

# Synergistic effect of STAT3-targeted small interfering RNA and AZD0530 against glioblastoma *in vitro* and *in vivo*

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**Abstract.** The aim of this study was to explore the synergistic effect of signal transducer and activator of transcription 3 (STAT3)-targeted small interfering (si)RNA and AZD0530 against glioblastoma *in vitro* and *in vivo*. Glioblastoma cell lines U87 and U251 were divided into four groups and treated with control, LV-STAT3 siRNA, AZD0530, and combined LV-STAT3 siRNA with AZD0530, respectively. The proliferation and apoptotic capacity of glioblastoma cells was assessed by Cell Counting Kit-8 and double staining flow cytometry assays, respectively. Additionally, the potential effect of LV-STAT3 siRNA and AZD0530 on glioblastoma was evaluated *in vivo*. Images were captured of the tumor formation in mice every week. Following three weeks of treatment, NMR scan and immunohistochemistry were performed. The treatment of combined LV-STAT3 siRNA and AZD0530 was more effective in inhibiting proliferation and inducing apoptosis of glioblastoma cells in comparison with the treatment of either LV-STAT3 siRNA or AZD0530 alone. Although LV-STAT3 siRNA or AZD0530 treatment alone suppressed tumor growth in mice, the combined treatment had a more significant effect than the treatment of LV-STAT3 siRNA or AZD0530 alone. According to the results of both *in vitro* and *in vivo* assays, a combined therapy of LV-STAT3 siRNA with AZD0530 could enhance therapeutic effects on glioblastoma, supporting the idea that the combination of LV-STAT3 siRNA and AZD0530 could serve as a novel and effective strategy to combat glioblastoma.

## Introduction

Glioblastoma is the most common malignant tumor that originates in the brain. Nowadays, glioblastoma becomes an intractable refractory tumor with high mortality, owing to its high degree of aggressiveness (1,2). Investigating its pathogenesis, administering appropriate therapy and improving the prognosis are essential but challenging in the field of neurosurgery and cancer research (3). Despite the development of traditional treatments, such as surgical tumor removal followed by the postoperative 40Gy-whole-brain, 15Gy-regional radiotherapy and chemotherapy (4), the resistance against chemotherapy and radiotherapy leads to the failure in obtaining satisfactory therapeutic benefits (5). Given the present data of clinical trials using molecular targets including monoclonal antibodies such as bevacizumab, or small molecule inhibitors such as bortezomib (6), more effective therapies are urgently needed to combat this disease. Such insight might be provided through proteomics and biomarker approaches that would simultaneously ascertain potential candidates for targeting therapy (7).

The signal transducer and activators of transcription (STAT) protein family is an ensemble of related protein receptors that could be triggered by specific cytokines. They serve as carriers in cytokine-receptor interaction and maintain specific intrinsic intracellular signaling (8,9). STAT3, as a member of STATs, could be phosphorylated by JAK. Additionally, STAT3 plays a critical role in a number of cellular processes such as cell growth and apoptosis. There exists an intimate relationship between STAT3 and multiple cancers. STAT3 is reported as being abnormally activated in glioblastoma, which therefore further activates multiple downstream genes and affects the growth, apoptosis and invasion of glioblastoma cells (10,11).

AZD0530 is a dual inhibitor of Src family kinase/Abl. A previous study indicated that in prostate cancer cell lines, AZD0530 suppressed the activation of Src and blocked cell growth by inducing G1/S cell cycle arrest (12). Additionally, AZD0530 could reduce the expression of c-Myc, cyclin D1 and  $\beta$ -catenin, and prevent the phosphorylation of extracellular signal regulated kinase 1/2 and glycogen synthase kinase 3 $\beta$ . AZD0530 is also known as a selective and high efficacy Src-kinase inhibitor (11,13). AZD0530 mechanistically features cooperation with the Src kinase and competition

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**Abbreviations:** STAT, signal transducer and activators of transcription; siRNA, small interfering RNA; STAT3, signal transducer and activator of transcription 3; RNAi, RNA interference; Bcl-2, B cell lymphoma-2

**Key words:** small interfering RNA, signal transducer and activator of transcription 3, AZD0530, glioblastoma, proliferation, apoptosis

of the kinase with the ATP binding site, thereby inhibiting the kinase activity, and impeding tumor migration, invasion, proliferation, and division (14). Upon the basis of the authors' preliminary findings (15), the present study used the STAT3 small interfering (si)RNA and Src protein inhibitor AZD0530 to specifically target Src-Abl, and evaluated the effects of combined therapy on glioblastoma.

## Materials and methods

**Ethics statement.** Animal studies were approved by the Tianjin Huanhu Hospital Institutional Animal Care and Use Committee.

**Reagents and antibodies.** AZD0530 was purchased from Selleck Chemicals and dissolved in dimethyl sulfoxide (DMSO) as a stock solutions of 10 mg/ml, then stored at -20°C. The anti- $\beta$ -actin, anti-STAT3, anti-p-STAT3, anti-SRC and p-SRC antibodies were bought from Abcam, the anti-B cell lymphoma-2 (Bcl-2) antibody was purchased from Signalway Antibody and the SABC Immunohistochemistry assay kit was provided by Abcam.

**Cell lines and cell cultures.** The human glioblastoma cell lines, U87 and U251, are glioblastoma of unknown origin and were purchased from the American type culture collection (catalogue number: ATCC HTB-14) and the European Collection of Authenticated Cell Cultures (cat. no. 09063001), respectively. Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 0.25  $\mu$ g/ml amphotericin B in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. All reagents were obtained from Invitrogen; Thermo Fisher Scientific, Inc.

**STAT3 siRNA lentivirus vectors.** Lentivirus vectors containing siRNA targeted STAT3 (LV-STAT3 siRNA) and an empty control of lentivirus vectortagged with green fluorescent protein (GFP) and luciferase (LV-empty) were purchased from Genbank. In brief, cells were infected with the luciferase lentivirus and implanted into mice. The activity of luciferase was measured using a small animal *in vivo* imaging system (In-Vivo Xtreme, Bruker Corporation). The STAT3 Gene Bank Accession Number is NC\_000017 and the siRNA targeted sense sequence was as follows (5'-CTTCAGACCCGTCAACAAA-3').

**Cell transfection.** The human glioblastoma cell lines U87 and U251 (2x10<sup>5</sup>) in serum-free DMEM medium were transfected with LV-STAT3 siRNA, LV-empty using Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific, Inc.) and treatment with 20  $\mu$ M AZD0530, respectively, or combined treatment with LV-STAT3 siRNA and AZD0530. Cells treated with DMSO served as a negative control. After 6 h, the serum-free culture medium was replaced with fresh complete DMEM and continued incubation for 36 h. The expression of GFP showed 80-90% infection efficiency.

**Cell proliferation assay.** Cell proliferation was measured by Cell Counting Kit-8 (CCK-8) assay. Briefly, glioblastoma cell

lines U87 and U251 were suspended and diluted to 5x10<sup>4</sup> cells/ml with DMEM, and 100  $\mu$ l cell solution (5,000 cells) were seeded into 96-well plates, then incubated overnight at 37°C. Next day, cells were transfected with LV, LV-STAT3 siRNA, LV-empty, treatment with AZD0530 or combined treatment with LV-STAT3 siRNA and AZD0530, respectively. During eight consecutive days, 10  $\mu$ l CCK-8 solution was added to each well for 3 h. The absorbance was measured at 450 nm using a microplate reader. For each group, three duplicate wells were set up and the data were summarized as mean  $\pm$  standard deviation (SD).

**Analysis of apoptosis.** Cell apoptosis was detected using Annexin V-PE Apoptosis Detection kit (cat. no. 559763, BD Biosciences). After treatment 96 h, cells from each group were harvested and adjusted concentration to 1x10<sup>6</sup>/ml, then 5  $\mu$ l Annexin V-Alexa Fluor 647, and 10  $\mu$ l propidium iodide (PI) were added. After 15 min in darkness under room temperature, 300  $\mu$ l PBS was added and analyzed with FACS Calibur (BD Pharmingen; BD Biosciences). Flow cytometry data were analyzed with CellQuest software (1998, BD Pharmingen; BD Biosciences).

**Immunoblot assays.** Glioblastoma cells were lysed in RIPA buffer (cat. no. 9800; Cell Signaling, Inc.). Then total proteins (30  $\mu$ g) were quantified by the BCA method and analyzed by 8% SDS-PAGE. Subsequently the polyvinylidene fluoride (PVDF) membranes were blocked with 5% milk buffer for 2 h at room temperature and then incubated with the primary antibodies (A11:500 dilution) at room temperature for 2 h. Then the PVDF membranes were incubated with HRP-conjugate secondary antibodies (Goat anti Rabbit and goat anti mice, cat. nos. 31430 and 31460, Thermo Fisher Scientific, Inc. 1:5,000 dilution) at room temperature for 45 min. Blots were detected with an ECL kit (ECL Substrate Kit, ab133406, Abcam). Image Pro software 6.0 (Media Cybernetics, Inc.) was used in this assay to calculate the intensity of each blot.

**Establishment of animal model.** A total of 130 BALB/c nude mice (half male and female; 10 weeks old; 20 g) were randomly assigned into five groups (n=26 for each group), including the negative control group, the empty vector group, the LV-STAT3 siRNA group, AZD0530 group and the LV-STAT3 siRNA-AZD0530 combination-therapy group. The mice were maintained in the place where environment temperature was 25°C, the humidity was 50%, the light and darkness were alternated for 12 h, and free water and food were given. Three group's mice were respectively subjected to LV, LV-STAT3 siRNA transfected U87 cells and LV-empty transfected U87 cells differential intracranial injection through the stereotactic approach (16,17). The AZD0530 group and for the combination groups, after LV-STAT3 siRNA transfected cell intracranial injection, 50 mg/kg AZD0530 was received via oral gavage 5 times per week for 3 weeks. Tumor sizes were measured by fluorescent images of whole mice at 7, 14 and 21 days. In view of humane endpoints, the mice whose tumors grew to a size >1,000 mm<sup>3</sup> were sacrificed. The volume (V) of tumors was calculated using the formula: V=0.5xW<sup>2</sup>xL. Additionally, the largest diameter of tumor was measured using a vernier caliper.

**Nuclear magnetic resonance (NMR) scanning.** Mice were imaged by a small animal *in vivo* imaging system. After the final luciferase imaging, the mice were anesthetized with isoflurane and situated in a magnetic coil in 3T NMR (GE Healthcare) for two-dimensional scans with T2-weighted imaging to further assess the position of the tumor and the extent of its spread. The tumor size was calculated according to previous studies (18,19). Scanning parameters: 1.2 mm isotropic spatial resolutions, repetition time=2,500 msec, band width=203 Hz/px, matrix=256x256x128. The first echo time=72 msec, echo spacing=12 msec, flip angle=1,200, the scan time=5 min 34 sec. Three days after the experiment, animals were sacrificed to extract tumor tissue for immunohistochemical analysis.

**Immunohistochemistry.** Non-necrotic and cystic tumor tissues of mice models were selected to make pathological sections (5  $\mu$ m thickness) and the sections were subsequently deparaffinized and rehydrated, then 3% hydrogen peroxide was used to remove endogenous peroxidase activity. Antigen retrieval was performed using 0.01 mol/l sodium citrate buffer at 92-98°C. The sections were blocked with 5% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.) in PBS at room temperature for 30 min to avoid non-specific binding, then anti-p-STAT3 (Tyr705; 0.5 mg/ml; BioLegend, Inc.) and anti-Bcl-2 (1:50; 1 mg/ml; BioLegend, Inc.) were added to sections, respectively and incubated overnight at 4°C. After washing with phosphate buffer, the sections were incubated in alkaline phosphatase-labeled streptavidin working solution (1:200) for 30 min at 37°C. The sections were visualized with DAB, finally, the sections were counterstained with hematoxylin for 2 min at room temperature, dehydrated, then mounted. For negative controls, normal rabbit serum replaced the primary antibody. Semi-quantitative analysis: Under high magnification (CX23, x400, Olympus Corporation), tumor regions were randomly selected for gray area examination (ImageJ 1.8.0, National Institutes of Health), with possible values ranging from 0 (black) to 255 (white) and a greater gray value denotes a higher protein expression, and the relative protein level was normalized to combined group.

**Statistical analysis.** Results were analyzed using SPSS software 13.0 and compared using one-way analysis of variance. Multi-group comparisons in this study were carried out by one-way analysis of variance test followed by a Student-Newman-Keuls post hoc test. Comparisons between the parametric groups were done using the LSD-t test. The data was presented as the mean  $\pm$  SD of three or six independent experiments was defined to be appropriate.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Combined treatment of STAT3 siRNA and AZD0530 inhibits the proliferation of glioblastoma cells.** The CCK-8 assays produced a stable growth curve for the transfected cell (Fig. 1). The expression of STAT3, SRC, pSRC decreased gradually within four days in AZD0530-treated U87 and U251 cells. The AZD0530 gave noticeable inhibition compared with the control groups.

Similarly, LV-STAT3 siRNA treatment had an obvious inhibition effect on the proliferation of U87 and U251 cells, compared with control groups. Interestingly, combination therapy had a significant inhibitory effect on U87 and U251 cell proliferation compared with other groups ( $P < 0.001$ ), and the mean inhibition of tumor growth rate were 28.60 and 36.36%, respectively. As a comparison, the curves for the negative control group and the empty vector cell growth were similar, displaying a modest effect on tumor growth. Based on this, the present study further detected the level of both STAT3 and phosphorylated STAT3 after siRNA and AZD0530 combined treatment. It was noticed that the expression levels of STAT3 was decreased in both U251 and U87 cells (Fig. 1B). The expression level of SRC and its phosphorylation level were also detected, and found that both was decreased in U251 cells (Fig. 1C). Therefore, these results suggest that the combined treatment inhibited the proliferation of glioblastoma cells in a SRC-dependent manner.

**Combined treatment shows pro-apoptotic effects on glioblastoma cells in vitro.** Through flow cytometry assays, it was demonstrated that the combined treatment with STAT3 siRNA and AZD0530 induced the apoptosis of glioblastoma cells. After 96 h incubation, glioblastoma U87 and U251 cells with AZD0530 and STAT3 siRNA treatment were analyzed through Annexin V-Alexa Fluor 647 and PI double staining flow cytometry assays, an increased rate of apoptosis was observed in combined treatment group compared with the STAT3 siRNA and AZD0530 alone group ( $P = 0.029$ ), and the rate of apoptosis in the STAT3 siRNA group was increased compared with AZD0530 treatment alone. There was a statistically significant difference among STAT3 siRNA group, AZD0530 treated group, and the combined treatment group ( $P < 0.05$ ). The combined treatment group, STAT3 siRNA and AZD0530 treated group exhibited a significantly increased-level of apoptosis compared with the empty-vector group and control group ( $P < 0.05$ ). Whereas no significant difference was found between the empty vector group and the control group ( $P = 0.554$ ), and there were similar results between U87 and U251 cell lines (Fig. 2). All these results indicate that the STAT3 siRNA and AZD0530 treatment groups showed significant effect on the apoptosis of glioblastoma cells, respectively. However, the effect granted by the combined treatment group was even more notable.

**Combined treatment with STAT3 siRNA and AZD0530 inhibits tumor growth in mice.** To further explore the potential synergistic role of LV-STAT3 siRNA and AZD0530 on glioblastoma, their effects on tumor growth were assessed *in vivo*. Aside from the negative control group, all mice showed xenograft formation in the right brain caudate nucleus (Fig. 3A-C). In the first week after the inoculation, tumor formation was detected and after 2 weeks, a period of accelerated growth was noted with the degree of growth proportional to time. Larger tumors may invade contralaterally via the corpus callosum (Fig. 3C). A comparison of the tumor fluorescence value (Fig. 3B) showed that for the first week, the LV-STAT3 siRNA group, the AZD0530 group and the combined treatment group had significantly lower luminosity compared with the empty

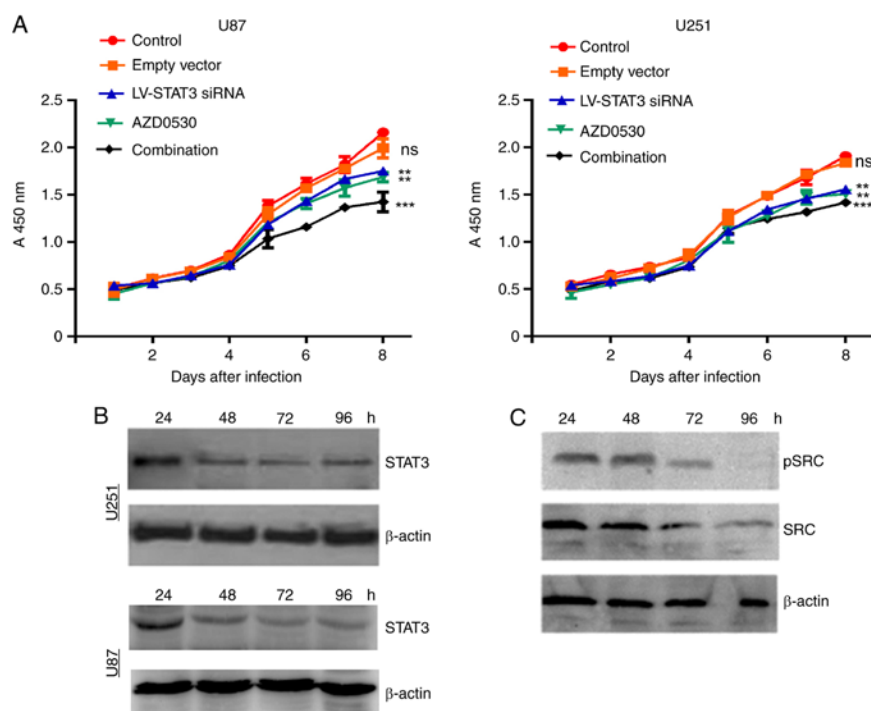


Figure 1. LV-STAT3 siRNA and AZD0530 combination therapy dramatically inhibits the proliferation of glioblastoma cells. (A) The growth curves of U87 and U251 cells upon the indicated treatment. (B) The expression levels of STAT3 upon AZD0530 treatment were detected through immunoblot assays after 24, 48, and 72 h in both U87 and U251 cells. (C) The expression levels of SRC and p-SRC upon AZD0530 treatment were detected through Immunoblot assays after 24, 48, and 72 h in U251 cells. Results are presented as mean  $\pm$  standard error of the mean, ns, not significant, \*\*P<0.01 vs. control \*\*\*P<0.001 vs. other groups. p-STAT3, phosphorylated signal transducer and activator of transcription-3; siRNA, small interfering RNA.

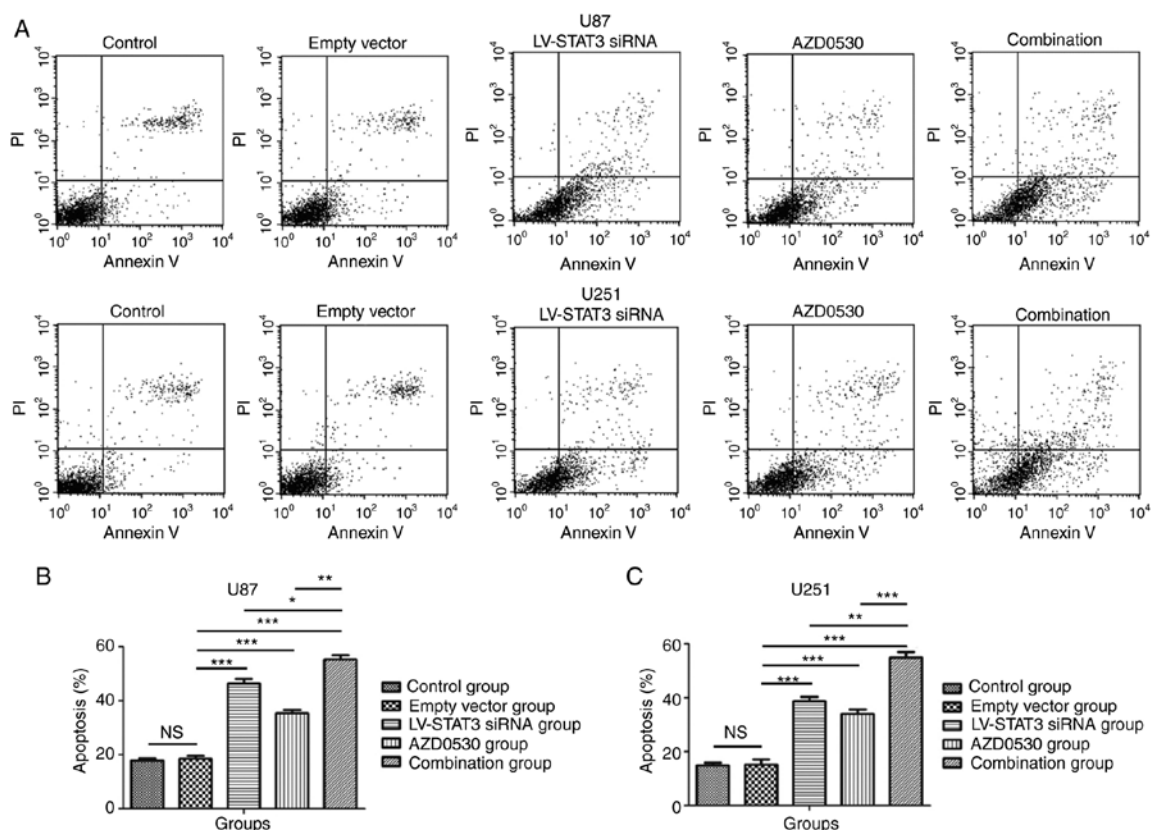


Figure 2. Combined treatment with LV-STAT3 siRNA and AZD0530 significantly induces apoptosis of glioblastoma cells. A total of 96 h after treatment, glioblastoma cells were analyzed by flow cytometry. (A) Data of Annexin V-Alexa Fluor 647 and PI double staining flow cytometry assays showed that the LV-STAT3 siRNA, AZD0530 and the combination groups induced significant apoptosis after treatment 96 h compared with the control and empty vector groups in U87 and U251 cells, respectively (n=3). The effects of the combination therapy are less than additive. (B and C) Bar charts of data presented in panel A. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001. p-STAT3, phosphorylated signal transducer and activator of transcription-3; siRNA, small interfering RNA; ns, not significant.



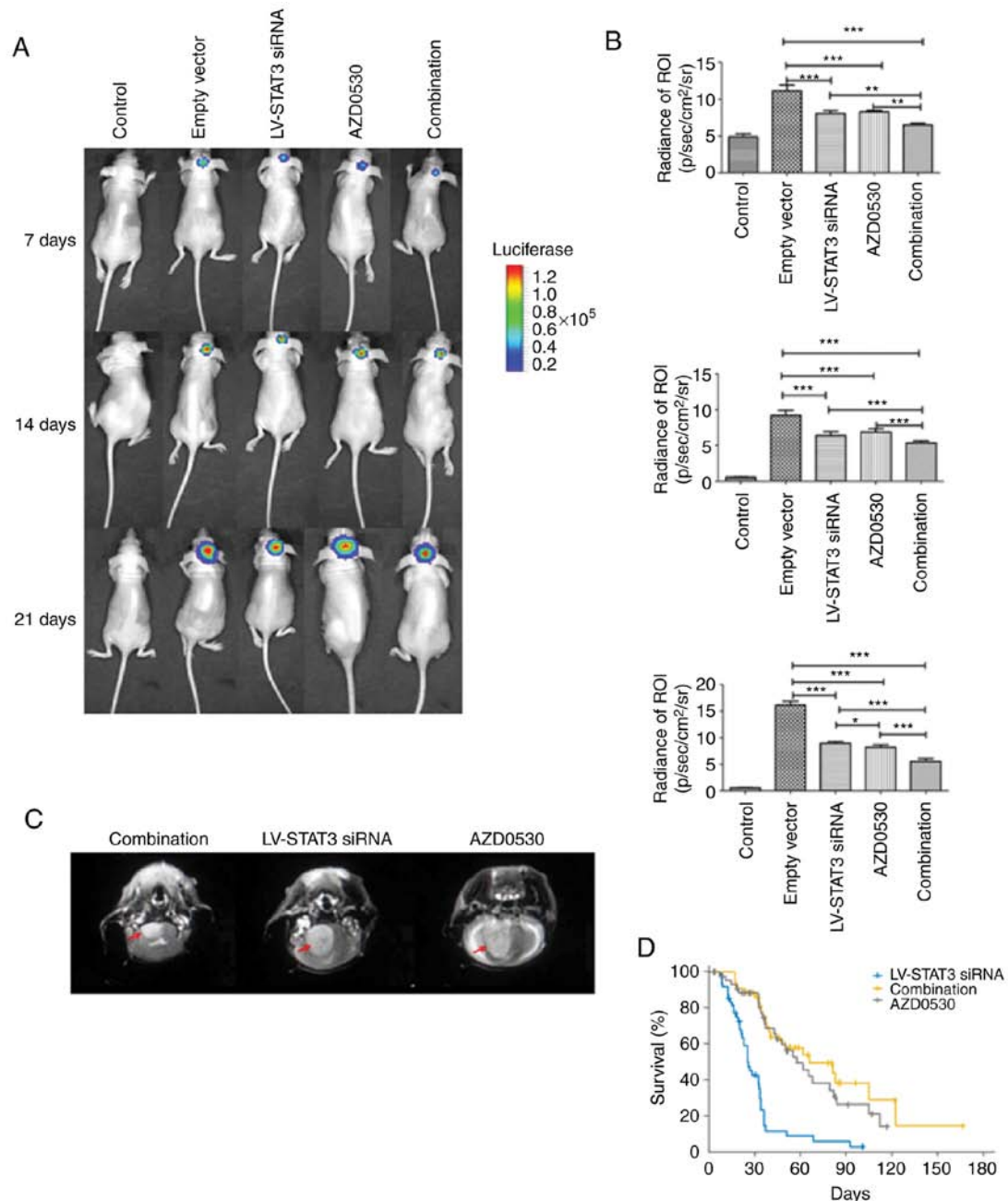


Figure 3. Combined treatment with LV-STAT3 siRNA and AZD0530 inhibits glioblastoma growth in mice. (A) Results of intracranial imaging for glioblastoma cells. (B) Bar charts of data presented in panel A. (C) Xenograft formation in the right brain caudate nucleus. (D) Survival time curves of different treatment groups. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . p-STAT3, phosphorylated signal transducer and activator of transcription-3; siRNA, small interfering RNA.

vector group ( $P < 0.001$ ). However, between any two of the three groups, there was no significant difference ( $P > 0.05$ ). The luminosity was the greatest for the empty vector group, bearing the largest tumor. Notably, this luminosity may reflect cellular activity but not tumor growth. The values of the first week and the second week were similar, but along with the enlargement of the tumor, the combinational therapy group showed the least luminosity, followed by the LV-STAT3 siRNA group and the AZD0530 group; the empty vector group had the maximum luminosity. The results were statistically significant ( $P < 0.001$ ). However, the effects of the LV-STAT3 siRNA group and the AZD0530 group were not statistically different ( $P = 0.517$ ).

*Combination therapy results in greater tumor inhibition.* According to the results of the *in vivo* assays, the present study found that the luminosity for LV-STAT3 siRNA, AZD0530, or combined treatment groups were decreased compared with the control (Fig. 3A and B). Additionally, with tumor enlargement, the luminosity for LV-STAT3 siRNA and AZD0530 treatment alone group was significantly decreased compared with the control, and the luminosity for the combined treatment group was significantly lower than that in other groups ( $P < 0.001$ ).

As treatment continued, the STAT3 targeted siRNA loaded lentivirus had a more pronounced and sustained anti-tumor effect. From the observation of weight changes in mice, the LV-STAT3 siRNA group had little influence on body weight

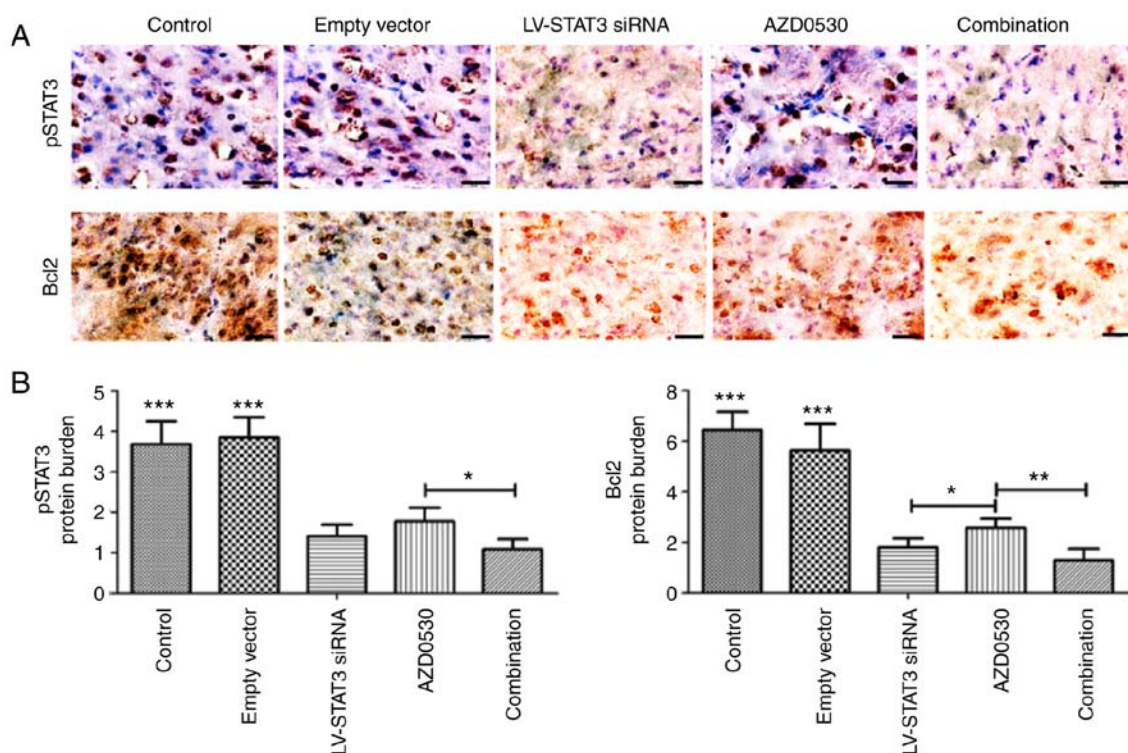


Figure 4. Combination treatment decreases the expression levels of p-STAT3 and Bcl-2. (A) Immunohistochemical staining of tumor tissue. p-STAT3 and Bcl-2 protein was highly expressed in the negative control group and the empty vector group, and relative less in the AZD0530 group, the LV-STAT3 siRNA group and the combined treatment group. (Scale bar, 100  $\mu$ M). (B) Bar charts of data presented in panel A. Results are presented as mean  $\pm$  standard error of the mean, \* $P < 0.05$ ; \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. combination group. p-STAT3, phosphorylated signal transducer and activator of transcription-3; siRNA, small interfering RNA; Bcl-2, B cell lymphoma-2.

(data not shown). While the negative control group had a significantly greater luminosity relative to the other groups ( $P < 0.05$ ; Fig. 3C). Interestingly, the data of K-M survival analysis further confirmed the improved survival of mice in the combined treatment group (Fig. 3D).

*Combined therapy decreases STAT3 phosphorylation and the expression of Bcl-2 in vivo.* According to the results of IHC assays, p-STAT3 and Bcl-2 was mainly localized in the cytoplasm of tumor cells, demonstrated by the light yellow, yellow, or tantint. There were sometimes mild coloration in the nuclei (Fig. 4A). The p-STAT3 was highly expressed in the control and empty vector group, but expression was less in the AZD0530 group, LV-STAT3 siRNA group and the combined treatment group. The p-STAT3 protein expression value for the negative control group and the empty vector group was ~three times that of the AZD0530 alone group, LV-STAT3 siRNA alone group and the combined treatment group. There were also a significant difference of values among the AZD0530 group and combined treatment group (Fig. 4B).

Bcl-2 expression was associated with p-STAT3, which suggested that lentiviral transfection with STAT3-targeted siRNA produced a significant reduction of p-STAT3 and Bcl-2 expression in tumor tissues ( $P < 0.05$ ).

## Discussion

STAT3 abnormal activation has a close relationship to cancer. Therefore, the abnormal activation of STAT3 could

encourage the genetic transcription of cyclin D1, Bcl-x1, c-Myc, interleukin (IL)-10, vascular endothelial growth factor (VEGF) (20), assisting the tumor in cellular survival, proliferation, angiogenesis and mitochondrial respiratory chain transfer (21).

STAT3 is abnormally expressed in human malignant glioblastoma tissues. Suppressing the expression of the STAT3 signal pathway or silencing STAT3 (22) could induce glioblastoma cell apoptosis and differentiation, and inhibit the proliferation and angiogenesis (23). siRNA through a DNA-directed RNA interference (RNAi) approach, enables gene silencing and expression attenuation, thereby creating a sustained block of the expression of a particular protein. Therefore, siRNA has become a potential new nucleic-acid drug (24). RNAi can be used to reduce STAT3 expression in glioblastoma cells and facilitate apoptosis (25). This study used STAT3-siRNA-lentivirus vectors with glioblastoma transfection and the subsequent luciferase imaging to monitor the dynamic changes of the tumor *in vivo*.

The JAK/STAT pathway is a major signaling pathway for relaying external signals into the nucleus (26). Cytokines IL-6, IL-10, growth factors epidermal growth factor, hepatocyte growth factor, HER2/Neu, VEGF and other mediators such as erythropoietin and granulocyte-macrophage-colony stimulating factor can induce STAT3 tyrosine phosphorylation and activation. STAT3 tyrosine can be phosphorylated by three kinds of protein kinases, such as Src, a non-receptor tyrosine kinases. After tyrosine phosphorylation, STAT3 forms homo- or heterodimers, enters the nucleus and binds to specific DNA sequences, and initiates the expression of the target gene (18).

Therefore, blocking the Src signaling pathway can indirectly compromise the STAT3 signaling pathway (27).

AZD0530 is a Src-Abl dual inhibitor that produces promising results in preclinical and clinical studies on solid tumors and osteolytic lesions (28). This study is the first time to the best of our knowledge that direct siRNA and the indirect AZD0530 was jointly used to block the STAT3 signaling pathway as a part of a therapeutic strategy.

AZD0530 is an inhibitor of SRC and therefore it could affect the phosphorylation of STAT3. However, the present study found that AZD0530 not only can affect the phosphorylation level of STAT3, but also has an effect on the expression of STAT3. In general, AZD0530 has a more significant effect on the phosphorylation of STAT3. The present study hypothesized that AZD0530 may lead to the ubiquitination degradation of STAT3 to some extent, but the finer molecular mechanism needs to be confirmed by further experiments.

Mitochondria are the crucial integrators and coordinators of both intracellular and extracellular signals that mediate caspase-dependent and caspase-independent cell death (29). Loss of the mitochondrial membrane potential is a prerequisite for mitochondrial-mediated apoptosis (27). In addition, members of Bcl-2 family are major regulators of Cytochrome C release from mitochondria. They participate in downstream caspase activation and play an important role in the regulation of glioblastoma cell apoptosis (30,31).

Through flow cytometry assays, it was found that the LV-STAT3 siRNA group, AZD0530 group and the combined treatment group had an inhibitory effect *in vitro*, with the combined treatment being the most effective. Meanwhile, apoptotic rates for these groups increased 2.51, 1.92 and 2.99 times in their respective orders. STAT3-targeting siRNA and Src-blocker AZD0530 lowered the expression of STAT3 in U87 and U251 cells, and in turn, promoted apoptosis in glioblastoma. Although currently there are multiple theories concerning the mechanism of apoptosis initiation, the present study revealed that LV-STAT3 siRNA might cause apoptosis through the mitochondrial pathway (15).

In this study, *in vivo* imaging was used after tumor inoculation to non-invasively detect the continuous growth of tumors. A week following the inoculation, obvious tumor formation and comparable luminescence values were found for the LV-STAT3 siRNA group, the AZD0530 group and the combined treatment group. In the second week, the empty vector group exhibited the greatest luminescence, while the combined approach showed the least. The Src signaling pathway inhibitor AZD0530 of the tumor had its effect dampened. Moreover, the third week had similar outcomes, with the combined treatment group giving greater cancer inhibition, while the influence by AZD0530 diminished further. Although the effect of STAT3 blocking is noticeable by dynamically observing tumor growth, the block of STAT3 is weakened owing to *in vivo* drug metabolism and tumor drug resistance, whereas STAT3-directed RNAi elicited a sustained inhibition on tumor growth. This calls for a stronger emphasis on the underlying mechanisms for AZD0530 resistance in future research endeavors. Immunohistochemistry assays indicated that p-STAT3 was highly expressed in the negative control group, the empty vector group and the AZD0530 group. The LV-STAT3 siRNA group and the combined treatment group

in turn showed a decline of expression. LV-STAT3 siRNA continued to block the STAT3 expression and achieved the purpose of undermining tumor growth. As the central target molecules of the mitochondrial apoptosis pathway, Bcl-2 protein (32) and STAT3 protein shared similar trends in expression. From a different standpoint, it validates the results from the authors' earlier study on the apoptosis effects of LV-STAT3 siRNA via the mitochondrial pathway (21).

Recent studies demonstrated that IL-8 derepression represents an critical and functionally-relevant consequence of deregulation of the PTEN-STAT3 tumor suppressive pathway in tumor cells (33,34). The downregulation of STAT3-IL-8 signaling pathway contributes to the proliferation and invasion of glioblastoma cells. Additionally, STAT3 is known to be activated in glioblastoma. These studies further confirmed the present study's hypothesis.

Integrated results from *in vitro* and *in vivo* studies suggest that *in vivo*, LV-STAT3 siRNA and AZD0530 individually caused significant clearance of glioblastoma while triggering apoptosis. However, a combination-therapeutic approach was able to render a more striking effect in eliminating cancer cells. LV-STAT3 siRNA in orthotopic glioblastoma was capable of continual expression by blocking the STAT3 expression. STAT3 was one of the targets for cancer therapy, and a recombinant lentivirus carrying siRNA that silences the STAT3 gene would yield a desired and sustained inhibition on tumor, and its combination with AZD0530 could augment the cancer inhibition even further. This method provides a novel and effective way to combat glioblastoma.

In conclusion, this study showed combined therapy of LV-STAT3 siRNA with AZD0530 could enhance the therapeutic effect against glioblastoma. Combined treatment inhibited proliferation and promoted apoptosis of glioblastoma cells *in vitro*, and inhibited tumor growth in mice. Mechanistic studies confirmed that the combined therapy decreased STAT3 phosphorylation and the expression of Bcl-2 *in vivo*. Taken together, this study supports the idea that combination of LV-STAT3 siRNA and AZD0530 could provide a novel and effective strategy to combat glioblastoma.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

QL designed the study; DL, JZ, SC, JL and LW searched and selected the articles, conducted the data extraction and did the statistical analysis; QL and LW wrote the manuscript. All authors read and approved the final manuscript.



## Ethics approval and consent to participate

This study was approved by the Ethics Committee at Tianjin Huanhu Hospital.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## References

- Niu G, Wright KL, Huang M, Song L, Haura E, Turkson J, Zhang S, Wang T, Sinibaldi D, Coppola D, *et al*: Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis. *Oncogene* 21: 2000-2008, 2002.
- Gray GK, McFarland BC, Nozell SE and Benveniste EN: NF- $\kappa$ B and STAT3 in glioblastoma: Therapeutic targets coming of age. *Expert Rev Neurother* 14: 1293-1306, 2014.
- Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, *et al*: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 352: 987-996, 2005.
- Roa W, Brasher PM, Bauman G, Anthes M, Bruera E, Chan A, Fisher B, Fulton D, Gulavita S, Hao C, *et al*: Abbreviated course of radiation therapy in older patients with glioblastoma multiforme: A prospective randomized clinical trial. *J Clin Oncol* 22: 1583-1588, 2004.
- Hide T, Takezaki T, Nakamura H, Kuratsu J and Kondo T: Brain tumor stem cells as research and treatment targets. *Brain Tumor Pathol* 25: 67-72, 2008.
- Yang F, Brown C, Buettner R, Hedvat M, Starr R, Scuto A, Schroeder A, Jensen M and Jove R: Sorafenib induces growth arrest and apoptosis of human glioblastoma cells through the dephosphorylation of signal transducers and activators of transcription 3. *Mol Cancer Ther* 9: 953-962, 2010.
- Liu Y, Li C and Lin J: STAT3 as a therapeutic target for glioblastoma. *Anticancer Agents Med Chem* 10: 512-519, 2010.
- Koskela HL, Eldfors S, Ellonen P, van Adrichem AJ, Kuusanmäki H, Andersson EI, Lagström S, Clemente MJ, Olson T, Jalkanen SE, *et al*: Somatic STAT3 mutations in large granular lymphocytic leukemia. *N Engl J Med* 366: 1905-1913, 2012.
- Zhang XP, Zheng G, Zou L, Liu HL, Hou LH, Zhou P, Yin DD, Zheng QJ, Liang L, Zhang SZ, *et al*: Notch activation promotes cell proliferation and the formation of neural stem cell-like colonies in human glioma cells. *Mol Cell Biochem* 307: 101-108, 2008.
- Fan QW, Cheng C, Knight ZA, Haas-Kogan D, Stokoe D, James CD, McCormick F, Shokat KM and Weiss WA: EGFR signals to mTOR through PKC and independently of Akt in glioma. *Sci Signal* 2: ra4, 2009.
- Molina JR, Foster NR, Reungwetwattana T, Nelson GD, Grainger AV, Steen PD, Stella PJ, Marks R, Wright J and Adjei AA: A phase II trial of the Src-kinase inhibitor saracatinib after four cycles of chemotherapy for patients with extensive stage small cell lung cancer: NCCTG trial N-0621. *Lung Cancer* 85: 245-250, 2014.
- Lin M, Yao Z, Zhao N and Zhang C: TLK2 enhances aggressive phenotypes of glioblastoma cells through the activation of SRC signaling pathway. *Cancer Biol Ther* 20: 101-108, 2018.
- Nygaard HB, Wagner AF, Bowen GS, Good SP, MacAvoy MG, Strittmatter KA, Kaufman AC, Rosenberg BJ, Sekine-Konno T, Varma P, *et al*: A phase Ib multiple ascending dose study of the safety, tolerability, and central nervous system availability of AZD0530 (saracatinib) in Alzheimer's disease. *Alzheimers Res Ther* 7: 35, 2015.
- De Luca A, D'Alessio A, Gallo M, Maiello MR, Bode AM and Normanno N: Src and CXCR4 are involved in the invasiveness of breast cancer cells with acquired resistance to lapatinib. *Cell Cycle* 13: 148-156, 2014.
- Liu J, Xu X, Feng X, Zhang B and Wang J: Adenovirus-mediated delivery of bFGF small interfering RNA reduces STAT3 phosphorylation and induces the depolarization of mitochondria and apoptosis in glioma cells U251. *J Exp Clin Cancer Res* 30: 80, 2011.
- Baumann BC, Dorsey JF, Benci JL, Joh DY and Kao GD: Stereotactic intracranial implantation and in vivo bioluminescent imaging of tumor xenografts in a mouse model system of glioblastoma multiforme. *J Vis Exp*: pii: 4089, 2012.
- Sansone P and Bromberg J: Targeting the interleukin-6/Jak/stat pathway in human malignancies. *J Clin Oncol* 30: 1005-1014, 2012.
- Wadghiri YZ, Li J, Wang J, Hoang DM, Sun Y, Xu H, Tsui W, Li Y, Boutajangout A, Wang A, *et al*: Detection of amyloid plaques targeted by bifunctional USPIO in Alzheimer's disease transgenic mice using magnetic resonance microimaging. *PLoS One* 8: e57097, 2013.
- Tomayko MM and Reynolds CP: Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother Pharmacol* 24: 148-154, 1989.
- Alvarez JV and Frank DA: Genome-wide analysis of STAT target genes: Elucidating the mechanism of STAT-mediated oncogenesis. *Cancer Biol Ther* 3: 1045-1050, 2004.
- Leslie K, Gao SP, Berishaj M, Podsypanina K, Ho H, Ivashkiv L and Bromberg J: Differential interleukin-6/Stat3 signaling as a function of cellular context mediates Ras-induced transformation. *Breast Cancer Res* 12: R80, 2010.
- Sanseverino I, Purificato C, Gauzzi MC and Gessani S: Revisiting the specificity of small molecule inhibitors: The example of statin in dendritic cells. *Chem Biol* 19: 1213-1216, 2012.
- Barre B, Vigneron A and Coqueret O: The STAT3 transcription factor is a target for the Myc and riboblastoma proteins on the Cdc25A promoter. *J Biol Chem* 280: 15673-15681, 2005.
- Rao DD, Vorhies JS, Senzer N and Nemunaitis J: siRNA vs. shRNA: Similarities and differences. *Adv Drug Deliv Rev* 61: 746-759, 2009.
- Gao LF, Xu DQ, Wen LJ, Zhang XY, Shao YT and Zhao XJ: Inhibition of STAT3 expression by siRNA suppresses growth and induces apoptosis in laryngeal cancer cells. *Acta Pharmacol Sin* 26: 377-383, 2005.
- Fire MA and Gellerman G: Targeted drug delivery for cancer therapy: The other side of antibodies. *J Hematol Oncol* 5: 70, 2012.
- Formisano L, Nappi L, Rosa R, Marciano R, D'Amato C, D'Amato V, Damiano V, Raimondo L, Iommelli F, Scorziello A, *et al*: Epidermal growth factor-receptor activation modulates Src-dependent resistance to lapatinib in breast cancer models. *Breast Cancer Res* 16: R45, 2014.
- de Wispelaere M, LaCroix AJ and Yang PL: The small molecules AZD0530 and dasatinib inhibit dengue virus RNA replication via Fyn kinase. *J Virol* 87: 7367-7381, 2013.
- Bourke LT, Knight RA, Latchman DS, Stephanou A and McCormick J: Signal transducer and activator of transcription-1 localizes to the mitochondria and modulates mitophagy. *JAKSTAT* 2: e25666, 2013.
- Chan SL and Yu VC: Proteins of the bcl-2 family in apoptosis signalling: From mechanistic insights to therapeutic opportunities. *Clin Exp Pharmacol Physiol* 31: 119-128, 2004.
- Gielen PR, Aftab Q, Ma N, Chen VC, Hong X, Lozinsky S, Naus CC and Sin WC: Connexin43 confers Temozolomide resistance in human glioma cells by modulating the mitochondrial apoptosis pathway. *Neuropharmacology* 75: 539-548, 2013.
- Coleman CB, McGraw JE, Feldman ER, Roth AN, Keyes LR, Grau KR, Cochran SL, Waldschmidt TJ, Liang C, Forrest JC and Tibbetts SA: A gammaherpesvirus Bcl-2 ortholog blocks B cell receptor-mediated apoptosis and promotes the survival of developing B cells in vivo. *PLoS Pathog* 10: e1003916, 2014.
- Abdalla F, Nookala A, Padhye SB, Kumar A and Bhat HK: 4-(E)-(p-tolylimino)-methylbenzene-1,2-diol (TIMBD) suppresses HIV1-gp120 mediated production of IL6 and IL8 but not CCL5. *Sci Rep* 7: 8129, 2017.
- Guo Y, Zang Y, Lv L, Cai F, Qian T, Zhang G and Feng Q: [Corrigendum] IL-8 promotes proliferation and inhibition of apoptosis via STAT3/AKT/NF- $\kappa$ B pathway in prostate cancer. *Mol Med Rep* 19: 2970, 2019.



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