hours, cells were treated with vehicle (0.1% dimethyl sulfoxide (DMSO)) or S3I-201 at required concentrations (0, 15, 25 and 50 μM) and incubated at 37˚C. The drugs were present in the medium throughout the whole incubation period. Once colony-formation (1 colony ≥50 cells) was observed in the control wells, all related wells were washed and stained with crystal violet. Images were taken by the Molecular Imager Gel Doc XR System (Bio-rad, Hercules, CA, USA).

Western blot analysis. Twenty-four hours after seeding in 6-well plates in normal culture medium, osteosarcoma cells were starved with medium containing 1% FBS for 24 h and then treated with vehicle (DMSO), 30 μM or 80 μM S3I-201 for another 24 hours. Cells were harvested immediately after 15 min of incubation with or without 100 ng/ml EGF (Sigma, Castle Hill, NSW, Australia) stimulation and total proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer (Sigma) with 1% protease and phosphatase inhibitor cocktails (#539131 Merck and #78420 Thermofisher, Scoresby, VIC, Australia). Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Thermofisher), according to the company's instruction. Subsequently, proteins were separated by 4-20% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-rad, Gladesville, NSW, Australia), which were then probed with primary antibodies overnight at 4˚C. The p^{Y705}STAT3, total STAT3, cyclin D1, survivin and cleaved caspase3 antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA) and the β-actin antibody from Santa Cruz Biotechnology (Dallas, TX, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were added and specific binding was detected by chemiluminescence agent Supersignal Western Dura Extended Duration (Thermofisher). Membranes were imaged by ImageQuant LAS4000 (GE healthcare, Uppsala, Sweden). Densitometric analysis was performed by the ImageQuant TL Software (GE healthcare) and presented as ratios of protein expression normalized to relevant β-actin loading control.

Real-time polymerase chain reaction (PCR). After 24 h of treatment, cells were harvested for total RNA extraction with the RNeasy plus kit (Qiagen). RNA concentration was determined using NanoDrop ND-1000 (NanoDrop Technologies, Delaware, USA) to measure the absorbance at 260 nM. The template RNA was mixed with gDNA wipeout buffer for 2 minutes at 42˚C and then mixed with Reverse-transcription master mix (Qiagen; Chadstone centre, VIC, Australia). The cDNA synthesis was performed in a Bio-rad C1000 Thermal cycler for 15 minutes at 42˚C and followed by 3 minutes at 95˚C. Hs_GAPDH_1_SG and Hs_STAT3_1_SG QuantiTect Primer assays were purchase from Qiagen. For quantitative real-time PCR, the mixtures of cDNA, QuantiFast SYBR green PCR master mix (Qiagen), QuantiTect Primers and RNase-free water were added into the PCR plate in duplicate and then placed into the Bio-rad CFX96 real-time cycler (95˚C for 5 min and then two-step cycling of 95˚C for 10 sec and 60˚C for 30 sec for 40 cycles). Two independent experiments were performed.

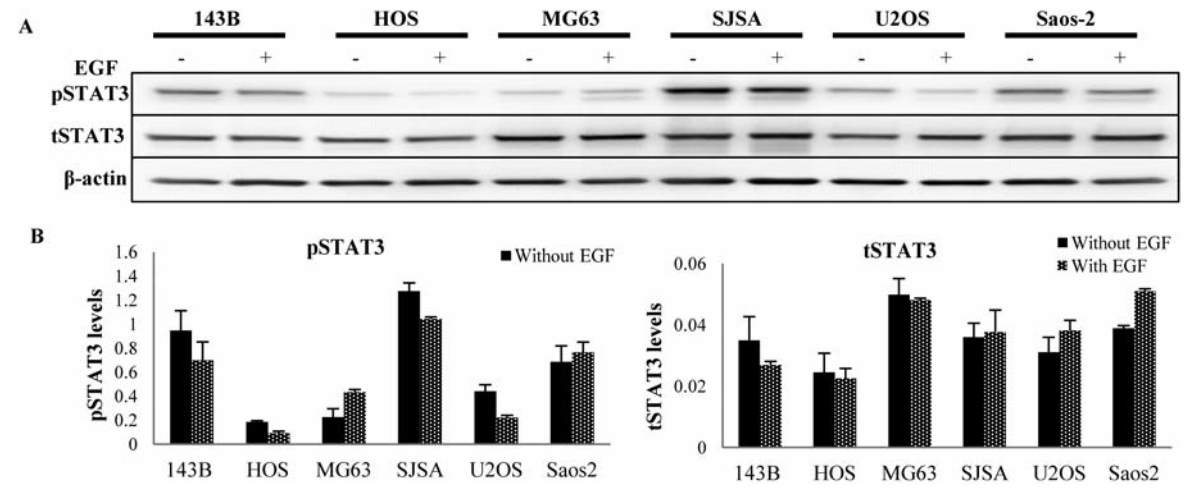

Figure 1. *Phosphorylated STAT3 (pSTAT3) and total STAT3 (tSTAT3) expression (A) and quantitation (B) in the absence and presence of EGF ligand in a panel of six osteosarcoma cell lines by western blot with β-actin serving as an internal protein loading control. (A) Representative images of western blots from duplicate experiments. (B) The mean relative value and standard deviation from duplicate experiments of pSTAT3 or tSTAT3 compared with relevant β-actin using the ImageQuant TL software.*

Statistical analysis. Growth inhibition data was calculated using the GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) software and IC_{50} curves were fitted using a nonlinear regression model with a sigmoidal dose-response. The relationship analysis was examined by the Pearson correlation coefficient. The statistical significance of the results was calculated using two-tailed Student's *t*-test and *p*-values of <0.05 were considered statistically significant.

Results

STAT3 expression and activation in osteosarcoma cell lines. We investigated the status of phosphorylated STAT3 at Tyrosine 705 (p^{Y705}STAT3) and total STAT3 (tSTAT3) expression in the absence and presence of EGF stimulation in a panel of six osteosarcoma cell lines (143B, HOS, MG163, SJSA, U2OS and Saos-2) using western blots with β-actin as an internal protein loading control, to determine whether STAT3 expression and activation is linked to osteosarcoma. As shown in Figure 1, our results indicated that STAT3 was phosphorylated in all osteosarcoma cell lines examined with variable levels of p^{Y705}STAT3, which were independent of EGF stimulation. SJSA showed the highest level of STAT3 phosphorylation followed by 143B, Saos-2, MG63, U2OS (21% of SJSA) and HOS (9% of SJSA). All osteosarcoma cell lines showed comparable total STAT3 expression levels.

Cell proliferation inhibition and apoptosis induction by STAT3 inhibitor S3I-201 in osteosarcoma.
(A) Inhibition of osteosarcoma cell growth and viability by S3I-201. To examine the potential anti-proliferative effects of S3I-201 in osteosarcoma, the crystal violet colorimetric assay was used to quantitate effects on osteosarcoma cell lines treated with 0 to 100 μM S3I-201 for 24, 48 or 72 h. Data in Figure 2 shows a significant time- and dose-dependent inhibition of proliferation in all six osteosarcoma cell lines. After 72 h of exposure to S3I-201 at 100 μM, the concentration used to evaluate growth inhibitory effect in most previous studies (16-18), osteosarcoma cell growth and viability were significantly suppressed (% growth to vehicle/untreated control, 143B: 11%, HOS: 5%, MG63: 9%, SJSA: 22%, U2OS: 16% and Saos-2: 13%). IC_{50}s of S3I-201 after 72 h of treatment from duplicate experiments are summarized in Table I. Using the same criteria, we previously reported in our soft tissue sarcoma study (15) that osteosarcoma cell lines with IC_{50}s ranging from 31.89 to 66.05 μM were all ranked as having moderate sensitivity to S3I-201 mono-therapy. Statistical analysis did not reveal any significant relationship between the sensitivities of osteosarcoma cell lines to STAT3 inhibitor treatment and the levels of pSTAT3, tSTAT3 or the ratio of pSTAT3/tSTAT3 irrespective of presence or absence of EGF stimulation (Table I, $p>0.05$). However, the degree of down-regulation after exposure to S31-201 was highly correlated with IC_{50}.

(B) Inhibition of colony formation by S3I-201. We also investigated the long-term treatment effect by clonogenic assay on osteosarcoma cell lines 143B and HOS (Figure 3A and B). Cells growing in a 6-well plate were treated with vehicle and different doses of S3I-201. Once colony formation (more than 50 cells) was observed in vehicle control plates, all cells were stained with crystal violet to determine the presence of colonies. After 6 days of

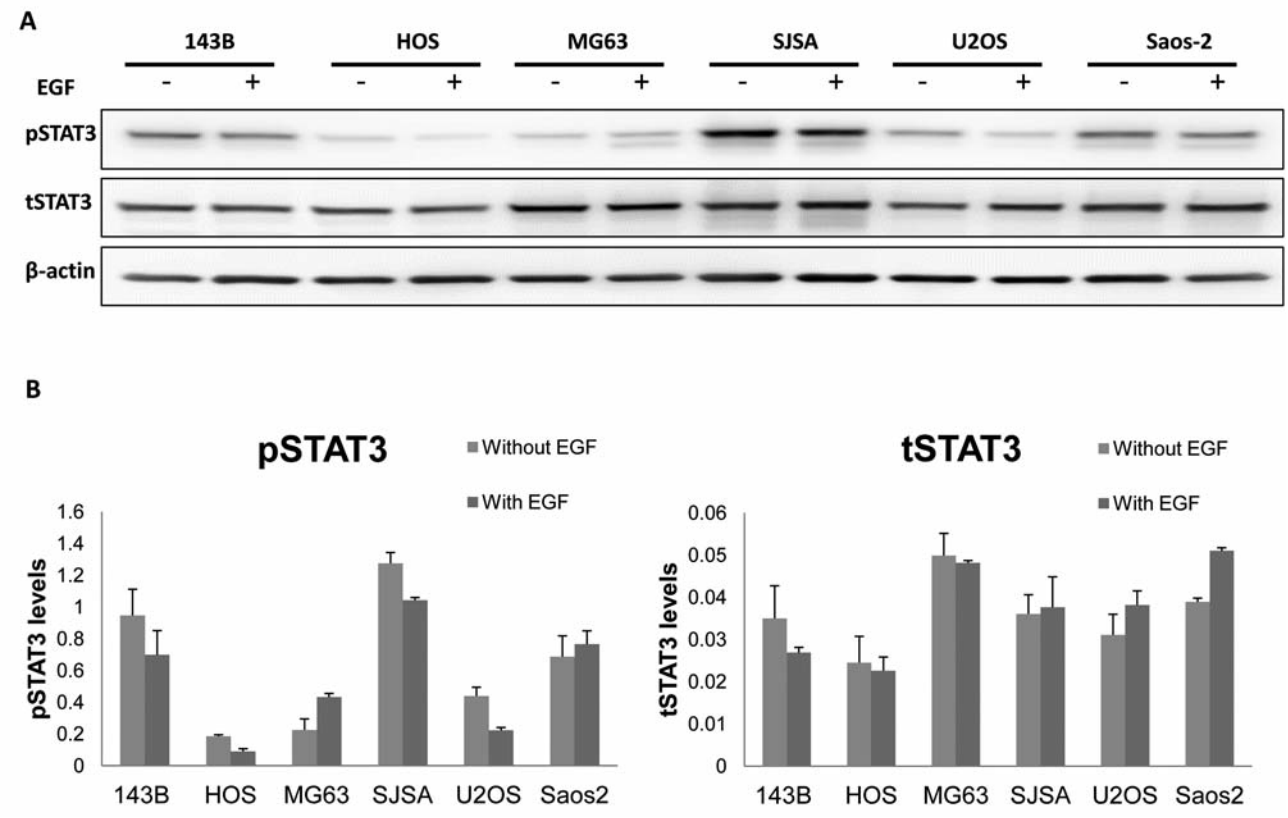

Figure 2. *S3I-201 induced time- and dose-dependent growth inhibitory effects on osteosarcoma cell lines. Osteosarcoma cells were treated with S3I-201 for 24 48 or 72 h. Cell viability was determined by the crystal violet colorimetric assay. Experiments were performed in duplicate, with samples in triplicate within each experiment. Each data shows mean±standard error of the mean (SEM).*

treatment, S3I-201 suppressed colony formation in both cell lines with IC_{50} less than 50 μM.

(C) Induction of apoptosis by S3I-201. To investigate whether apoptosis was also involved after S3I-201 treatment, we examined caspase-3 cleavage by western blot assay. The expression of cleaved caspase-3 (cCaspase-3) was evaluated in the Saos-2 cell line treated with vehicle (DMSO) or S3I-201. At 24 h post-treatment, the pro-apoptotic effect of S3I-201 was observed and levels of cCaspase-3 were increased by 58% compared to the vehicle control (Figure 3C and D).

Molecular mechanisms of S3I-201-mediated anti-tumour effect on osteosarcoma cell lines.

(A) Suppression of STAT3 phosphorylation and mRNA. We investigated the protein levels of total and activated STAT3 before and after S3I-201 treatment in a panel of six osteosarcoma cell lines using western blot assays (Figure 4A and B). Our data demonstrated that S3I-201 down-regulated significantly STAT3 phosphorylation in all six osteosarcoma cell lines with the most down-regulation seen in Saos-2 (91% pSTAT3 expression was suppressed after 80 μM S3I-201 treatment for 24 hours). To determine whether there was any statistical relationship between the efficacy of S3I-201 and the potential degree of activated STAT3 down-regulation in osteosarcoma cell lines, a xy scatter graph was plotted as shown in Figure 4C. Utilizing Pearson correlation coefficient analysis, we showed a strong inverse correlation between IC_{50} and the down-regulation of pSTAT3 in the EGF stimulation-independent manner (Table I, in the absence of EGF: p=0.0029, r=–0.9236; in the presence of EGF: p=0.0040, r=–0.9145). After 24 h of treatment with vehicle or S3I-201, MG63 cells were harvested for quantitative real-time PCR to examine the alteration of STAT3 mRNA. Data in Figure 4D shows that 80 μM S3I-201 significantly inhibited *STAT3* mRNA (Student's *t*-test, p=0.03).

(B) Anti-proliferative effect and apoptosis induction by regulating STAT3-dependent genes encoding cyclin D1 and surviving. To further explore the potential molecular mechanisms underlying SI-201-induced anti-proliferation and apoptosis, we investigated the protein expression of STAT3-

Table I. *Osteosarcoma cell line growth inhibition and correlation analysis before and after S3I-201 treatment.*

Osteosarcoma cell lines	IC_{50} (μM) from duplicate experiments with triplicate samples within each experiment	
143B	51.12±7.69	
HOS	54.23±3.67	
MG63	32.79±1.48	
SJSA	66.05±3.38	
U2OS	52.73±0.37	
Saos-2	31.89±3.03	
Pearson correlation coefficient before treatment		
IC_{50} *versus* pSTAT3 expression	Without EGF	p=0.295; r=0.497
	With EGF	p=0.792; r=0.137
IC_{50} *versus* tSTAT3 expression	Without EGF	p=0.156; r=–0.630
	With EGF	p=0.123; r=–0.667
IC_{50} *versus* pSTAT3/tSTAT3 expression	Without EGF	p=0.179; r=0.605
	With EGF	p=0.458; r=0.369
Pearson correlation coefficient after treatment		
IC_{50} *versus* % down-regulation of pSTAT3 to control	Without EGF	p=0.0029; r=–0.9236
	With EGF	p=0.0040; r=–0.9145

dependent genes before and after 24 h exposure to S3I-201 in these six osteosarcoma cell lines by western blot. Our data showed that survivin (a member of the inhibitor of apoptosis family of anti-apoptotic proteins) was positively expressed in all six osteosarcoma cell lines and cyclin D1 (a member of the cyclin proteins family that are involved in regulating cell cycle progression) in five of them except for Saos-2, which did not express cyclin D1. S3I-201 suppressed cyclin D1 in all those 5 osteosarcoma cell lines with positive cyclin D1 expression (% down-regulation to relevant control: 143B: 43%; HOS: 46%; MG63: 61%, SJSA: 38% and U2OS: 74%) as shown in Figure 5. Treatment of S3I-201 also resulted in reduction of survivin in 5 out of 6 osteosarcoma cell lines (% down-regulation to relevant control: HOS: 24%, MG63: 54%, SJSA: 38%, U2OS: 38% and Saos-2: 49%).

Discussion

Our study demonstrated a high incidence of STAT3 tyrosine phosphorylation in osteosarcoma cell lines. Targeting STAT3 using STAT3 inhibitor S3I-201 in osteosarcoma cell lines showed significant inhibition of cell growth and colony formation, as well as apoptosis enhancement *via* the caspase-3 pathway *in vitro*. Our results also demonstrated that treatment with S3I-201 inhibited STAT3 phosphorylation, and the degree of inhibition was correlated with the extent of cell growth inhibition. We also found that reduction of STAT3-dependent genes encoding cyclin D1 and survivin may mediate the effect of S3I-201-induced down-regulation of STAT3 signalling pathways. To clarify the effect of growth factor EGF stimulation on osteosarcoma cell lines, we included EGF stimulation in this study. Western blot data showed that EGF did not have any significant reproducible effect on basic levels of STAT3 phosphorylation or the alteration of protein markers after S3I-201 treatment in osteosarcoma cell lines.

We showed STAT3 tyrosine phosphorylation in all six osteosarcoma cell lines, which is consistent with similar discoveries in a broad range of cancers and other sarcomas (19-22). In several studies of patient tissues, elevated STAT3 phosphorylation was reported in 27% of rhabdomyosarcoma, 15-34% of soft tissue sarcomas and 19% of osteosarcoma, and was significantly associated with clinicopathological parameters (19, 23-25). This overexpression of activated STAT3 in human tumour tissue together with our findings *in vitro* that STAT3 was expressed, functional and activated in osteosarcoma cell lines suggests that inhibition of STAT3 is a rational therapeutic target in osteosarcoma.

Various approaches have been developed to target the STAT3 pathway (9, 12, 26-33). Although some of these have been tested in both pre-clinical models and clinical trials (9) in variety of human cancers, there are limited reports on osteosarcoma due to the low incidence of this disease. We therefore investigated the role of inhibition of activated STAT3 signalling using STAT3 inhibitor S3I-201. S3I-201 was identified from the National Cancer Institute chemical libraries and inhibited STAT3 dimerization and STAT3 DNA-binding and transcriptional activates (16). Confirmed by experimental analysis using a STAT3 DNA-binding assay and electrophoretic mobility shift assay (EMSA) analysis, S3I-201 specifically inhibited STAT3 DNA-binding activity (IC_{50}: STAT3-STAT3, 86±33 μM), rather than STAT1 (STAT1-STAT1, >300 μM) and STAT5 (166±17 μM) (16). S3I-201 at 100 μM was selected as the cutoff concentration to evaluate the growth inhibitory effect

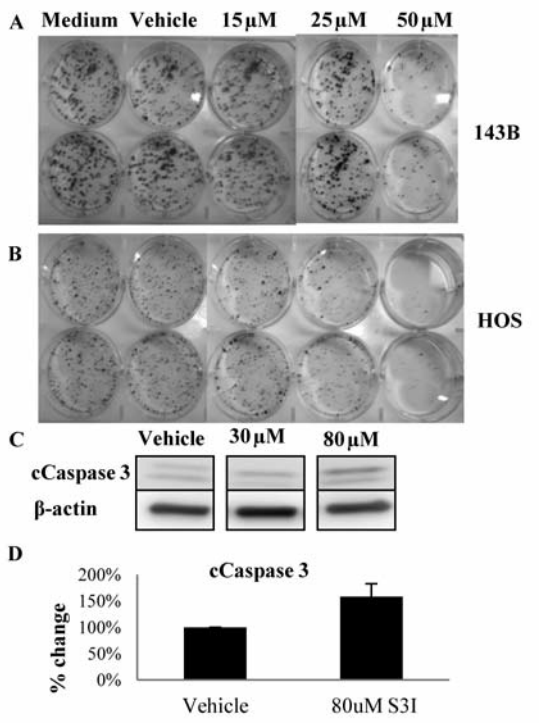

Figure 3. *S3I-201 induced anti-colony formation and apoptosis through caspase-3 pathway on osteosarcoma cell lines. The 143B (A) and HOS (B) cells were treated with vehicle and different doses of S3I-201. Experiments were duplicated, with duplicate samples within each experiment. (C) After starving 24 h, the osteosarcoma cell line Saos-2 was treated with vehicle (DMSO), 30 μM and 80 μM S3I-201 for 24 h, and then were lysed for western blot to analyze expression levels of cleaved caspase-3 (cCaspase-3). (D) Expression levels of cCaspase-3 were quantified using the ImageQuant software, normalized to β-actin and shown as percentage change (%) to vehicle control after cells treated with 80μM S3I-201. Error bars represent standard error of means from duplicate experiments.*

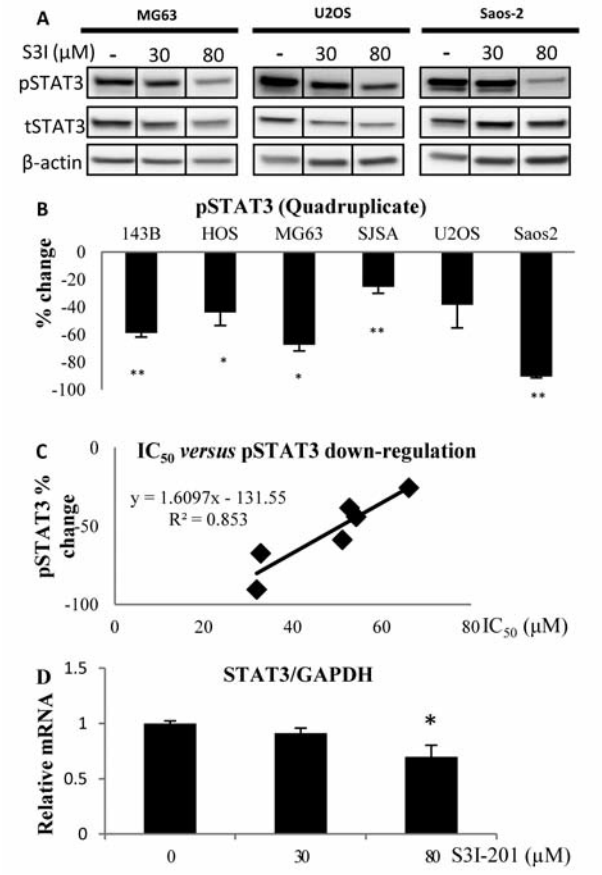

Figure 4. *S3I-201 suppressed STAT3 phosphorylation and mRNA levels in osteosarcoma cell lines. (A) 24-h starved osteosarcoma cells were treated with vehicle (DMSO), 30 μM and 80μM S3I-201 for 24 h. Then, cells were lysed and subjected to western blot to analyze expression levels of phosphorylated (pSTAT3) and total STAT3 (tSTAT3). Representative images are shown here. (B) Expression levels of pSTAT3 were quantified using the ImageQuant software. pSTAT3 was normalized to β-actin and was shown as percentage down-regulation (%) to relevant control after osteosarcoma cells treated with 80μM S3I-201. Error bars show standard error of means from quadruplicate experiments. (C) xy scatter plot was plotted to show the correlation between IC50 and pSTAT3 down-regulation. (D) 24 h treatment with S3I-201 inhibited STAT3 mRNA levels in MG63 by quantitative real-time PCR assay. *p<0.05, **p<0.01.*

in most previous studies (16-18). In addition, S3I-201, as a single agent, reduced cell proliferation and viability and induced apoptosis in human breast cancer and hepatocellular carcinoma cell lines that expressed pSTAT3 both *in vitro* and in xenografts (16, 34). To our knowledge, S3I-201 has not yet been evaluated in osteosarcomas.

In our panel of six osteosarcoma cell lines, S3I-201 demonstrated significant inhibition of both cell growth and colony formation. We discovered a strong inverse correlation between IC_{50} and the down-regulation of pSTAT3 in osteosarcoma cell lines. This relationship suggested that inhibition of osteosarcoma cell growth by S3I-201 was due to the specific blocking of STAT3 phosphorylation pathway. STAT3 pull-down assays have previously demonstrated that S3I-201 specifically blocked STAT3-STAT3 dimerization and thus prevent *de novo* phosphorylation and activation of STA3 monomer (16).

We found that S3I-201 induced apoptosis by increasing caspase-3 cleavage, which is in agreement with studies targeting STAT3 pathway in prostate cancers using dnSTAT3 plasmid and antisense STAT3 oligonucleotide (35, 36), as well as in endometrial and cervical cancers using dnSTAT3 and a JAK/STAT3 inhibitor JSI-124 (37). Caspase, a family of aspartate-specific cysteine proteases, including apoptosis activator (caspase-2, 8, 9 and 10) and apoptosis executioner (caspase-3, 6 and 7), are situated at pivotal junctions in apoptosis pathways (38). The involvement of both caspase-8

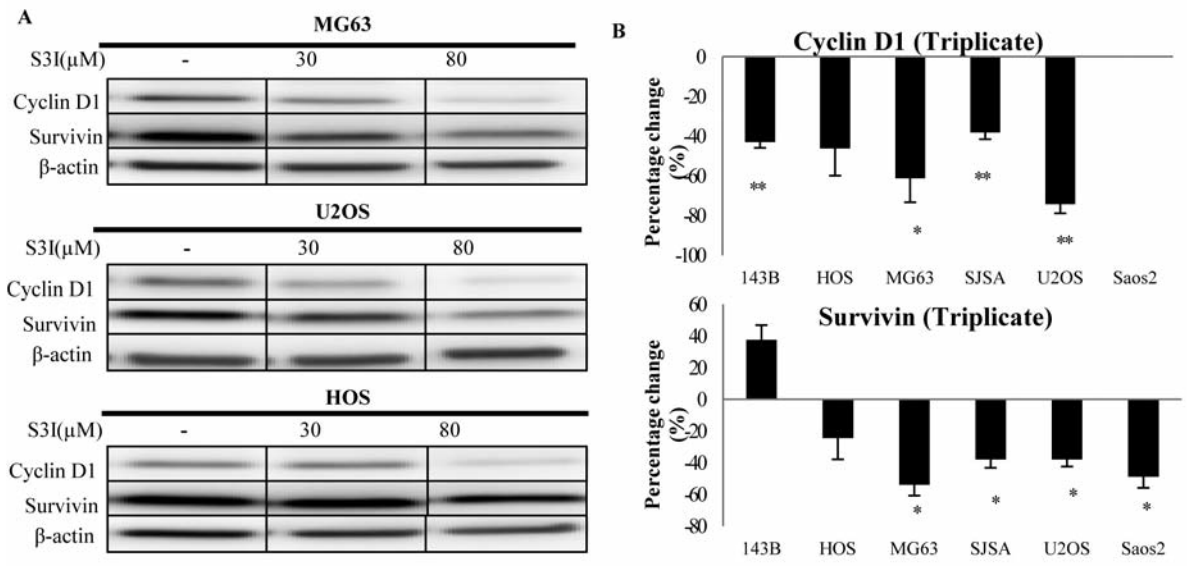

Figure 5. *S3I-201 regulated protein expression of STAT3-dependent genes in osteosarcoma cell lines. (A) 24-h starved osteosarcoma cells were treated with vehicle (DMSO), 30 μM and 80 μM S3I-201 for 24 h. Then, cells were lysed and subjected to western blot to analyze expression levels of cyclin D1 and survivin. Representative images are shown. (B) Expression levels of the indicated proteins were quantified using the ImageQuant software. Protein expression levels after osteosarcoma cells exposure to 80 μM S3I-201 were normalized to β-actin and shown as percentage change (%) to relevant control. Error bars represent standard error of means from triplicate experiments. *p<0.05, **p<0.01.*

and 9 pathways has been reported along with dnSTAT3, expression leading to induction of apoptosis that ultimately resulted in downstream caspase-3 cleavage (19). These specific RNA inhibition studies support our contention that this effect on the apoptosis pathway is a specific on target effect of S3I-201 and collectively support the notion that STAT3 plays a role in osteosarcoma cell survival through an anti-apoptotic pathway.

Activated STAT3 is known to regulate many downstream target genes associated with uncontrolled proliferation and defective apoptosis (9). We selected two key STAT3-regulated genes to demonstrate the effect of S3I-201 on transcription. Increased expression of downstream genes cyclin D1 (up-regulation of cell proliferation) and survivin (prevention of apoptosis) has been observed in endometrial, cervical and head and neck in association with elevated pSTAT3 (37, 39). In our current study, survivin was expressed in 6/6 (100%) osteosarcoma cell lines and cyclin D1 in 5/6 (83%), both proteins were suppressed by STAT3 inhibition in the majority of the osteosarcoma cell lines. Similar results using other small-molecular STAT3 inhibitors, LLL12 and STX-0119, in a broad range of other human cancer and osteosarcoma cell lines also down-regulated the same STAT3-related genes, cyclin D1 and survivin, at both mRNA and protein levels (40-45). Collectively these findings suggest that the inhibitory effects on these downstream target genes may be the molecular mechanism for the inhibition of cell proliferation and induction of apoptosis seen in our panel of osteosarcoma cell lines.

In conclusion our current study on the anti-proliferative and pro-apoptotic effects of S3I-201 through inhibition of the JAK/STAT signaling pathway and its downstream effectors, proliferation-related cyclin D1 and anti-apoptosis-related survivin, gives support to the potential role of STAT3 inhibition in osteosarcoma treatment.

Conflicts of Interest

The Authors declare no conflicts of interest.

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