Overcoming Chemoresistance of Pediatric Ependymoma by Inhibition of STAT3 Signaling

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Abstract
The long-term clinical outcome of pediatric intracranial ependymoma is poor with a high rate of recurrence. One of the main reasons for this poor outcome is the tumor’s inherent resistance to chemotherapy. Signal transducer and activator of transcription 3 (STAT3) is overactive in many human cancers, and inhibition of STAT3 signaling is an emerging area of interest in oncology. In this study, the possibility of STAT3 inhibition as a treatment was investigated in pediatric intracranial ependymoma tissues and cell lines. STAT3 activation status was checked in ependymoma tissues. The responses to conventional chemotherapeutic agents and a STAT3 inhibitor WP1066 in primarily cultured ependymoma cells were measured by cell viability assay. Apoptosis assays were conducted to reveal the cytotoxic mechanism of applied agents. Knockdown of STAT3 was tried to confirm the effects of STAT3 inhibition in ependymoma cells. High levels of phospho-STAT3 (p-STAT3) expression were observed in ependymoma tissue, especially in the anaplastic histology group. There was no cytotoxic effect of cisplatin, ifosfamide, and etoposide. Both brain tumor-initiating cells (BTICs) and bulk tumor cells (BCs) showed considerably decreased viability after WP1066 treatment. However, BTICs had fewer responses than BCs. No additive or synergistic effect was observed for combination therapy of WP1066 and cisplatin. WP1066 effectively abrogated p-STAT3 expression. An increased apoptosis and decreased Survivin expression were observed after WP1066 treatment. Knockdown of STAT3 also decreased cell survival, supporting the critical role of STAT3 in sustaining ependymoma cells. In this study, we observed a cytotoxic effect of STAT3 inhibitor on ependymoma BTICs and BCs. There is urgent need to develop new therapeutic agents for pediatric ependymoma. STAT3 inhibitors may be a new group of drugs for clinical application.

Introduction
Ependymoma is an intra-axial brain tumor that predominantly develops in young children. In the histological grading system of brain tumors proposed by the World Health Organization (WHO), ependymomas correspond to either grade 2 (classic) or grade 3 (anaplastic variant) [1]. However, the prognosis of ependymoma is dire despite its modest histological grade. Long-term survival rates of pediatric ependymoma patients range from 50 to 70% [2–5]. In a recently published analysis of our patients’ outcome, we observed a high rate of tumor recurrence (73% of patients). The five-year overall survival rate was only 71% [6]. The poor treatment outcome of
ependymoma patients urges clinicians to speculate on the legitimacy of current treatment protocols.

Current treatment protocols for intracranial ependymoma consist of a maximal surgical resection followed by local radiation therapy (RT). Ependymoma is intrinsically resistant to chemotherapy and currently no reliable and uniform chemotherapy regimen exists for this tumor. RT has a moderate effect on tumor control of intracranial ependymomas and most treatment protocols advocate early postoperative RT. However, the average age of ependymoma patients is younger than that of medulloblastoma. In our previous study, 42% of patients were under the age of three at diagnosis—the conventional age threshold to allow safe delivery of high-dose radiation to developing brains [6]. Recently, clinicians in the St. Jude Children’s Research Hospital reported that early application of conformal RT, even to patients under one year, could raise the local control rate of intracranial ependymoma to over 80% [7]. This should be considered as a true improvement, but RT for such a young-age group needs to be more sophisticated in application. In this context, development of a new therapeutic agent can provide a robust means to prevent surgical complications, to postpone RT until brain maturation, and to enhance the survival of the children.

Signal transducer and activator of transcription 3 (STAT3) is a crucial intracellular signal transducer that, once activated by various growth factors and cytokines, binds to DNA and guides specific gene expression [8]. STAT3 protein expression has been known to be elevated in many kinds of human cancers [9]. Furthermore, in some cancers such as colorectal, ovarian, and renal cell carcinomas, high STAT3 expression was associated with poor prognosis of patients [10–12]. Especially, STAT3 may play a role in augmenting chemo-resistance in many types of human cancers [13–15]. Many kinds of STAT3 inhibitors have been developed [8,9]. WP1066 is a potent inhibitor of JAK2 and its downstream STAT3 pathways. WP1066 showed marked suppressive activities on many type of potential inhibitor of JAK2 and its downstream STAT3 pathways. Many STAT3 inhibitors for pediatric ependymoma.

During the last decade, tumor-initiating cells have been found in many solid cancers including brain tumors [19]. These tumor-initiating cells have demonstrated stem cell–like characteristics in gene expression, sphere-formation in specialized culture media, and serial tumor formation after transplantation into immune-deficient mice. Tumor-initiating cells may have an important role in chemoresistance, tumor dormancy, and metastasis [20,21]. Interestingly, in colon cancer cells, ALDH+/CD133+ tumor-initiating cells showed higher STAT3 activation levels and these cells were sensitive to STAT3 inhibition [22]. Brain tumor–initiating cells (BTICs) are also found in ependymoma [23]. In this study, the potential of a STAT3 inhibitor, WP1066, to suppress ependymoma cell growth and to overcome chemo-resistance was evaluated in the context of BTICs and bulk tumor cells (BCs) to test the therapeutic potentials of STAT3 inhibitors for pediatric ependymoma.

Materials and Methods

Human Brain Tissues

Fresh human brain tumor specimens were collected from patients undergoing respective surgery at the Seoul National University Children’s Hospital. Eligible patients or their parents provided written informed consent to provide the tumor tissues. The institutional review board of the Seoul National University Hospital approved the tissue banking and this study protocol. None of the patients received neoadjuvant therapies. Non-tumor brain tissues were obtained from patients receiving surgery for pharmacologically intractable epilepsy. Tissues used for experiments are as follows: epilepsy (N = 5), ependymoma (WHO grade 2; N = 6), and anaplastic ependymoma (WHO grade 3; N = 6). Tissue selection was determined by tissue availability and performed by a person blinded to the treatment outcome of the patients.

Primary Cell Culture

Fresh tumor tissues were mechanically chopped in Dulbecco’s phosphate-buffered saline (Invitrogen, Grand Island, NY) without calcium and magnesium before enzymatic digestion. Within 6 h after resection, tissues were washed and enzymatically dissociated into single cells and filtered through a 40 μm filter. Red blood cells were removed. Live cells were seeded at a standard density of 2 × 10^5 cells/cm². Primary tumor cell cultures were maintained in Neurobasal Medium (Invitrogen) containing 2 mM l-glutamine, N2 supplement, B27 supplement, and 20 ng/ml of human recombinant epidermal growth factor (Chemicon, Temecula, CA) and a basic fibroblast growth factor (Chemicon). To generate tumor spheres, the cells were seeded at a standard density of 2 × 10^5 cells/cm² at each passage. Spheres were maintained and subcultures were performed accordingly. All primarily cultured cells were used for in vitro experiments in less than four cell passages. For BC culture, tumor cells were transferred to Dulbecco’s Modified Eagle’s Medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and penicillin–streptomycin (× 1 final concentration; Invitrogen). All cells were maintained at 37°C with 5% CO₂ in a humidified atmosphere. Neural stem cells (NSCs) were cultured in the same media condition.

Because we used only early-passage (<4) cells for all in vitro experiments, different primary ependymoma cell lines were used (designated as SNU-EP1, SNU-EP2, SNU-EP3, SNU-EP4, and SNU-EP5, respectively). SNU-EP1, SNU-EP2, SNU-EP3, SNU-EP4 cells originated from grade 3 anaplastic ependymomas (patient age 16 years, 3 years, 5 years, and 25 months, respectively). SNU-EP5 cells were from WHO grade 2 ependymoma (patient age 25 months). Neural stem cells were primarily cultured from the human neocortex obtained during epilepsy surgery. A human astrocyte cell line (Life Technologies, Carlsbad, CA) further immortalized with SV40 and hTERT was used (courtesy of Professor Jong Bae Park at National Cancer Center, Korea).

In Vitro Sphere Formation Assay

For tumor-sphere formation assay, dissociated cells (1 × 10^3 cells, N = 3) were plated onto poly-l. lysine-coated eight-well plates (Nunc, Rochester, NY) in serum-free Neurobasal medium with B27 supplement (Invitrogen) for 2 to 7 days. The number of tumor-spheres with a diameter >40 μm were counted with an inverted
Characterization of Spheroid Cells

For characterization of tumor-spheres, spheroids were fixed in 2% paraformaldehyde. Immunofluorescence was performed using the following antibodies: Nestin (Chemicon; 1:200) or Musashi (Neuromics, Bloomington, MN; 1:100). The secondary antibodies Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated goat anti-rabbit (Invitrogen; 1:200) were used. The cells were mounted with an anti-fading solution containing 4′-6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Fluorescent images were obtained using a confocal microscope (Zeiss, Oberkochen, Germany). All experiments were conducted in triplicate.

Cell viability assay

The cytotoxic effect of WP1066, cisplatin, ifosfamide, or etoposide was determined using the colorimetric cell counting kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD) that shows good correlation with the [3H]-thymidine incorporation assay. Dissociated spheres were plated in 96-well plates in 100 μl of serum free media supplemented with growth factors or Dulbecco’s Modified Eagle’s Medium supplemented with fetal bovine serum, at a density of 5 × 10^3 cells per well. F3 cells used as a control cell for detecting cytotoxic effect to normal stem cells. After exposure to drugs (at concentrations from 0 μM to 50 μM) for 48 hours, the colorimetric cell counting kit-8 reagent was added to each well. The absorbance of the samples against a background control was measured using a Microplate Reader (Molecular Devices, Sunnyvale, CA). Fluorescent images were obtained using a confocal microscope (Zeiss, Oberkochen, Germany). All experiments were conducted in triplicate.

Statistical Analysis

Unpaired t test was applied to compare continuous variables between two groups. A Kruskal-Wallis test was used for comparison of continuous variables in three groups. IBM SPSS version 19.0 software (IBM, Armonk, NY) was used for statistical analyses.

Results

STAT3 Activation in Ependymoma Tissues

The status of STAT3 activation was assessed using western blotting in fresh-frozen tissues of ependymoma (WHO grade 2) and anaplastic ependymoma (WHO grade 3). Brain tissues from focal cortical dysplasia patients were used as controls. The STAT3 expression was highly elevated in tumor tissues compared with controls (Figure 1A). Tumor tissues also showed high level of STAT3 activation with thick bands for p-STAT3. Three of six cases of ependymoma showed high p-STAT3 expression and five of six cases of anaplastic ependymoma revealed high p-STAT3 expression. In semi-quantitative measuring, there was a marginal difference in STAT3 band intensities among the three categories (P = .0546; Kruskal-Wallis test), but a significant difference was noted in p-STAT3 band intensity (P = .0298; Kruskal-Wallis test; Figure 1B). Immunohistochemistry of paraffin-embedded tissue revealed nuclear labeling of p-STAT3 in tumor cells and confirmed increased p-STAT3 expression in anaplastic ependymoma (Figure 1D).

Culture of BTICs and BCs

Four fresh tumor tissues were chopped, dissociated, and cultured into two different conditions. One is a culture method optimized for BTICs and the other is a serum-added condition for BCs. BTICs formed multiple characteristic spheroids, whereas BCs showed an elongated morphology (Figure 2A). In immunofluorescence, BTICs
were strongly positive for neural stem cell markers, Nestin and Musashi (Figure 2B). These markers were not expressed in BCs (data not shown).

**Sensitivity of Ependymoma Cells to Various Chemotherapeutic Agents**

To assess the cytotoxic effect of chemotherapeutic agents currently used for intracranial ependymomas, cell viability assay was conducted for SNU-EP1 cells after treatment of cisplatin, ifosfamide, etoposide, or WP1066 in serial concentrations. Drugs were delivered to either BTICs or BCs of SNU-EP1 cells, respectively. There was virtually no cytotoxic effect of cisplatin and ifosfamide (Figure 3, A and B). Etoposide showed a modest decrease of cell viability (Figure 3C). In contrast, WP1066 showed smaller IC50 value for BCs than all chemotherapeutic agents tested above. The IC50 value of WP1066 for BTICs was similar to that of etoposide (Figure 3D). The IC50 values of drugs for SNU-EP1 cells were summarized in Table 1.

**WP1066 Treatment to Ependymoma Cells, NSCs, and Astrocytes**

For four other primary ependymoma cell lines (SNU-EP2, SNU-EP3, SNU-EP4, and SNU-EP5 cells), treatment of WP1066 resulted in cytotoxicity to both BTICs and BCs (Figure 4). The IC50 values for the cell lines are summarized in Table 2. Inhibition of BCs were more effective that suppression of BTICs in all ependymoma cell lines except for in SNU-EP3 cells. Interestingly, BTICs of SNU-EP3 cells escaped the suppressive effect of WP1066 at higher concentrations. It is noteworthy that primary NSCs and astrocytes exhibited higher IC50 values than ependymoma cell lines (Table 2).

**Combination Treatment of WP1066 and Conventional Chemotherapeutic Agents**

Cell viability was assessed after various combinations of WP1066 and conventional chemotherapeutic agents in two ependymoma cell lines (SNU-EP1 and SNU-EP2). As shown in Table 1, cisplatin and ifosfamide alone showed no cytotoxic effects and etoposide exhibited modest cytotoxic effects on SNU-EP1 cells. Combination of conventional agents with WP1066 yielded no additive or synergistic effect for all cell lines compared with the response to WP1066 alone (Figure 5). Indeed, cytotoxic effects of WP1066 seemed to be hampered by adding conventional agents. There were no meaningful differences in the response patterns between day 2 and day 3 after drug treatment.

**Increased Apoptosis After WP1066 Treatment**

TUNEL and Annexin V assays were performed for SNU-EP1 and SNU-EP2 cells to verify whether WP1066 induces apoptosis in
ependymoma cells. In TUNEL assay, apoptotic cells increased significantly at 48 hours from WP1066 treatment (Figure 6). In Annexin V assay, augmentation of early apoptosis was more prominent than increase of late apoptosis after a 24 hours WP1066 treatment (Figure 7). In SNU-EP1 cells, there was significant increase of early apoptosis ($P = .0468$; unpaired $t$ test) and marginally significant increase of late apoptotic cells ($P = .0590$; unpaired $t$ test) after WP1066 treatment. In SNU-EP2 cells, significant augmentation of both early and late apoptosis was observed ($P = .0016$ and $P < .0001$, respectively; unpaired $t$ test).

Figure 2. Distinct morphological divergence of ependymoma cells in two different culture conditions. A. Upper row is BCs cultured in serum-containing media and the lower row is BTICs cultured in serum-free media (original magnification, $\times 200$). (B) Expression of stem cell markers in ependymoma BTICs. Immunofluorescence images showed intense expression of Nestin and Musashi in the tumor spheres of ependymoma BTICs.

Figure 3. Responses of ependymoma cells (SNU-EP1) to conventional chemotherapeutic agents and WP1066. Conventional chemotherapeutic agents, cisplatin and ifosfamide showed little cytotoxic effect and only etoposide exhibited modest cytotoxicity to SNU-EP1 cells. WP1066 showed better cytotoxic effects to both BTIC and BC of SNU-EP1 cells than cisplatin and ifosfamide. WP1066 showed smaller $IC_{50}$ value for BCs than all chemotherapeutic agents tested. The $IC_{50}$ value of WP1066 for BTICs was similar to that of etoposide.
Expression of Apoptosis-Related Proteins After WP1066 Treatment

We observed the expression of cleaved caspase-3 and anti-apoptotic factors, Bcl-xl and Bcl-2, after WP1066 treatment in SNU-EP1 and SNU-EP2 cells. At 72 hours after WP1066 treatment, p-STAT3 expression was nadir in both cell lines. Cleaved caspase-3 expression was paradoxically decreased, indicating that a caspase-independent mechanism may be working in the apoptosis of ependymoma cells after STAT3 inhibition. The expression of anti-apoptotic proteins of Bcl-2 family, Bcl-xl and Bcl-2 was assessed. The level of Bcl-xl was not changed with WP1066 treatment. Instead, there was a decrease of Bcl-2 expression in both cell lines. However, the decrease of Bcl-2 expression was observed 72 hours after WP1066 treatment (Figure 8A). As we observed increase of apoptosis at 24 to 48 hours from WP1066 treatment (Figures 6 and 7), it is unlikely that the delayed decrease of Bcl-2 was the main factor of apoptosis augmentation. We next checked the expression of Survivin, a member of other anti-apoptotic protein family. The levels of Survivin

Table 1. Comparison of the half maximal inhibitory concentration (IC₅₀) values for drugs in ependymoma cells (SNU-EP1)

| Drugs    | BC   | BTIC  |
|----------|------|-------|
| Cisplatin| 95.5 ± 19.9 | 213.6 ± 149.6 |
| Ifosfamide| 152.1 ± 47.3 | 113.0 ± 12.3 |
| Etopside | 49.7 ± 2.9 | 49.4 ± 1.5 |
| WP1066   | 39.4 ± 2.2 | 55.9 ± 1.9 |

BC, BTIC, the IC₅₀ value, relative to untreated control. IC₅₀ ± SD (μM).

Table 2. Comparison of the half maximal inhibitory concentration (IC₅₀) for WP1066 in various tumor and normal cell lines

| Cells    | BC   | BTIC  |
|----------|------|-------|
| SNU-EP2  | 3.1 ± 0.5 | 12.3 ± 1.3 |
| SNU-EP3  | 20.4 ± 1.9 | 5.6 ± 0.8 |
| SNU-EP4  | 21.5 ± 0.9 | 104.5 ± 5.1 |
| SNU-EP5  | 1.3 ± 0.1 | 8.9 ± 5.2 |
| NSC      | 43.1 ± 2.1* | 26.9 ± 2.0* |
| Astrocyte|       |       |

BC, BTIC, NSC. The IC₅₀ value, relative to untreated control. IC₅₀ ± SD (μM).
*NSCs were sphere-forming cells and astrocytes were adherent cells.

Figure 4. Cell viability of other ependymoma primary cell lines, NSC, and astrocytes to WP1066. A–D. WP1066 showed stable cytotoxic effects on BCs of SNU-EP2, SNU-EP3, SNU-EP4, and SNU-EP5 cell lines. BTICs of these cell lines were also suppressed by WP1066, but their responses were variable in the cell lines tested. NSCs derived from the neocortex and immortalized astrocytes were also suppressed by WP1066 treatment to some degree, but they were more resistant to WP1066 than ependymoma cell lines. NSCs exhibited a higher IC₅₀ value than immortalized astrocyte cell line.
expression were decreased at 24 h after WP1066 treatment in two cell lines (SNU-EP1 and SNU-EP2 cells).

**Suppression of Ependymoma Cell Viability With STAT3 Knockdown**

To verify the important role of STAT3 suppression by WP1066 in ependymoma cells (SNU-EP1 and SNU-EP2 cells), we tried STAT3 knockdown with specific siRNAs (STAT3-siRNA1 and STAT3-siRNA2). A decrease of STAT3 expression and further suppression of p-STAT3 expression were confirmed by western blot with both STAT3-siRNA1 and STAT3-siRNA2 (Figure 9A). A decrease of Survivin expression was also observed with STAT3 knockdown in SNU-EP2 cells. After knockdown of STAT3, the cell viability was significantly decreased in both cell lines (Figure 9B). These results further demonstrated the critical role of STAT3 in maintaining ependymoma cells.

**Discussion**

STAT3 is an important cellular signal transducer interconnecting many receptors, signaling pathways, and target genes. *In vitro* studies showed that activation of STAT3 pathway leads to enhanced cell survival, cell cycle progression, and cellular migration, all of which are cardinal features of malignancy. Indeed, many human cancers showed overexpression of STAT3 and activation of its signaling that has prognostic correlation in many instances [10,12]. Therefore, suppression of the activated STAT3 pathway has been a topic of investigation for many types of cancer. Inhibition of STAT3 is possible by administration of specific siRNA, decoy oligonucleotide, or synthetic or natural inhibitors [9,24]. There are many kinds of STAT3 inhibitors and WP1066 has been known as a potent inhibitor of STAT3 pathway [16]. STAT3 is constitutively activated in malignant glioma and WP1066 have shown growth-suppressing ability in U87-MR and U373-MG cells *in vitro* and *in vivo* [18]. The cellular effects of STAT3 pathway inhibition are diverse, but the induction of apoptosis is regarded as the main consequence [16]. Therefore, STAT3 inhibitors can acts as a cytotoxic agent. Silencing of STAT3 resulted in reduction of glioma cell infiltration into the mouse brain, thereby prolonging the survival of the animal model [25]. This finding presents another promising role of STAT3 inhibitors as an anti-invasive agent for malignant brain tumors.

In this study, a STAT3 inhibitor, WP1066 treatment led to robust suppression of ependymoma cell growth. Conventional chemotherapeutic agents (cisplatin, ifosfamide, and etoposide) showed insufficient cytotoxic effects to ependymoma cells *in vitro*. It has been postulated that STAT3 signaling is deeply involved in the acquisition of chemo-resistance of tumor cells [26,27]. In gastric cancer cells, it was shown that treatment of STAT3 siRNA increased sensitivity to cisplatin in drug-resistant cell populations [14]. Monoclonal antibody to IL-6, siltuximab, increased the cytotoxicity of paclitaxel in drug-resistant ovarian carcinoma cell lines by inhibiting the STAT3 signaling [28].

However, we observed no synergistic effect of combined treatment of WP1066 and chemotherapeutic agents. Limited efficacy to a certain group of patients and the emergence of resistance in initially
responsive patients are major obstacles frequently encountered during application of new molecular-targeted agents. At present, we may have to proceed on trying a single STAT3 inhibitor or to search for an optimal combination of drugs for ependymoma to maximize the effect of STAT3 inhibitors.

We demonstrated that anaplastic ependymoma had higher p-STAT3 expression than grade 2 ependymoma. However, it is not conclusive whether WP1066 is more effective in suppressing the viability of anaplastic ependymoma cells, because only one grade 2 ependymoma cell line was used for this study and the cells (SNU-EP5) exhibited low IC50 values to WP1066.

The last decade witnessed the emerging concept of cancer stem cells in various solid tumors [21]. The cancer stem cell concept has been criticized mainly for the lack of out-and-out definitions and criteria, and for brain tumors, BTIC may be more appropriate term. BTICs are thought to be dormant in nature and more resistant to chemotherapy than BCs [20]. Our results showed that BTICs of ependymoma were more resistant to WP1066 in four out of the five ependymoma cell lines tested. Only one cell line (SNU-EP3) showed more effective suppression of BTICs than BCs by WP1066, but BTICs escaped the suppressive effect at high concentrations. Nonetheless, WP1066 showed sufficient cytotoxicity to suppress ependymoma BTICs, considering the inefficiency of conventional chemotherapeutics on ependymoma cells.

The viability of human NSC and astrocyte cell lines were also affected by WP1066 treatment, raising some concerns about clinical application of STAT3 inhibitors. However, their IC50 values were higher than those of BCs of ependymoma cell lines. Furthermore, NSCs were more resistant to WP1066 than astrocytes. The astrocytes used in this study had been artificially immortalized after primary culture, while the NSCs were primarily cultured early-passage cells. Immortalization is a hallmark phenotype of cancer and immortalized cells could be more vulnerable to STAT3 inhibition. In this regards, naïve neurons and glial cells might be protected when STAT3 inhibitors were administrated.

Previous studies suggested that STAT3 inhibitors including WP1066 augment apoptosis of tumor cells especially through suppression of anti-apoptotic factors, such as survivin or Bcl-xl [28]. We also observed a dramatic increase of apoptosis after WP1066 treatment in ependymoma cell lines. Bcl-2 and Bcl-xl are anti-apoptotic proteins that belong to Bcl-2 family. Decrease of Bcl-2 expression was observed, but it was evident at 72 h after WP1066 treatment, whereas robust apoptosis was observed in TUNEL staining and Annexin V assay within 24 to 48 hours. Survivin is a member of other anti-apoptotic protein family. A decrease of Survivin was observed at 24 h after WP1066 treatment, making it a candidate mechanism of WP1066 treatment in ependymoma cells.

The central role of STAT3 in maintaining ependymoma cell survival was further confirmed by knockdown experiments with specific STAT3-siRNAs. Knockdown of STAT3 yielded cytotoxic effects on ependymoma cells, supporting the critical role of STAT3 in these tumor cells and the therapeutic potentials of STAT3-targeting strategy in ependymomas.

At present, several human clinical trials are ongoing that target refractory solid tumors with novel STAT3 inhibitors (OPB-31121 and OPB-51602) and chronic lymphocytic leukemia with an
anti-protozoal medication (pyrimethamine) that has STAT3-inhibiting actions [29]. In our study, we observed cytotoxic effect of STAT3 inhibitor to ependymoma BTICs and BCs. We have urgent needs to develop new therapeutic agents for pediatric ependymoma patients. STAT3 inhibitors may emerge as a new group of drugs for clinical application for pediatric intracranial ependymomas.

**Figure 7.** Annexin V assays in two ependymoma cell lines after WP1066 treatment. Increase of apoptotic cell proportion was more prominent in early apoptosis. In SNU-EP1 cells, the proportions of early apoptotic cells were 0.96 ± 0.27% in control cells and 7.7 ± 2.66% in WP1066-treated cells \( (P = .0468; \text{unpaired } t \text{ test}) \). There was no significant difference in the proportions of late apoptotic cells (9.87 ± 0.80% in control cells and 13.1 ± 1.65% in WP1066-treated cells; \( P = .0590; \text{unpaired } t \text{ test} \)). In SNU-EP2 cells, the proportions of early apoptotic cells were 2.55 ± 0.45% in control cells and 18.8 ± 1.56% in WP1066-treated cells \( (P = .0016; \text{unpaired } t \text{ test}) \). The proportions of late apoptotic cells were 4.30 ± 0.32% in control cells and 12.6 ± 0.24% in WP1066-treated cells \( (P < .0001; \text{unpaired } t \text{ test}) \). *\( P < .05 \), **\( P < .01 \).

**Figure 8.** Expression of apoptosis-related proteins. (A) The expression of cleaved caspase-3 and anti-apoptotic proteins (Bcl-xl and Bcl-2) was assessed with western blot. Cleaved caspase-3 expression was paradoxically decreased, indicating that a caspase-independent mechanism may be working. A decrease of Bcl-2 expression was observed in SNU-EP1 and SNU-EP2 cells, but it appeared at 72 hours after WP1066 treatment which was not compatible with the early increase of apoptosis in TUNEL and Annexin V assays. (B) Expression of survivin was decreased after a 24 hours WP1066 treatment in SNU-EP1 and SNU-EP2 cells.


Figure 9. STAT3 knockdown and cell viability. (A) Decrease of STAT3 expression was observed with STAT3-siRNA treatment in SNU-EP1 and SNU-EP2 cells. Expression of p-STAT3 was further suppressed. A decrease of Survivin expression was also observed in SNU-EP2 cells which exhibited strong suppression of Survivin expression after WP1066 treatment. (B) Cell viability was significantly decreased in SNU-EP1 cells after treatment of two different STAT3-siRNAs (control vs siRNA1: 100.0 ± 2.5% vs 79.3 ± 12.3%, P = 0.0081; control vs siRNA2: 67.9 ± 5.3%, P < .0001; unpaired t test). Significant decrease of cell viability was also observed in SNU-EP2 cells (control vs siRNA1: 100.0 ± 9.6% vs 72.7 ± 9.3%, P = .0032; control vs siRNA2: 67.7 ± 1.4%, P = 0.0033). NC-siRNA = negative control siRNA, *P < .05, **P < .01, ***P < .001.

Conclusions

In vitro studies indicated that a STAT3 inhibitor, WP1066 has cytotoxic effect to ependymoma cells. Considering the high resistance of ependymoma cells to various chemotherapeutic agents, this is a promising result. STAT3 inhibitors may be a new group of drugs for intracranial ependymomas.

Acknowledgements

This study was jointly supported by a grant (No. 0420120910) from the Seoul National University Hospital and a fund from the National Research Foundation of Korea (No. 2014R1A1A1005765).

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