Abstract. Signal transducers and activators of transcription 3 (STAT3) signaling is persistently activated in many types of cancer cells, and represents a valid target for anticancer drug design. However, few reports have described the constitutive activation of STAT3 in human sarcoma cells. In this study, we demonstrate that the STAT3 signaling pathway is constitutively activated in human rhabdomyosarcoma cells (RH28, RH30, and RD2). We also investigated the inhibitory effects of two newly developed small molecules, LLL12 and FLLL32, on the STAT3 signaling pathway in human rhabdomyosarcoma cells. Both LLL12 and FLLL32 downregulated STAT3 constitutively and interleukin-6 (IL-6) stimulated phosphorylated STAT3 (p-STAT3). The inhibition of STAT3 via LLL12 and FLLL32 was confirmed by the inhibition of STAT3 DNA binding activity. The downstream targets of STAT3, cyclin D1, Bcl-xL, and survivin were also downregulated by LLL12 and FLLL32 at both messenger RNA and protein levels. The potency of LLL12 and FLLL32 to inhibit proliferation/viability in human rhabdomyosarcoma cells (RH28, RH30, and RD2) was higher than that of the 5 previously reported Janus kinase 2 (JAK2)/STAT3 inhibitors (LLL3, WP1066, Static, S3I-201, and AG490) and curcumin. Thus, in this study, we investigated the inhibitory effects of two STAT3 inhibitors, LLL12 and FLLL32, on the STAT3 signaling pathway in human rhabdomyosarcoma cells; we also demonstrated their higher potency in inhibiting proliferation on human rhabdomyosarcoma cells as compared to other five JAK2/STAT3 inhibitors and curcumin.

Introduction

Rhabdomyosarcoma, a highly malignant mesenchymal tumor, originating from immature striated muscle, is the most common soft tissue sarcoma in children (1). Patients with rhabdomyosarcoma have a high relapse rate of 30% (1,2). Despite aggressive therapy, incorporating surgery, radiation therapy and dose-intensive chemotherapy, mortality rate of relapsing rhabdomyosarcoma is still 50-80% (2,3). Thus, development of higher potency and less toxicity anticancer drugs is pivotal to improve survival for patients with rhabdomyosarcoma.

Signal transducers and activators of transcription 3 (STAT3) signaling is constitutively activated in various types of cancer cells, such as breast cancer, prostate cancer, leukemia and lymphomas (4). Activated STAT3 binds the promoters of the targeted genes to control cell growth and survival. Thus, inhibition of STAT3 signaling pathway seems to be an attractive strategy for anticancer drug design (5,6). For the past few years, several small molecules of the JAK2/STAT3 inhibitors, such as WP1066 (7-9), LLL3 (10) and AG490 (11-13), Static (14,15) and S3I-201 (16,17), have been reported with a wide range of antitumor effects. Although persistent activation of STAT3 has been detected in diverse cancer cell lines and tissue, only few reports have described elevated p-STAT3 in rhabdomyosarcoma.

Two small molecule compounds, LLL12 and FLLL32, exhibit potent inhibitory activity on STAT3 in human rhabdomyosarcoma cells

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Received August 10, 2010; Accepted October 22, 2010

DOI: 10.3892/ijo_00000848

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Abbreviations: STAT3, signal transducers and activators of transcription 3; JAK2, Janus kinase 2; IL-6, interleukin-6; DMEM, Dulbecco’s modified Eagle’s medium; PARP, Poly(ADP-ribose) polymerase; pY705, phosphoryl tyrosine 705

Key words: signal transducers and activators of transcription 3, IL-6, rhabdomyosarcoma
In this study, we present two newly developed small molecule STAT3 inhibitors, LLL12 and FLLL32, and analyzed their effects on reducing proliferation and inducing apoptosis, compared to the 5 previously reported JAK2/STAT3 inhibitors (LLL3, WP1066 and AG490, Statistic, and S31-201) and curcumin in human rhabdomyosarcoma cells (RH28, RH30 and RD2). Both LLL12 and FLLL32 displayed high potency in inhibiting proliferation/viability over the other 6 compounds on human rhabdomyosarcoma cells.

Materials and methods

Antibodies and reagents. STAT3 inhibitors, LLL3, LLL12 and FLLL32, were synthesized in the Laboratory of Dr Pui-Kai Li (College of Pharmacy, The Ohio State University). The powder was dissolved in sterile dimethyl sulfoxide (DMSO) to make a 20 mmol/l stock solution. Aliquots of the stock solution were stored at -20°C. STAT3 inhibitors (S31-201 and Statistic), JAK2 inhibitors (AG490 and WP1066) and curcumin were purchased from Calbiochem.

Cell lines and culture. Human rhabdomyosarcoma cell lines (RD2) was purchased from American Type Culture Collection. Human rhabdomyosarcoma cell lines (RH28 and RH30) were gifts from Peter Houghton. All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin/amphotericin B (Fisher Scientific International) in a 5% CO2 incubator at 37°C.

MTT cell viability assay. Approximately 3,000 cells of RH28, RH30, and RD2 were seeded in 96-well plates (3,000 cells/well) in triplicates with 100 μl 10% FBS-supplemented DMEM medium overnight. Each cell line was treated with target medications with a series of concentrations for 72 h. Cell viability was analyzed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) assay in three replicates. At the end-point, 25 μl of MTT was added to each well of the plate and incubated for 3.5 h. Cells were treated with MTT (1 mg/ml) for 3-4 h and then 100 μl of N,N-dimethylformamide (DMSO) was added to each well. Colorimetric quantification was determined by an EL808 Ultra Micro-plate Reader (Bio-Tek Instruments, Inc.) after the addition of formazan dissolved in 25% N,N-dimethylformamide and 10% SDS under light-proof conditions overnight. Microsoft excel was used to analyze the cell viability data. The viability of the untreated cells was arbitrarily set at 100% and compared with the viability of cells treated with LLL12, FLLL32, selected JAK2/STAT3 inhibitors (LLL3, WP1066 and AG490, Statistic, and S31-201), and curcumin. Error bars represent one standard deviation. Half-maximal inhibitory concentrations (IC50) were calculated using Sigma Plot 9.0 software (Systat Software Inc.) using the 4 parameter logistic function standard curve analysis for dose response.

Western blot analysis. Rhabdomyosarcoma cells (RH28, RH30, and RD2) were treated with LLL12 or FLLL32 at a concentration of 5 μM or 10 μM or a DMSO vehicle control for 24 h. For Western blotting, 60 μg of total cell lysate was subjected to SDS polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membrane. The membranes were blocked with 5% non-fat dry milk in TBST for 30 min and then blotted overnight with the following antibodies purchased from Cell Signaling Technologies: phospho-STAT3Y705 antibody (#9131); STAT3 antibody (#9132); cleaved Poly(ADP-ribose) polymerase (PARP) antibody (#9546); cleaved caspase-3 antibody (#9661) and GAPDH antibody (#2118). Membranes were analyzed with enhanced chemiluminescence Plus reagents (GE Healthcare) and scanned with a Storm PhosphorImager (Amersham Pharmacia Biotech Inc.).

IL-6 induction of STAT3 phosphorylation. Rhabdomyosarcoma (RH28) cells were seeded in 10-cm plates and allowed to adhere overnight. The following night, the cells were serum-starved. The cells were then left untreated or were treated with LLL12 (5 and 10 μM), FLLL32 (10 and 20 μM), or DMSO. After 3 h, the untreated and LLL12-treated cells were stimulated by IL-6 (10 ng/ml). The cells were harvested at 30 min and analyzed by Western blotting.

DNA binding activity. Rhabdomyosarcoma cells (RH30) at 60-80% confluence were treated with LLL12 (2.5 or 5 μM), 5 μM FLLL32, or DMSO in the presence of 10% FBS for 24 h. The nuclear extracts were analyzed for STAT3 DNA binding activity using STAT3 Transcription Factor Kits following the manufacturer's protocol (Clontech, Inc.).

RT-PCR analysis. RNA was collected from human rhabdomyosarcoma cells (RH28, RH30, and RD2) with RNeasy Kits (Qiagen) following 24 h of treatment with LLL12 and FLLL32 at concentration of 10 μM. cDNA was generated from 500 ng sample RNA using Omniscript RT (Qiagen). Two μl of cDNA was subsequently used for PCR. Primer sequences were as follows: (forward/reverse sequences) Bcl-xL (5'-TTGGACAAATGAGCCTTGTGA/TTAGAGTTGGATTGCAGTG-3'); Cyclin D1 (5'GCTGGAGCCCGTGTAAAGA/CTCCGGCTTCTGCACTTTTGT-3'); Survivin (5'-ACCAGGTTGAGGAAGGTTGACAGTGAGGGA/AACAGTGAGGACGAGGGA-3'); GAPDH (5'-TGATGACATGAGGATGGGT-3'); GAPDH (5'-TTGGACAATGGACTGGTTGA/GTAGAGTGGATGGTCAGTG-3'). Amplifications were performed as follows: 5 min at 94°C followed by 25 cycles of (30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C) and a final extension at 72°C for 5 min. The PCR products were then run on 2% agarose gels, stained with ethidium bromide and visualized under UV light.

Results

LLL12, a STAT3 inhibitor. Phosphoryl tyrosine 705 (pY705) is crucial for the dimerization of STAT3 (18-20). Using a structure-based drug design, we developed a compound, LLL12 (Fig. 1A), which interferes with STAT3 dimerization by competitively binding to pY705. The phosphoryl tyrosine 705 is located on a loop segment of the SH2 domain and binds with several adjacent amino acid residues (leucine 706, threonine 708, and phenylalanine 710) to a cavity on the SH2 domain of the other STAT3 monomer. To optimize potency and selectivity, the main scaffold of LLL12 contains fragments
that directly contact the pY705 binding site of STAT3. A simulated docking model shows that the carboxylic and sulfonamide tails of LLL12 occupy the pY705 binding pocket of STAT3. The simulated binding energies of LLL12 to STAT3 predict it to be a potent inhibitor of the constitutive STAT3 pathway. The detail of LLL12 synthesis was described in our previous report (21).

FLLL32, a JAK2/STAT3 inhibitor. FLLL32 is a diketone analogue of curcumin (Fig. 1B). The central ß-dicarbonyl moiety of curcumin is subjected to keto-enol tautomerization, which is hypothesized to influence its target selectivity by virtue of its biological activity. Replacing the 2 hydrogen atoms on the central carbon of curcumin with a spiro-cyclohexyl ring in FLLL32, eliminated the ability of curcumin to enolize. These FLLL32 modifications are predicted to make it interact better with key binding sites of JAK2 and the SH2 domain of STAT3 than the keto-enol form of curcumin (22).

In addition, FLLL32 is characterized as a 3,4-dimethoxy substituent that mimics dimethoxycurcumin, which has shown increased stability, higher plasma concentration, and greater efficacy against cancer cells than standard curcumin (22).

Inhibition of cell viability by LLL12 and FLLL32 in human rhabdomyosarcoma cells. STAT3 activation is important for tumor cell proliferation and survival (19,23). We observed the total cell number reduction of human rhabodomyosarcoma cells (RH30 and RD2) after treatment with LLL12 5 μM or FLLL32 5 μM for 24 h.

LLL12 and FLLL32 inhibited STAT3 phosphorylation and induced apoptosis in human rhabdomyosarcoma cells. Firstly, we detected constitutively activated STAT3 in human

Figure 1. (A) The chemical structure of (A) LLL12 and (B) FLLL32.

Figure 2. Inhibition of cell proliferation on human rhabdomyosarcoma cells (RH30 and RD2) was observed under phase contrast microscope (x100) after treatment with LLL12 5 μM or FLLL32 5 μM for 24 h.
rhodomyosarcoma cells (RH28, RH30, and RD2) by Western blotting with GAPDH as an internal control and IFN-γ stimulated HeLa cells as a positive control (data not shown). Then, RH28, RH30, and RD2 cells were separated into the first 3 groups treated with DMSO, 5 μM LLL12 and 10 μM LLL12 and into further 3 groups treated with DMSO, 5 μM FLLL32 and 10 μM FLLL32. Protein samples were collected for Western blot analysis after treatment for 24 h. Both LLL12 and FLLL32 inhibited STAT3 phosphorylation at tyrosine residue 705 (Tyr 705) in the 3 cells lines. Upregulation of cleaved PARP and cleaved caspase-3 suggested that inhibition of STAT3 phosphorylation induced apoptosis (Fig. 4). Combined with cell viability assay data, LLL12 and FLLL32 not only suppressed cell growth/viability of rhodomyosarcoma cells expressing constitutively active STAT3 but also induced cell apoptosis resulting a reduction of total cell number.

LLL12 and FLLL32 inhibited STAT3 phosphorylation induced by IL-6. STAT3 activation can be induced by IL-6 (24-26). Human rhodomyosarcoma (RH28) cells were used to determine if LLL12 and FLLL32 can inhibit IL-6-induced STAT3 phosphorylation. We found that the basal levels of STAT3 phosphorylation are high even at serum-free medium for 24 h. IL-6 slightly increased STAT3 phosphorylation in RH28 cells and the increasing was blocked by LLL12, and was blocked to a lesser degree by FLLL32 because FLLL32 is diketone analogues of curcumin, which is less soluble in serum-free medium (22). This inhibitory effect was dose-dependent (Fig. 5).

LLL12 and FLLL32 inhibited STAT3 DNA binding. To confirm the inhibition of STAT3 signaling by LLL12 and FLLL32, we examined the inhibition of STAT3 DNA binding activity. Both LLL12 and FLLL32 caused a statistically significant inhibition of STAT3 DNA binding activity in human rhodomyosarcoma cells (RH30) (Fig. 6).

LLL12 and FLLL32 inhibited the transcription of downstream targets of STAT3 on human rhodomyosarcoma cells. To further analyze the mechanism of inhibition of STAT3 by LLL12 and FLLL32 on the inhibition of STAT3, we examined the transcription of downstream target genes of STAT3, such as cyclin D1, survivin, and Bcl-xL, by reverse transcriptase

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**Figure 3.** (A) LLL12 and (B) FLLL32 inhibited proliferation/viability of human rhabdomyosarcoma cells (RH28, RH30 and RD2), as determined by the MTT assay. A dose-dependent inhibition in tumor cell proliferation/viability was observed after 72-h treatment.

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**Figure 4.** Western blot analysis showed that LLL12 and FLLL32 inhibited STAT3 phosphorylation on human rhabdomyosarcoma cells (RH28, RH30, and RD2) and induced apoptosis through the induction of cleaved caspase-3 and cleaved PARP.
PCR (RT-PCR). STAT3 is known to upregulate cyclin D1, which promotes cell cycle progression and growth factor independence, and to upregulate Bcl-xL and survivin, which inhibit apoptosis. We treated human rhabdomyosarcoma cells (RH28, RH30, and RD2) with FLLL32 (10 μM), LLL12 (10 μM), or DMSO for 24 h. We found that LLL12 or FLLL32 resulted in downregulation of transcription of STAT3-regulated genes, cyclin D1, survivin, and Bcl-xL in 3 cell lines (Fig. 7).

LLL12 and FLLL32 showed higher potency in inhibiting proliferation/viability compared to selected JAK2/STAT3 inhibitors and curcumin in human rhabdomyosarcoma cells. We analyzed the inhibitory effects of LLL12 and FLLL32 on proliferation/viability and compared their inhibitory effects with other previously reported JAK2/STAT3 inhibitors (LLL3, WP1066, Stattic, S3I-201, and AG490) and curcumin in human rhabdomyosarcoma cells (RH28, RH30 and RD2). A dose-dependent inhibition in cell proliferation/viability was observed after 72-h of treatment. Based on IC50 values calculated for LLL12, FLLL32, and other JAK2/STAT3 inhibitors (Table I), both LLL12 and FLLL32 showed superior growth suppressive activity compared to other JAK2/STAT3 inhibitors (LLL3, WP1066, Stattic, S3I-201, and AG490) and curcumin on RH28, RH30, and RD2.

Discussion

To our knowledge, this is the first study to demonstrate the inhibitory effects of 2 newly developed small molecule compounds, LLL12 and FLLL32, on STAT3 signaling pathway in human rhabdomyosarcoma cells (RH28, RH30 and RD2). Both LLL12 and FLLL32 presented more potent antiproliferative effects than the 5 previously reported drugs targeting the JAK2/STAT3 signaling pathway (LLL3, WP1066, Stattic, S3I-201, and AG490) and curcumin in human rhabdomyosarcoma cells. Our study is one of the few showing the constitutive activation of STAT3 in human rhabdomyosarcoma cell lines as well as demonstrating the effects of reducing proliferation and inducing apoptosis by treating these cells with STAT3 inhibitors.

Recent studies have successfully demonstrated the role of aberrant STAT3 in malignant transformation and tumorigenesis (19,23,27). Activation of the upstream cytokine and growth factor receptors induces STAT3 phosphorylation at tyrosine residue 705, dimerization through reciprocal SH2 domains, and translocation into the nucleus. Then, phosphorylated STAT3 binds target genes to control cell growth and survival (28). Growing evidence from both cellular and animal systems
has shown that the inhibition of constitutively active STAT3 induces apoptosis in cancer cells (29-32). STAT3 has such crucial roles in human cancer formation as to be a valid target for novel anticancer drug design (28,33). Although various compounds have been reported to inhibit the STAT3 signaling pathway, such as DNA decoys, dominant-negative STAT3 mutants, STAT3 small interfering RNAs (siRNAs), or antisense STAT3 oligonucleotides, the development of smaller and more effective compounds by either manipulating the potency or improving the pharmacokinetics by reducing the molecule weight is a feasible strategy in drug design (34). In addition, targeting central protein directly to avoid adverse effects is also important in developing new anticancer agents. Several target sites have been reported in designing STAT3 inhibitors. Selective inhibition of JAK2 can prevent STAT3 phosphorylation and dimerization. Several JAK2 inhibitors, such as AG490, WP1066, and SD-1029 have been reported in recent studies (7-13). Selective competition of SH2 domain which is crucial for STAT3 dimerization can disrupt STAT3 signaling. Several non-peptide small molecule SH2 inhibitors, such as Static and S3I-201, have also been recently reported (14,16).

We have been developing a series of compounds that competitively bind to pY705 of STAT3 and then interfere with STAT3 dimerization and activation. Interestingly, LLL2 has been the most potent inhibitor of the STAT3 signaling pathway among these compounds. Our previous findings have shown the inhibitory effects on STAT3 activation and cell proliferation of some of these series compounds on different cancer cells, such as STA-21 on human breast, prostate, and bladder cancers (35-37), and LLL3 on human prostate cancer and glioblastoma (10,36). The main scaffold of LLL3 only binds to the side pocket in close proximity to the pY705 binding site of STAT3 monomer but does not bind directly to the dimerization site, pY705. To optimize the potency and selectivity, we further design a compound attached to the main scaffold that contact pY705 (LLL2; Fig. 1A). The simulated docking mode of LLL12 is similar to LLL3 but with the carboxylic and sulfonamide tails occupying the pY705 binding pocket of STAT3. For simulated binding energies to STAT3, LLL12 is 10-fold better than LLL3 and is predicted to be more potent than LLL3 in inhibiting constitutive STAT3 pathway. Recently, we reported the potent inhibition of STAT3 phosphorylation and growth-suppression of LLL12 in human breast cancer and glioblastoma cells (21).

A newly developed small molecule STAT3 inhibitor, FLLL32, has recently been developed from curcumin in our laboratory. Curcumin (diferuloylmethane) is a leading compound of perennial herb Curcuma longa (22). Almost 3,000 studies carried out with curcumin suggest that this natural agent has a diverse range of molecular targets, including transcription factors, growth factors, cytokines, enzymes, and genes regulating cell proliferation and apoptosis (38-45). The complex effects of curcumin allow it to inhibit multiple oncogenic processes, including those associated with the JAK2/STAT3 pathway. Although curcumin is pharmacologically safe in humans based on nearly 40 clinical trials, its low bioavailability is a concern. FLLL32 shares many of the bioactive properties of curcumin based on their similar structures. Designed to preferentially interact with both the SH2 domain of STAT3 and its upstream activator JAK2, FLLL32 has greater stability and higher selectivity to critical domains in JAK2 and STAT3 compared to curcumin. We also demonstrated the potent inhibition of STAT3 phosphorylation and growth-suppression of FLLL32 in human breast and pancreatic cancer cells (21).

In this study, constitutive activation of STAT3 was observed in human rhabdomyosarcoma cells, and inhibition of STAT3 activity reduced proliferation and induced apoptosis on these cells. Although STAT3 is found to be constitutively activated in human tumors, reports on activated STAT3 in sarcoma are rare. One study showed nearly 50% Ewing sarcoma tissue presenting STAT3 activation (46). We recently analyzed sarcoma tissue microarray slides and sarcoma cell lines and found elevation of activated STAT3 detected in approximately 25% sarcoma cells, including human rhabdomyosarcoma, osteosarcoma, and other soft tissue sarcomas (47). We also treated the human rhabdomyosarcoma cells with a low molecular weight compound STA-21 or block STAT3 signaling by dominant-negative STAT3 mutant, both of which induced rhabdomyosarcoma cell growth inhibition and apoptosis (37,47). Persistently activated STAT3 was also found in canine osteosarcoma (48). Our current study extended previous findings to show that blocking constitutive STAT3 activation in rhabdomyosarcoma cells inhibited cell growth and induced apoptosis through newly developed small molecule inhibitors and other JAK2/STAT3 inhibitors. Furthermore, LLL12 and FLLL32 inhibited the expression of the STAT3-regulated genes encoding cyclin D1, Bcl-xL, and survivin and inhibited the growth of human rhabdomyosarcoma cells in vivo (5).

In conclusion, the 2 small-molecular STAT3 inhibitors, LLL12 and FLLL32, have higher potency in inhibiting tumor growth compared to the 5 previously reported JAK2/STAT3 inhibitors (7-13). Selective competition of SH2 domain which is crucial for STAT3 dimerization can disrupt STAT3 signaling. Several non-peptide small molecule SH2 inhibitors, such as Static and S3I-201, have also been recently reported (14,16).

Table 1. Half-maximal inhibitory concentrations (IC₅₀) (µM) obtained for LLL12, FLLL32, JAK2/STAT3 inhibitors (LLL3, WP1066, Static, S3I-201, and AG490), and curcumin in human rhabdomyosarcoma cells (RH28, RH30, and RD2).

<table>
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All values reflect concentrations calculated after 72-h treatment in a MTT viability assay.
inhibitors and curcumin on rhabdomyosarcoma cells (RH28, RH30 and RD2). Further in vivo mouse tumor model, pharmacodynamic, and pharmacokinetic studies should be conducted to explore the future clinical potential of STAT3 inhibitors in rhabdomyosarcoma cells harboring aberrant STAT3 signaling.

Acknowledgements

This study was funded by China Medical University Hospital (DMR-94-023).

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