Original Article
WP1066 prevents retinoblastoma cell growth in vitro and in vivo

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Abstract: The compound WP1066, a novel small molecule STAT3 inhibitor, was originally synthesized by modifying the structure of AG490, which inhibits the activation of signal transducer and activator of transcription 3 (STAT3) by directly targeting Janus kinases (JAKs). WP1066 exhibits stronger anti-cancer activity than AG490 against malignant glioma and other cancer cells and is regarded as a promising therapeutic agent. Whether WP1066 has antitumor effect by targeting STAT3 signaling in retinoblastoma (RB) cells is unclear. In the present study, we found that WP1066 inhibited persistent STAT3 phosphorylation and induced apoptosis in human RB Y79 cell lines. In addition, WP1066 also inhibited colony formation and decreased the viability of Y79 cells. Furthermore, WP1066 suppressed RB tumor growth in a mouse model in vivo and suggested that WP1066 is a promising therapeutic drug candidate for RB by inhibiting persistent STAT3 activity.

Keywords: Retinoblastoma (RB), signal transducer and activator of transcription 3, WP1066

Introduction
Retinoblastoma (RB) is a malignant tumor of the developing retina, and the most common intraocular cancer in children, with 80% of cases occurring before the age of three years [1]. The most common presenting signs are leukocoria, strabismus, and poor vision [2]. Advanced stage retinoblastoma is challenging to treat because retinoblastomas can rapidly fill the eye, invade the optic nerve, and eventually spread to the central nervous system, thus becoming fatal. Importantly, a favorable outcome is dependent upon an early diagnosis, in addition to the success of broad-based treatments such as chemotherapies, enucleation, laser therapy, or cryotherapy [3]. Children diagnosed with retinoblastoma tend to present before 2 years of age with one or both eyes affected [4]. Most retinoblastomas initiate from a bi-allelic loss of the RB1 gene, which encodes a critical regulator (pRb) of the cell cycle. Retinoblastoma progression involves inactivating pathways such as, Arf-MDM2/MDMX-p53, while maintaining a relatively low mutation rate [5]. The identification of genetic changes that occur after the initiating genetic lesion is necessary to further define the aggressive proliferative ability of retinoblastoma tumors.

Signal transducer and activator of transcription-3 (STAT3), also known as acute phase response factor (APRF), is a DNA-binding transcription factor [6]. STAT3 is frequently constitutively activated in many human cancers and with bad prognosis [7-10]. Tumorigenic STAT3 activation has been frequently linked to more malignant cancer behaviors, including growth, epithelial-mesenchymal transition, migration, invasion and metastasis [11-13].

Once activated, STAT3 plays a critical role in oncogenesis, proliferation, survival, invasion, and inflammation of various human cancers and cancer cell lines. The persistently activated Stat3 up-regulates its downstream gene expression such as cyclin D1, c-myc, bcl-2, survivin, bcl-XL and VEGF, which contribute to uncontrolled proliferation of cancer cells through promoting cell cycle progression, inhibiting apoptosis, stimulating tumor angiogenesis, and metastasis [14-16]. STAT3 has a oncogenic
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WP1066, a novel small molecule STAT3 inhibitor, was first introduced in 2007 [24, 25] as a JAK kinase inhibitor in acute myelogenous leukemia and glioma, and now it is used as a STAT3 phosphorylation (Tyr 705 residue) blocker. Horiguchi et al has reported that WP1066 prevented the phosphorylation of STAT3, and significantly inhibited renal cancer cell survival and proliferation in vitro and in vivo [26]. In mantle cell lymphoma cells, combination of WP1066 with SAHA inhibited the constitutive STAT3 activation and modulated mRNA expressions of anti- and pro-apoptotic genes, resulted in antiproliferative and proapoptotic activity [27]. However, WP1066 suppressed macrophage cell death independently of its inhibitory effect on STAT3, suggesting that WP1066 differentially modulates cell death in a context-dependent manner [28]. Whether WP1066 has antitumor effect by targeting STAT3 signaling in RB cells is unclear.

In the present study, we evaluated the inhibitory effects of WP1066 on STAT3 phosphorylation in RB cells. Furthermore, we explored the role of WP1066 on proliferation, apoptosis and colony formation in RB cells in vitro and in vivo.

Materials and methods

Cell and cell culture

Y79 cells (American Type Culture Collection, Shanghai, China) were maintained in RPMI-1640 (Thermo) with 10% fetal bovine serum (FBS) at 37°C in the humidified atmosphere of 95% air and 5% CO2.

STAT3 inhibitors

Inhibitor WP1066 was purchased from Santa Cruz (Shanghai, China) and dissolved in DMSO (Solorbo, China) for use and storage at -20°C.

Cell viability assays

Cell viability was assessed by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions. Briefly, Y79 cells were incubated overnight in 96-well plates (5 x 10^3 cells/well) and then treated for 24-96 h with the indicated concentration of WP1066 (0.1, 1 or 5 µM) (in DMSO) or with the corresponding amount of DMSO. Absorbance at 570 nm was measured using a microtiter plate reader (Multiskan MK3, Thermo Electron Corporation, USA). Data were obtained from three separate experiments. The percentage of decrease in proliferation in the WP1066 groups was determined by comparing the absorbance values in the DMSO controls. Each experiment was repeated at least three times in quadruplicate wells.

Colony formation assay

Forty-eight hrs treatment of WP1066 (0.1, 1 or 5 µM), the Y79 cells were seeded at a density of 300/mL on 35-mm dishes. Colonies were allowed to grow for 10-14 days. The medium was discarded and each well was washed twice with phosphate buffered saline (PBS) carefully. The cells were fixed in methanol for 15 min, and then stained with crystal violet for 20 min. Finally, positive colony formation (more than 50 cells/colony) was counted.

Flow cytometry

WP1066-induced apoptosis of Y79 cells was examined using a PE Annexin V Apoptosis Detection Kit (BD Biosciences, Shanghai, China) according to the protocol provided. Cells

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were washed once with RPMI-1640 medium containing serum, and incubated with PE-conjugated Annexin V. Apoptosis was analyzed by flow cytometer (FACS-Calibur).

**TUNEL analysis of cell apoptosis**

TdT-UTP nick end labeling (TUNEL) assays were performed with the one-step TUNEL kit according to the manufacturer’s instructions. Cells grown in 6-well culture clusters were treated with different concentrations of WP1066 (0.1, 1 or 5 µM) for 48 h or treated with WP1066 (5 µM) for 6-48 h. Treated cells were fixed onto poly-(L-lysine)-coated slides with 4% paraformaldehyde/PBS. Slides were rinsed with PBS, and cells were then permeabilized with 0.1% Triton X-100. Slides were washed with PBS, and cells were incubated in 50 µL TUNEL reaction mixture for 1 h at 37°C in the dark. Next, 50 µL DAPI was added and incubated for 2 min at room temperature. Cells were imaged by fluorescent microscopy using 488 nm excitation and 530 nm emission. Cells exhibiting green fluorescence were defined as TUNEL positive, apoptotic cells.

The extent of apoptosis in the tumor specimens of mouse models from *in vivo* study was evaluated by TUNEL method using an *in situ* Cell Death Kit (Roche, USA). Cell nuclei were counterstained with Hoechst 33342 and visualized by fluorescent microscopy and analyzed by IPP5.1 (Olympus, Japan).

**Western blot**

Total protein was extracted using lysis buffer supplemented with 1% PMSF, and the concentration was determined using a BCA protein assay kit (Sigma, St Louis, MO, USA). Approximately 20 µg of total protein from each sample was loaded onto 10% SDS-polyacrylamide gels and subsequently transferred to nitrocellulose membranes (Millipore, Massachusetts, USA). The membranes were incubated with rabbit antibody against cleaved caspase-3, human Phospho-STAT3 (Y705) antibody and STAT3 Antibody (1:200, Santa Cruz, Shanghai, China) at 4°C overnight. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard to normalize protein expression, and the membranes were incubated with GAPDH monoclonal antibody (1:2500) simultaneously. The membranes were subsequently washed with TBST (Tris-HCl+Tween) and incubated with HRP-conjugated secondary goat anti-rabbit or anti-mouse antibody (1:2000) at 37°C for 1 h. The protein-antibody complexes were analyzed using chemiluminescence. All antibodies used in this study were purchased from Tuoran Biotechnology Inc. (Shanghai, China).

**Tumor models in mice**

All animal experimental protocols were approved by the Animal Care and Use Committee of the Affiliated Hospital of Qingdao University. The NU/NU nude mice at age of 4 weeks, female, SPF grade (Vital River Laboratories, China) (*n* = 30, 10 for each group) were implanted the right back subcutaneously with 5 × 10⁶ Y79 cells. The tumor volume was measured with a caliper every 3 days using a formula (volume = long diameter × short diameter²/2). When the volume of xenograft tumors was approximately 250 mm³, the tumors was injected with DMSO, WP1066 (40 mg/kg, local injection) every 3 days. After 3 weeks, the mice were sacrificed and the xenograft tumors were removed for formalin fixation and preparation of paraffin-embedded sections. Apoptosis (programmed cell death) in the tumor specimens of
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**Figure 2.** WP1066 enhances apoptosis in Y79 cells as assessed by TUNEL and Flow cytometry assay. Y79 cells was treated with 0, 0.1, 1 or 5 µM WP1066 for 48 hs and 5 µM WP1066 for 12 h, 24 h, 36 h and 48 h. The TUNEL assay was performed using the TUNEL Apoptosis Assay Kit. Images were captured by fluorescence microscopy. The green color is indicative of TUNEL-positive cells, and the blue color marks the presence of all cells. A and B. The percentage of apoptotic cells is reported. vs control, *p < 0.05; **p < 0.01; Cells were stained with Annexin V-FITC/PI, and apoptosis was analyzed by flow cytometry. C and D. The percentage of apoptotic cells is reported. vs control, *p < 0.05; **p < 0.01.
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the murine model was examined by TUNEL method as above.

**Immunohistochemistry**

Immunohistochemical studies were performed on paraffin-embedded tissue sections. Serial sections (5-mm thick) of paraffin-embedded tissues were fixed on silane coated glass slides, deparaffinized, and rehydrated in tap water. Antibodies used was human pSTAT3^Y705^ antibody at dilution 1:200. Antigen was retrieved using Target retrieval system (TRS, DAKO S1700) for 20 minutes at 95°C. Sections were blocked with Avidin-Biotin blocking system (DAKO) for 10 minutes and the endogenous peroxidase was quenched with 3% H$_2$O$_2$ for 15 minutes. Background was blocked using DAKO protein block for 30 minutes followed by primary antibody for 1 hr (2 mg/ml). Sections were exposed to secondary donkey anti-body (30 min) and horseradish peroxidase-linked DAKO streptavidin for 30 minutes in 3,30-diaminobenzyl tetrahydrocholoride (DAB) as chromogen and counterstained with hematoxylin. The extent and intensity of staining in the cancer cells were evaluated by a single investigator blinded to the treatment arm. Microscopic fields were evaluated and those with the highest degree of immunoreactivity were pictured.

**Statistical analysis**

SPSS13.0 software was used. Each assay was performed at least three times. The data were expressed as mean ± SD and Student’s t-test was used to determine the significance of differences in multiple comparisons, p < 0.05 was considered to be statistically significant.

**Results**

**WP1066 inhibited STAT3 phosphorylation**

Previous study has shown that highly positive nuclear staining of pSTAT3^Y705^ was found in the RB tissues and Y79 cells [23]. So we used Y79 cells for further investigation. WP1066 dose-response and time-response experiments were carried out by treating Y79 cells with 0, 0.1, 1 or 5 µM WP1066 for 48 h and 5 µM WP1066 for 12 h, 24 h, 36 h and 48 h and measuring pSTAT3^Y705^ . As expected, pSTAT3^Y705^ was strongly inhibited by WP1066 at all concentrations tested and by > 80% at 5 µM at 48 h (Figure 1A), suggesting that pSTAT3^Y705^ was dose-dependently inhibited after WP1066 administration. In addition, pSTAT3^Y705^ was time-response inhibited by 5 µM WP1066 for 12 h, 24 h, 36 h and 48 h (Figure 1B).

**WP1066 induces apoptosis in Y79 cells**

Y79 cells was treated with 0, 0.1, 1 or 5 µM WP1066 for 48 h and 5 µM WP1066 for 12 h, 24 h, 36 h and 48 h. The resulting fragmented DNA of apoptotic cells were labeled using the TUNEL assay. Increasing concentrations of WP1066 significantly increased the number of apoptotic cells (Figure 2A). In addition, increasing time of WP1066 treatment also significantly increased the number of apoptotic cells (Figure 2B). To further confirm that WP1066 promotes apoptosis, we analyzed cells treated with increasing concentrations of WP1066 for 48 h by FACS. The number of apoptotic cells increases by 10.55-44.38% at 0.1-5 µM WP1066 treatment (Figure 2C). In addition, increasing time of WP1066 treatment also significantly increased the number of apoptotic cells (Figure 2D). This finding shows that WP1066 enhances apoptosis in Y79 cells by dose-dependent and time-dependent pathway. Our results also showed that WP1066 induced the cleavage of caspase-3, indicating apoptosis in Y79 cells (Figure 1A, 1B).

**WP1066 inhibited Y79 cell viability and decreased colony formation**

Forty-eight hrs treatment of WP1066 (0.1, 1 or 5 µM), the Y79 cells were seeded at a density of 300/ml on 35-mm dishes. Colonies were allowed to grow for 10-14 days. Our results demonstrated that WP1066 remarkably reduced the colony formation capacity in Y79 cell lines (Figure 3A).

Treatment with WP1066 for 24-96 hours resulted in a dramatic decrease in Y79 cell viability in a dose-dependent and time-dependent manner (Figure 3B).

**WP1066 suppressed tumor growth of Y79 cells in vivo**

Finally, we tested the effect of WP1066 on suppressing Y79 cells in nude mice xenograft models in vivo. As shown in Figure 4A, WP1066 suppressed tumor growth compared to the
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WP1066 inhibited STAT3 phosphorylation and induced caspase-3 cleavage, indicating apoptosis in vivo (Figure 4B). WP1066 also induced apoptosis by using TUNEL assay in xenograft tumors (Figure 4C). These results demonstrated that inhibition of STAT3 by WP1066 resulted in the suppression of tumor growth in mice, suggesting WP1066 might be a potent compound in suppressing tumor growth in vivo.

Discussion

Constitutive activation of STAT3 signaling is crucial to tumorigenesis, proliferation, survival, and invasion of various human cancers and cancer cell lines, promoting it as a very attractive drug development target for tumor treatment [29-31]. The first phosphotyrosyl peptide was reported as a STAT3 inhibitor in 2001 to inhibit STAT3 activity in vitro and in vivo by competing with the native phosphopeptide of STAT3 protein [32]. This peptide and subsequently developed peptidomimetics inhibitors elucidate the role of STAT3 and the impact of the Stat3 inhibitor. Because of the challenge of the metabolic instability and poor cell permeability, the use of peptide and peptidomimetics inhibitors was limited. Therefore, additional research works focus on developing non-peptide small molecule inhibitors of STAT3, which were mostly designed to directly inhibit STAT3 activity through disrupting STAT3-STAT3 dimerization.

Priebe and his colleagues synthesized WP1066 by modifying the structure of AG490, and WP1066 has been shown to inhibit STAT3 activity and to have a potent antitumour effect on malignant glioma cells, both in vitro and in vivo [25]. Marked antitumour activity of WP1066 has also been shown against gastric cancer [33], melanoma [34], renal cell carcinoma [35] and oral squamous cell carcinoma [36]. In this study, we evaluated the inhibitory efficacy of WP1066 in RB Y79 cells with persistent STAT3 signaling. The results demonstrate that WP1066 is capable of inhibiting STAT3 phosphorylation, resulting in the induction of apoptosis. In addition to its anti-apoptotic function, STAT3...
also stimulates proliferation. The results indicated that WP1066 inhibited cell viability and colony-forming ability in vitro. Previously study has reported that WP1066 shows a strong trend to inhibit Reg1 in gastric tumours of the gp130<sup>757FF</sup> mouse along with IL-6 and IL-11 inhibition. Such inhibition would contribute to the reduced tumour burden, and increased apoptosis observed following WP1066 application [33]. In our study, we found that the staining intensity of p-STAT3 in tumours treated with WP1066 was much reduced than that in control tumours by immunostaining, suggesting that WP1066 inhibited STAT3 phosphorylation.

**Figure 4.** WP1066 inhibited tumor growth in mouse xenografts with Y79 cells. A. WP1066 decreased tumor volumes compared with vehicle group. B. WP1066 inhibited STAT3 phosphorylation and induced caspase-3 cleavage in mouse xenografts in vivo by immunohistochemical assay. C. WP1066 induced apoptosis by using TUNEL assay in xenograft tumors (*p* < 0.05).
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in vivo. Furthermore, WP1066 significantly inhibited the growth of Y79 xenografts.

In summary, we showed that WP1066 inhibits STAT3 dependent growth and blockade of apoptosis, both in vitro in human RB Y79 cells, and in vivo in an established mouse model of RB tumourigenesis. This data support efforts to further develop and apply inhibitors of STAT3 pathway as therapeutic agents for RB treatment.

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Disclosure of conflict of interest

None.

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