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Targeting Upstream Kinases of STAT3 in Human Medulloblastoma Cells

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Abstract

Background: Medulloblastoma is the most common malignant brain tumor in children. Despite improvement in overall survival rate, it still lacks an effective targeted treatment strategy. The Janus family of cytoplasmic tyrosine kinases (JAKs) and Src kinases, upstream protein kinases of signal transducer and activator of transcription 3 (STAT3), play important roles in medulloblastoma pathogenesis and therefore represent potential therapeutic targets.

Methods: In this report, we examined the inhibitory efficacy of the JAK1/2 inhibitor, ruxolitinib, the JAK3 inhibitor, tofacitinib and two Src inhibitors, KX2–391 and dasatinib.

Results: These small molecule drugs significantly reduce cell viability and inhibit cell migration and colony formation in human medulloblastoma cells *in vitro*. Src inhibitors have more potent efficacy than JAK inhibitors in inhibiting medulloblastoma cell migration ability. The Src inhibitors can inhibit both phosphorylation of STAT3 and Src while JAK inhibitors reduce JAK/

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

STAT3 phosphorylation. We also investigated the combined effect of the Src inhibitor, dasatinib with cisplatin. The results show that dasatinib exerts synergistic effects with cisplatin in human medulloblastoma cells through the inhibition of STAT3 and Src.

Conclusion: Our results suggest that the small molecule inhibitors of STAT3 upstream kinases, ruxolitinib, tofacitinib, KX2-391, and dasatinib could be novel and attractive candidate drugs for the treatment of human medulloblastoma.

Keywords

Medulloblastoma; Src inhibitor; JAK inhibitor; STAT3; dasatinib; cisplatin

1. INTRODUCTION

Medulloblastoma (MB) is a malignant pediatric brain tumor, accounting for about 63.7% of all embryonal tumors in children and adolescents [1]. Due to its complexity and heterogeneity, the traditional classification of MB was based on four molecular subgroups: wingless (WNT), sonic hedgehog (SHH), Group 3 and Group 4 with WNT group having the best and Group 3 medulloblastoma having the worst clinical prognosis [2]. Recently, the revised 2016 WHO classification of CNS tumors defines medulloblastoma both histologically and genetically [3]. The WHO classification divides tumors into WNT-activated, SHH-activated-TP53 wildtype, SHH-activated-TP53 mutant and non-WNT-SHH, where group 3 and group 4 medulloblastomas are provisional entities in order to form the basis for the next generation of clinical trials and targeted therapy [4]. Current treatment choice for medulloblastoma is based on the age of the patients at the diagnosis, the risk classification and the genetic background [4, 5]. Tremendous improvements in therapy using a combination of surgery, craniospinal radiotherapy and chemotherapy with vincristine, cisplatin and cyclophosphamide have yielded an overall survival of 70–80%, however, radio-polychemotherapy increases the risk of significant toxicities including neurocognitive impairment, endocrinopathy and neurologic deficits [6, 7]. In addition, many patients need to receive craniospinal irradiation to protect against developing metastasis, which places patients at the risk for significant long-term neurocognitive side-effects and sequelae that may impair their quality of life [8]. Targeted treatment is hence strongly desired based on the aggressive nature of the tumor and treatment-related side effects [9, 10].

Signal transducers and activators of transcription 3 (STAT3) signaling are persistently activated in medulloblastoma and could serve a crucial role in its tumorigenesis [11, 12]. Once activated, they may contribute to uncontrolled proliferation of cancer cells by promoting cell cycle progression, the inhibition of apoptosis and tumor metastasis [13–16]. Excessive activation of the Janus family of cytoplasmic tyrosine kinases (JAK) is associated with STAT3 activation, and JAKs are key participants in signaling networks fueled by an oversupply of cytokines secreted from both tumor cells and cells in the tumor environment [17]. The activation of JAK/STAT signaling has been found in many types of solid tumors including medulloblastoma [18]. We have previously detected high expression of p-STAT3 in human medulloblastoma cell lines (UW426, UW288 and DAOY cells) [19] or induced at the presence of cytokines (IL-6) [20]. Based on this, inhibition of JAK/STAT3 signaling may show its highest clinical relevance as adjuvant therapy, and JAKs are regarded as potential

drug targets for solid tumors. Ruxolitinib, a JAK1/2 inhibitor, can improve symptoms and prolong survival of patients with primary myelofibrosis secondary to polycythemia vera and essential thrombocythemia [21]. Tofacitinib, a JAK3 inhibitor, has recently been recommended as a treatment option for adults with active rheumatoid arthritis [22]. Although the cause of JAK/STAT activation in solid malignancies is less well defined, both ruxolitinib and tofacitinib have now been approved in phase I~III clinical trials in metastatic pancreatic cancer, breast cancer, lung adenocarcinoma and ulcerative colitis [17, 23, 24]. These two JAK inhibitors have been noted to cross the blood-brain barrier [25, 26], making them attractive potential therapies for brain tumors, however, their effects on human medulloblastoma have not been fully investigated.

Besides JAK, the Src family kinase, involved in regulating important mechanisms of receptor tyrosine kinases, has also been found to activate STAT3 and linked to drug resistance in some cancer cells [27, 28]. It is an integrator of multiple signals facilitating the action of other signaling proteins, making it a potential target for the treatment of human tumors [29]. High Src activity has been documented in medulloblastoma, so targeting Src may be a novel therapeutic strategy [30]. The FDA-approved small molecule, KX2-391, a non-ATP-competitive inhibitor of Src kinase and tubulin polymerization [31], has been tested in phase I~II clinical trials for prostate cancers and advanced malignancies [31, 32]. It can also induce cell death in dasatinib-sensitive T-cell acute lymphoblastic leukemia (T-ALL) patients [33]. It was evaluated in mouse tumor model and has an excellent brain penetration in mice [34]. However, its effects on medulloblastoma have not been investigated. Dasatinib, another potent Src inhibitor, which is now being used as a standard treatment of chronic myelogenous leukemia and Philadelphia-chromosome-positive ALL, proves to be well tolerated and can cross the blood-brain-barrier in pediatric patients [35, 36]. Several studies have demonstrated the efficacy of dasatinib in solid tumors and clinical trials are in progress [37–39]. Previous reports have already found the potential inhibitory effect of dasatinib against certain medulloblastoma cells [40, 41]; however, the effects of dasatinib in combination with cisplatin the latter frequently used in induction or adjuvant chemotherapy regimens in medulloblastoma [42, 43], has not yet been investigated. If this drug is effective in medulloblastoma cells especially when in combination with conventional chemotherapeutic drugs, it may be a promising candidate for combined therapy in the future to reduce the risk of side effects due to conventional chemotherapy and radiotherapy.

Since all the four small molecule inhibitors mentioned above can cross the blood-brain barrier and have excellent pharmacokinetic profiles and antitumor activities with a good safety profile in patients with different types of solid tumors, it makes them potential candidates for MB therapeutics. In this study, we examined the efficacy and underlying inhibitory mechanism of two JAK inhibitors and two Src inhibitors in human medulloblastoma cells in an effort to select a candidate targeted treatment for MB. We also investigated the synergistic effects of cisplatin with dasatinib in human medulloblastoma cells.

2. MATERIALS AND METHODS

2.1. Cell Culture and Reagents

Human medulloblastoma cell lines (UW426, UW288 and DAOY) were provided by Dr. Corey Raffel (The Research Institute at Nationwide Children's Hospital, USA). Normal Human Astrocytes (NHA) were purchased from ATCC (the American Type Culture Collection, Manassas, VA, USA). Cells were maintained in Dulbecco's Modification of Eagle's Medium (DMEM, Hyclone, USA) with 4.5 g/L, L-glutamine and sodium pyruvate (Mediatech, USA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, USA), and 1% Penicillin/Streptomycin (P/S) (Sigma, USA) in incubators set at 37 °C and aired with 5% CO₂.

All the reagents in the study were purchased as follows: dimethyl sulfoxide (Sigma, USA), ruxolitinib (LC Laboratories, Woburn, MA, USA), tofacitinib (LC Laboratories, Woburn, MA, USA), KX2-391 (Selleckchem, Woburn, MA, USA), dasatinib (LC Laboratories, Woburn, MA, USA) and cisplatin (Sigma, USA). All the drugs were stored at -20 °C with 20 mM stock solution.

2.2. MTT Cell Viability Assay

Human medulloblastoma cancer cell lines (UW426, UW288, and DAOY) were seeded in 96-well plates at a density of 3000 cells per well. The next day, different concentration of JAK, Src inhibitors with or without cisplatin were added in triplicate to the plates in the presence of 10% FBS in human medulloblastoma cells. The cells were incubated at 37°C for a period of 48 to 72 hours. Then 25µl of 3-(4, 5-Dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma) was added to each sample in a volume of 100 µl and incubated for 4 hours. After that, 150 µl of N, N-dimethylformamide (Sigma, USA) solubilization solution were added to each well. The absorbance was read at a wavelength of 595 nm. Half-maximal inhibitory concentrations (IC₅₀) were determined by GraphPad Prism software7.0.

Combination index (CI) was determined using data obtained from the MTT assay with CompuSyn software [44]. CI values indicate a synergistic effect when <1, an antagonistic effect when >1, and an additive effect when equal to 1.

2.3. Colony-forming Cell Assay

Clonogenic capacity was evaluated according to the protocol of Franken *et al.* [45]. Cells were treated with JAK inhibitors or Src inhibitors alone or in combination with cisplatin. After treatment for 72 hours, 1000 cells were harvested and reseeded on 6-cm plates with a drug-free medium for an additional incubation of one to two weeks. Colonies were fixed with ice-cold methanol for 30 minutes and then stained with 1% crystal violet dye for two to three hours. After staining, the plates were washed with distilled water and dried. To determine the relative number of clones, 10% acetic acid was used to elute the crystal violet and the absorbance was detected at 590 nm wavelength light in a spectrophotometer.

2.4. Wound Healing/Cell Migration Assay

When human medulloblastoma cells (UW426, UW288, and DAOY) were 100% confluent, the monolayer was scratched in a uniform width using a pipette tip. After washing, the cells

were then treated with different concentrations of JAK inhibitors or Src inhibitors, or cisplatin alone or in combination. After scratching the cells with a yellow tip pipette, UW426, UW288 and DAOY cells could migrate within 24 hours to fill the scratched area completely. At 24 hours after scratching, images were captured by an inverted microscope (Nikon, Eclipse TS100, Japan). The percentage of wound healing was measured by software ImageJ (National Institutes of Health, USA) and calculated by the equation: percent wound healing = average of (gap area before treatment - gap area after treatment)/ gap area before treatment.

2.5. Western Blotting Assay

Medulloblastoma cell lines (UW426, UW288, and DAOY) or NHA cells were washed with cold PBS and harvested with a rubber scraper alone or after the desired treatment. Cell plates were kept on ice and lysed for 20 minutes in cell lysis buffer (Cell Signaling Technology, USA) with protease inhibitors cocktail and phosphatase inhibitors. The lysates were cleared by centrifugation, and the supernatant fractions were collected. Cell lysates were then separated by 10% SDS-PAGE and subjected to western blot analysis detected using a 1:1000 or 1:2000 dilution of primary antibodies according to the protocols and a 1:10000 dilution of horseradish peroxidase-conjugated secondary antibodies. Antibodies against the following were used for western blotting: phosphorylated STAT3 (Y705), phosphorylated Src (Tyr416), phosphorylated JAK2 (Tyr1007/1008), phosphorylated JAK3 (Tyr980/981), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), phosphorylated AKT (Ser473), E-Cadherin, N-Cadherin, PTEN, cleaved Caspase-3, AKT, ERK, JAK2, STAT3, GAPDH and secondary antibody (all from Cell Signaling Technology, USA). Membranes were analyzed using enhanced chemiluminescence plus reagents and scanned with the Storm Scanner (Amersham Pharmacia Biotech Inc., USA). The relative protein levels were quantified by densitometry with ImageJ software (National Institutes of Health, Bethesda, USA) according to the manufacturer's instructions.

2.6. Statistics

The significance of correlations was assessed using GraphPad Prism software 7.0 (GraphPad Software, Inc, USA). Unpaired t tests were used for analyses assuming Gaussian populations with a 95% confidence interval. Data are presented as mean $\pm$ standard deviation (SD). Differences were analyzed with the Student t test, and significance was set at $p < 0.05$. *, ** and *** indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

3. RESULTS

3.1. JAK/STAT3 and Src was Highly Activated in Human Medulloblastoma Cells

To determine the expression of JAK/STAT3 and Src activation in human medulloblastoma cells, we have compared the basal activation level of p-JAK2, p-JAK3, p-STAT3 and p-Src in three human medulloblastoma cell lines (UW426, UW288, and DAOY) with NHA cell line. The results indicated that all three human medulloblastoma cells had higher basal level of p-JAK2, p-JAK3, p-STAT3 and p-Src. The level of p-JAK2, p-JAK3, p-STAT3 and p-Src was higher than NHA normal astrocyte cell line (Fig. 1A–B).

3.2. JAK/Src Inhibitors Significantly Reduce Viability of Human Medulloblastoma Cells

First, we tested the effects of JAK inhibitors (ruxolitinib and tofacitinib) and Src kinase inhibitors (KX2-391 and dasatinib) on human medulloblastoma cell lines. The IC₅₀ values for JAK and Src inhibitors are listed in Table 1. Our MTT array showed that ruxolitinib and tofacitinib could significantly reduce cell viability of UW426, UW288, and DAOY cell lines (Fig. 2A–B). Ruxolitinib and tofacitinib were most potent in DAOY cell line than the other two cell lines with IC₅₀ 0.93±0.93 μmol/L (μM) and 2.77±0.51 μM respectively. KX2-391 and dasatinib could also reduce cell viability of UW426, UW288, and DAOY cell lines (Fig. 2C–D). KX2-391 was most sensitive in UW288 cells with IC₅₀ 0.021±0.019 μM. Dasatinib was most sensitive in UW426 cells with IC₅₀ 3.52±0.48 μM.

3.3. JAK/Src Inhibitors Inhibit Colony Formation and Cell Migration of Human Medulloblastoma Cells

We next sought to investigate whether these drugs might inhibit the colony formation capability. As shown in (Fig. 3A), the UW426, UW288, and DAOY cells showed a decreased ability to recover and form colonies following treatment with ruxolitinib and tofacitinib. (Fig. 3B) showed the results of colony formation assay treated with KX2-391 and dasatinib. As expected, these two Src inhibitors could also inhibit cell colony formation ability in human medulloblastoma cells and KX2-391 showed more potent activity than JAK inhibitors.

Cell migration ability is an important process for cancer cell metastasis. In order to assess the effect of JAK and Src inhibitors on medulloblastoma cell migratory ability, a wound healing assay was performed. After treatment with 10 μM, 20 μM and 40 μM of ruxolitinib or tofacitinib, the migration abilities of UW426, UW288 and DAOY cells were inhibited (Fig. 4A–F). Src inhibitors, however, showed much more potent ability than JAK inhibitors to inhibit the migration of UW426, UW288, and DAOY cells. As shown in (Fig. 5A–C), only 50nM dasatinib could markedly inhibit the movement of UW426, UW288, and DAOY cells. Similarly, 0.25 μM KX2-391 was sufficient to inhibit the migration of UW426, UW288, and DAOY cells (Fig. 5D–F). Dasatinib appears to be the most potent candidate drug among these four drugs in inhibiting cell migration in medulloblastoma cells.

3.4. JAK/Src Inhibitors Induce Apoptosis, Inhibit JAK/STAT3 or Src/STAT3 Phosphorylation and Regulate Epithelial-Mesenchymal Transition (EMT) in Human Medulloblastoma Cells

We first tested the effect of the different inhibitors on apoptosis of the medulloblastoma cell lines and the results showed that all four candidate drugs can induce the expression of cleaved caspase-3 in UW288, UW426, and DAOY cells (Fig. 6A–C).

Since JAK/STAT3 and SRC was highly activated in UW288, UW426, and DAOY cells and activation of STAT3 could occur through JAK2, JAK3 or SRC family kinase, we then assessed the phosphorylation of JAK2, JAK3, STAT3, SRC, ERK1/2 and AKT after treatment of UW426, UW288 and DAOY cells with those kinases inhibitors. Ruxolitinib could reduce the expression of p-JAK2, p-STAT3 and p-AKT in UW288, UW426, and DAOY cells. Tofacitinib could reduce the expression of p-JAK3, p-STAT3 and p-AKT in

UW288, UW426, and DAOY cells. KX2-391 and dasatinib can reduce the activation of SRC, STAT3, AKT and ERK in UW288, UW426, and DAOY cells (Fig. 6A–C).

We also investigated the major epithelial-mesenchymal transition markers. As shown in (Fig. 6A–C), E-Cadherin can be induced by ruxolitinib and tofacitinib in UW426 and DAOY cells, and by KX2-391 and dasatinib in UW288, UW426, and DAOY cells. N-Cadherin can be reduced by ruxolitinib and tofacitinib in UW426 and UW288 cells and by KX2-391 and dasatinib in UW288, UW426, and DAOY cells. The PTEN protein level did not show significantly change with the treatment of all the candidate drugs in DAOY cells.

3.5. Dasatinib Shows a Synergistic Effect with Cisplatin in Human Medulloblastoma Cells

Since Src inhibitors show potent efficacy in the treatment of medulloblastoma cells, we then tested whether dasatinib or KX2-391 had a synergistic effect with the combination of cisplatin. The results showed that either dasatinib or KX2-391 had significantly greater synergistic effects than a single drug alone in all three human medulloblastoma cells. All CI values in the three groups were less than 1 (Fig. 7A). Since dasatinib can penetrate the blood-brain barrel following oral intake, we mainly focused on synergistic effects with dasatinib. Our results showed that the combination of dasatinib and cisplatin could significantly reduce colony formation compared with single drug alone (Fig. 7B). The cell migration ability was also markedly inhibited when 25nM dasatinib was combined with cisplatin (Fig. 7C). When cells were treated with 5μM cisplatin alone, the p-STAT3, p-Src and c-myc levels remained unchanged. With the combination treatment of 25nM dasatinib, the p-STAT3, p-Src, p-ERK expression as well as c-myc was greatly reduced compared with cisplatin alone (Fig. 7D).

4. DISCUSSION

Finding novel molecular targeted drugs in medulloblastoma is desired to avoid the increased risk of neurotoxicity-associated side effects and/or improve the relatively poor prognosis in this disease. In this study, we tested the efficacy of four small molecule drugs which target STAT3 upstream kinases. The JAK1/2 inhibitor ruxolitinib, the JAK3 inhibitor tofacitinib and two Src inhibitors, KX2-391 and dasatinib, were tested on human medulloblastoma cell lines *in vitro*. Our preliminary results showed that all four small molecular drugs have prominent anti-tumor effects on human medulloblastoma *in vitro*. The dosages of the selected drugs in this research were determined by other *in vitro* experiments in other solid tumors [46–48]. As our results showed, JAK or Src inhibitors showed different sensitivities on different cell lines indicating the heterogeneous nature of this tumor type.

Src is a well-known oncogenic tyrosine kinase and p-Src, which is essential for Src kinase activity, is detectable in 44% of medulloblastoma cells [40]. Finding drugs that target Src has generated considerable interest in many solid cancers. We showed that p-SRC are aberrantly activated in three SHH medulloblastoma cell lines (UW426, UW288 and DAOY cells) compared with normal astrocyte cell line making Src as a potential target for SHH MB. Dasatinib, which has been approved for clinical use for chronic myeloid leukemia in patients resistant to or intolerant of imatinib, has shown promising results in clinical studies of some solid tumors [49, 50]. The advantage of this drug is that it can penetrate the blood-

brain barrier following oral administration, offering a potential promising future in the treatment of medulloblastoma. Our results and those of others are similar and confirming the potent efficacy of dasatinib in medulloblastoma [40]. The underlying mechanism mediating the antitumor effects by either KX2-391 or dasatinib was through inhibition of activated SRC/STAT3 pathway and reducing the level phosphorylation of AKT and ERK. However, based on clinical practice in other solid tumors, better outcomes may not be achieved without combination with other drugs in therapeutic regimens. Cisplatin is currently considered a backbone of chemotherapy for medulloblastoma [42, 43] and significant concentrations of cisplatin can be obtained in human CSF following systemic administration [51]. However, some types of medulloblastoma have shown resistance with this drug, with an elevated level of p-STAT3 [52]. Increased tumor invasion and upregulated STAT3 can be detected in cisplatin-selected resistant pediatric tumor of the central nervous system (CNS) [53]. The combination therapy of cisplatin and dasatinib can markedly inhibit cell migration and cell colony formation. Our results showed dasatinib can greatly enhance the efficacy of cisplatin in medulloblastoma treatment. Some researchers have tried to combine dasatinib with cisplatin in human esophageal squamous cell carcinoma *via* suppression of the PI3K/Akt and STAT3 pathways [54]. Our results showed that the mechanism for this synergy is through the inhibition of p-Src, p-STAT3 as well as p-ERK.

For decades, medulloblastoma has been recognized as an extraordinarily heterogeneous disease [55]. Not all types of medulloblastoma were correlated with high expression levels of p-Src. Src inhibitors hence may show drug resistance in this group, so we also tested JAK inhibitors. JAK kinases belong to the upper kinases of STAT3. Active JAKs phosphorylate tyrosine residues in the cytoplasmic region of the receptor creating binding sites that recruit STATs [18]. The evidence that the JAK/STAT pathway is activated in a large proportion of solid tumors and that its activation contributes to the malignant properties of cancer cells makes the JAK/STAT pathway a promising target for the development of new therapies [17]. Since developing drugs that can directly target STAT3 has been challenging, small molecule JAK inhibitors provide a possible clinical alternative to block STAT3 signaling [56, 57]. Theoretically, inhibition of JAK/STAT3 pathway can suppress the activity of STAT3 and induce the suppression of medulloblastoma cells. Our results support this hypothesis. The JAK1/2 inhibitor, ruxolitinib and JAK3 inhibitor, tofacitinib can reduce cell viability, inhibit colony formation and cell migration by inhibiting excessive p-JAK2 or p-JAK3 and p-STAT3 in human medulloblastoma cells. Although Src inhibitors seem more potent than JAK inhibitors in suppressing cell migration in medulloblastoma, JAK inhibitors can still yield therapeutic benefits if p-Src expression is absent in some types of medulloblastoma. Our findings further confirmed that ruxolitinib and tofacitinib could be used to kill human medulloblastoma cells by direct targeting the JAK/STAT3 signal pathway. These two small molecule target drugs may serve together with surgery, radiation therapy and standard chemo-therapy in the management of medulloblastoma.

Metastatic dissemination is a major treatment challenge and cause of death in patients with medulloblastoma. Although MB SHH is not the more metastatic subgroup but some high-risk SHH patients can still develop metastasis and predict the poor prognosis [58]. Since Epithelialmesenchymal transition (EMT) is a common manifestation of tumor, we hence investigated the candidate drugs in the context of EMT. As shown, all the four candidate

drugs can inhibit the cell migration ability by inducing the epithelial marker, E-Cadherin and reducing the mesenchymal marker, N-Cadherin. It is therefore likely that these four small molecules targeting JAK/STAT3 or SRC/STAT3 may serve as a promising therapeutic strategy for the treatment of medulloblastoma.

So far, no clinical trials for medulloblastoma are available either with JAK inhibitors ruxolitinib and tofacitinib or Src inhibitors KX2–391 and dasatinib. Ruxolitinib was repurposed as an anti-cancer agent for solid tumors including medulloblastoma *in vitro* [59]. For Src inhibitors, KX2–391 has only proven to be a favorable pharmacokinetic profile and be well tolerated in patients with advanced malignancies in phase I trial [32] but not been reported in any brain tumors. Dasatinib has been reported to undertake phase I trial in children with relapsed or refractory central nervous system tumors and preliminary evidence of clinical benefit was seen [60].

In conclusion, our results suggested that FDA-approved small molecule drugs ruxolitinib, tofacitinib, KX2–391 and dasatinib could be novel attractive candidates for the future treatment of medulloblastoma through inhibiting JAK/STAT3 or Src/STAT3. Dasatinib can enhance efficacy of cisplatin through abrogating activation of STAT3 and Src. The transition from preclinical models into the early phase of clinical trials are needed to validate our findings for clinical use soon.

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Fig. (1). JAK/STAT3 and Src is highly activated in human medulloblastoma cells.

A: The basal activation level of p-JAK2 (Tyr1007/1008), p-JAK3 (Tyr980/981), p-STAT3 (Y705), and p-SRC (Tyr416) was evaluated in three human medulloblastoma cells (UW288, UW426, and DAOY) and one normal human astrocyte cell line (NHA). Cells were harvested and the protein expression was detected by western blot. JAK2, STAT3 and GAPDH was served as loading control. **B:** The relative protein expression level was quantified by image J. (***, $p < 0.001$).

Fig. (2). JAK and Src inhibitors decrease viability of human medulloblastoma cell lines.

A-B: Human medulloblastoma cells (UW288, UW426, and DAOY) were seeded in 96-well plates at a density of 3000 cells per well overnight and then treated with ruxolitinib and tofacitinib at the indicated concentration for 72 hours. Cell viability was measured by MTT assay. **C-D:** Human medulloblastoma cells (UW288, UW426, and DAOY) were seeded in 96-well plates at a density of 3000 cells per well overnight and then treated with KX2-391 and dasatinib at indicated concentration for 72 hours. Cell viability was measured by MTT assay. Error bars indicate SD of mean. (**, $p < 0.01$ and ***, $p < 0.001$).

Fig. (3). JAK and Src inhibitors significantly inhibit the colony forming ability of human medulloblastoma cell lines.

A: UW288, UW426, and DAOY cells were treated with ruxolitinib and tofacitinib (10 μ M and 25 μ M) for 72 hours. After treatment, 1000 cells were reseeded on 6-cm plates with a drug-free medium for an additional incubation of one to two weeks. Colonies were fixed by ice-cold methanol and stained by 1% crystal violet. 10% acetic acid was used to elute the crystal violet and the absorbance was detected at OD590 nm. **B:** UW288, UW426, and DAOY cells were treated with KX2-391 (2.5 μ M and 5 μ M) and dasatinib (5 μ M, 10 μ M and 25 μ M) for 72 hours. After treatment, 1000 cells were reseeded on 6-cm plates with a drug-free medium for an additional incubation of one to two weeks. Colonies were fixed by ice-cold methanol and stained by 1% crystal violet. 10% acetic acid was used to elute the crystal violet and the absorbance was detected at OD590 nm. (***, $p < 0.001$).

Fig. (4). JAK inhibitors significantly inhibit the migration of human medulloblastoma cell lines. A-C: Representative pictures are shown of UW426 (A), UW288 (B) and DAOY (C) cells in a wound healing assay. The assay was conducted by scratching the cells with yellow pipette tip when cells grew to monolayer. Then cells were then treated with ruxolitinib (10, 20 and 40 μ M) or DMSO and allowed to migrate into the scratched area for 24 hours. The percentage of migrating area in wound healing assay was quantified in UW426, UW288, and DAOY cells. D-F: Similarly, representative pictures are shown of UW426 (D), UW288 (E) and DAOY (F) cells in a wound healing assay. Cells were treated with tofacitinib (10, 20 and 40 μ M) or DMSO and allowed to migrate into the scratched area for 24 hours. The percentage of migrating area in wound healing assay was quantified in UW426, UW288, and DAOY cells. (Red arrows indicate gap of scratched area, ***, p < 0.001).

Fig. (5). Src inhibitors significantly inhibit the migration of human medulloblastoma cells.

A-C: Representative pictures are shown of UW426 (**A**), UW288 (**B**) and DAOY (**C**) cells in a wound healing assay. This was conducted by scratching the cells with a yellow pipette tip when cells grew to monolayer. Cells were then treated with dasatinib (50 and 100nM) and allowed to migrate into the scratched area for 24 hours. The percentage of the migrating area in wound healing assay was quantified in UW426, UW288, and DAOY cells. **D-F:** Similarly, representative pictures are shown of UW426 (**D**), UW288 (**E**) and DAOY (**F**) cells in a wound healing assay. Cells were treated with KX2-391 (250 nM, 500 nM and 1000 nM) and allowed to migrate into the scratched area for 24 hours. The red arrows indicated the gap of the scratched area. The percentage of the migrating area in wound healing assay was quantified in UW426, UW288, and DAOY cells. (Red arrows indicate gap of scratched area, ***, $p < 0.001$).

Fig. (6). JAK/Src inhibitors induce apoptosis, inhibit JAK/STAT3 or Src/STAT3 phosphorylation and regulate Epithelial-Mesenchymal Transition (EMT) in human medulloblastoma cells.

A: UW426 cells were treated with ruxolitinib (2.5, 5 and 10 μ M), tofacitinib (2.5, 5 and 10 μ M), KX2-391 (0.1, 1 and 5 μ M) and dasatinib (0.1, 1 and 5 μ M) for 24 hours. The expression of p-JAK2 (Tyr1007/1008), p-JAK3 (Tyr980/981), p-STAT3 (Y705), p-SRC (Tyr416), p-AKT (S473), p-ERK1/2 (T202/Y204), E-Cadherin, N-Cadherin, cleaved caspase-3, JAK2, ERK, AKT, STAT3 and GAPDH was detected by Western blots. B: UW288 cells were treated with ruxolitinib (2.5, 5 and 10 μ M), tofacitinib (2.5, 5 and 10 μ M), KX2-391 (0.1, 1 and 5 μ M) and dasatinib (0.1, 1 and 5 μ M) for 24 hours. The expression of p-JAK2 (Tyr1007/1008), p-JAK3 (Tyr980/981), p-STAT3 (Y705), p-SRC (Tyr416), p-AKT

(S473), p-ERK1/2 (T202/Y204), E-Cadherin, NCadherin, cleaved caspase-3, JAK2, ERK, AKT, STAT3 and GAPDH was detected by western blots. C: DAOY cells were treated with ruxolitinib (1, 2.5, 5 and 10 μ M), tofacitinib (1, 2.5, 5 and 10 μ M), KX2-391(0.1, 1 and 5 μ M) and dasatinib (0.1, 1 and 5 μ M) for 24 hours. The expression of p-JAK2 (Tyr1007/1008), p-JAK3 (Tyr980/981), p-STAT3 (Y705), p-SRC (Tyr416), p-AKT (S473), p-ERK1/2 (T202/Y204), PTEN, E-Cadherin, N-Cadherin, cleaved caspase-3, JAK2, ERK, AKT, STAT3 and GAPDH was detected by western blots. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Fig. (7). Dasatinib shows synergistic effects when combined with cisplatin.

A: Human medulloblastoma cells (UW288, UW426, and DAOY) were seeded in 96-well plates at a density of 3000 cells per well for 24 hours and then treated with KX2-391, dasatinib, cisplatin, KX2-391 and cisplatin combined or dasatinib and cisplatin combined at the indicated doses for 72 hours. Cell viability was performed by MTT. The CI values of all the combination treatments were calculated by CompuSyn software. **B:** UW426, UW288, and DAOY cells were treated with dasatinib, cisplatin, dasatinib and cisplatin combined at the indicated doses for 72 hours. After treatment, the same number of cells were seeded and cultured in a drug-free medium for 1–2 weeks. Colonies were fixed by ice-cold methanol and stained by 1% crystal violet. **C:** Representative pictures are shown of UW426, UW288 and DAOY cells in a wound healing assay. It was acquired by scratching the cells with a yellow pipette tip when cells grew to monolayer. Then the cells were treated with dasatinib (25nM), cisplatin (5 μ M) and dasatinib (25nM)+cisplatin (5 μ M) in combination. Cells were allowed to migrate into the scratched area for 24 hours. The red arrows indicate the gap of the scratched area. The percentage of the migrating area in wound healing assay was quantified in UW426, UW288, and DAOY cells. **E:** DAOY cells were treated with dasatinib (25 nM), cisplatin (5 μ M) or in combination of both for 48 hours. The expression of p-

STAT3, p-Src, p-ERK and STAT3 was detected on western blots. GAPDH served as loading control. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

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Table 1.

IC50 for JAK and Src inhibitors in human medulloblastoma cells.

	Ruxolitinib	Tofacitinib	KX2-391	Dasatinib
UW288 (μ M)	27.73 $\pm$ 3.73	39.37 $\pm$ 2.88	0.021 $\pm$ 0.019	30.17 $\pm$ 8.24
UW426 (μ M)	92.81 $\pm$ 12.83	64.91 $\pm$ 12.35	0.062 $\pm$ 0.034	3.52 $\pm$ 0.48
DAOY (μ M)	0.93 $\pm$ 0.93	2.77 $\pm$ 0.51	10.24 $\pm$ 0.55	8.06 $\pm$ 0.71