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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 *ascyclin 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

potential to be involved in cell malignant transformation. Compelling evidence has confirmed that aberrant activated STAT3 participated in the process of oncogenesis in tumor cells by oncogenic tyrosine kinases [17-19]. Further support comes from animal experiments that a constitutively activated STAT3 mutant alone can directly induce tumor formation in nude mice, which suggested that STAT3 activation plays a critical role in oncogenesis [20].

Given the important role of constitutive STAT3 signaling in tumors, it provides a potential therapeutic target in treatment of this tumor. Mounting evidence has shown that disrupting STAT3 activation can inhibit the growth of tumor cells and induce apoptosis in tumor cells [21, 22]. Based on these findings, devising an inhibitor of STAT3 is becoming more and more attractive for development of cancer therapeutic drugs. It has recently found that STAT3 inhibition by targeted siRNA suppressed the proliferation of retinoblastoma cells, and effectively inhibited the formation of *in vivo* orthotopic tumors [23]. Based onthe data, we suggested that targeting STAT3 could be a potential therapeutic approach in retinoblastoma.

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% CO_2.

STAT3 inhibitors

Inhibitor WP1066 was purchased from Santa Cruz (Shanghai, China) and dissolved in DMSO (Solarbo, 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 (5×10^3 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

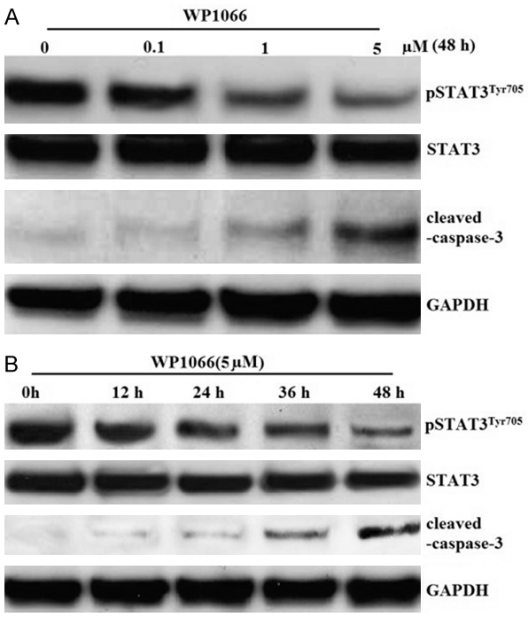


Figure 1. Effect of WP1066 on STAT3 phosphorylation and caspase-3 activity. Y79 cells was treated with 0, 0.1, 1 or 5 μM WP1066 for 48 hs and 5 μM WP1066 for 12 h, 24 h, 36 h and 48 h. pSTAT3^{Tyr705} and cleaved-caspase-3 expression was detected by western blot assay.

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 hours 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.

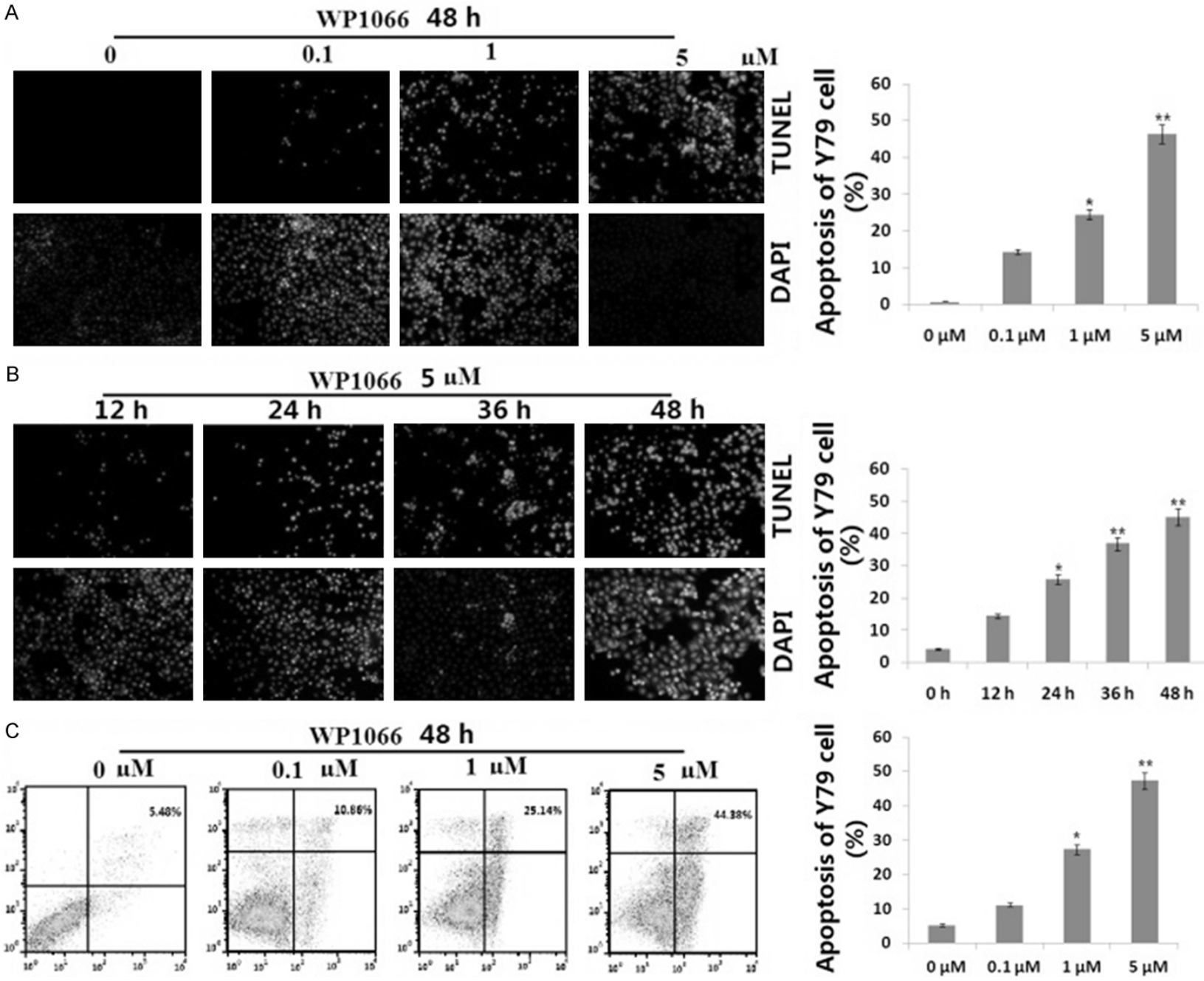
The extent of apoptosis in the tumor specimens of mouse models from *in vivo* study was evaluated by TUNEL method using an *in situ* Cell Death Kit (Roche, USA). Cell nuclei were counterstained with Hoechst 33342 and visualized by fluorescent microscopy and analyzed by IPP5.1 (Olympus, Japan).

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 capase-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 5×10^6 Y79 cells. The tumor volume was measured with a caliper every 3 days using a formula (volume = long diameter × short diameter^2/2). When the volume of xenograft tumors was approximately 250 mm^3, 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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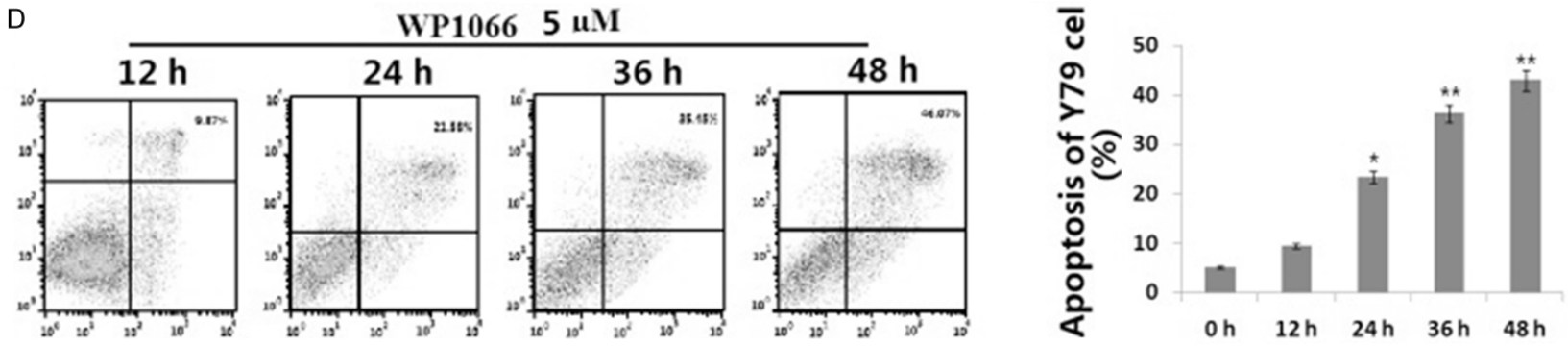

Figure 2. WP1066 enhances apoptosis in Y79 cells as assessed by TUNEL and Flow cytometry assay. Y79 cells was treated with 0, 0.1, 1 or 5 μM WP1066 for 48 hs and 5 μM WP1066 for 12 h, 24 h, 36 h and 48 h. The TUNEL assay was performed using the TUNEL Apoptosis Assay Kit. Images were captured by fluorescence 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, $^{*}p < 0.05$; $^{**}p < 0.01$.

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_2O_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-diaminobenylin 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^{Tyr705} 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^{Tyr705}. As expected, pSTAT3^{Tyr705} was strongly inhibited by WP1066 at all concentrations tested and by > 80% at 5 μM at 48 h (**Figure 1A**), suggesting that pSTAT3^{Tyr705} was dose-dependently inhibited after WP1066 administration. In addition, pSTAT3^{Tyr705} 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 WP-1066 promotes apoptosis, we analyzed cells treated with increasing concentrations of WP-1066 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**, WP10-66 suppressed tumor growth compared to the

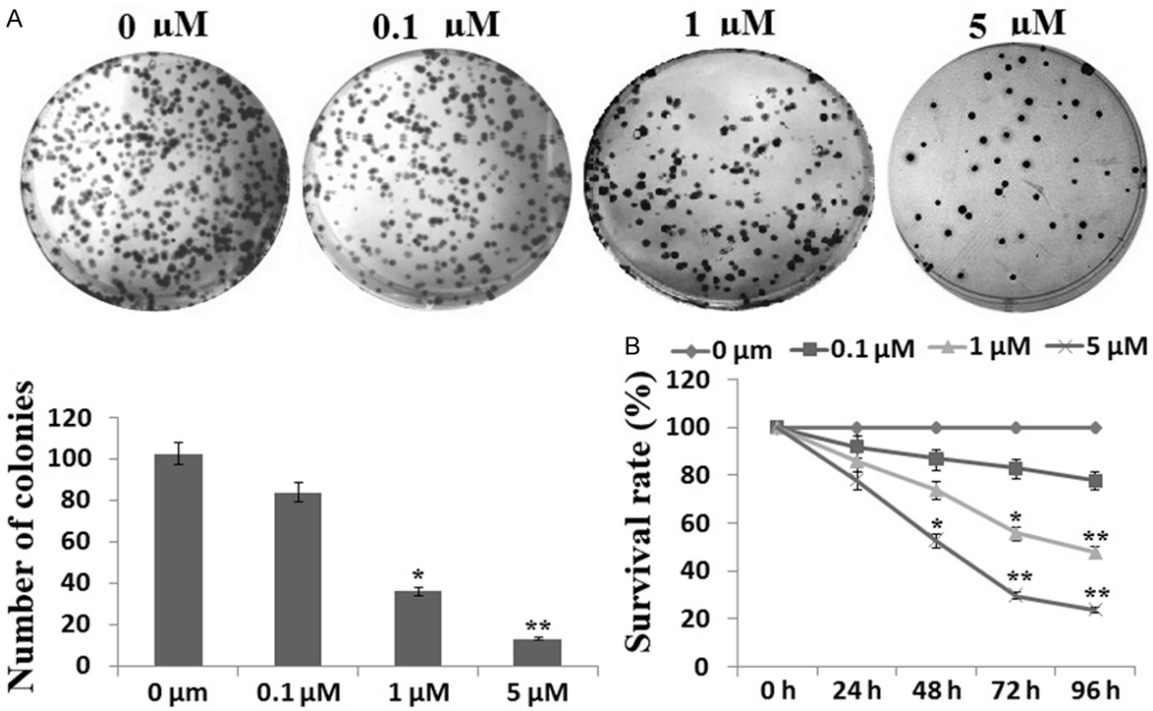

Figure 3. WP1066 inhibited the cell viability and colony formation in Y79 cells. A. Colony formation assay was used to evaluate the viability of Y79 cells treated with WP1066 (0.1, 1 or 5 μM). Represent pictures of colony formations in different groups of triplicate experiments were shown. WP1066 resulted in inhibition of clone formation in a does-dependent matter. B. MTT was done to analyze the viability of Y79 cells. The treatment of WP1066 reduced the viability of Y79 cells in a dose-dependent and time-dependent pathway. *vs* control, $^{*}p < 0.05$; $^{**}p < 0.01$.

vehicle-treated controls in Y1066 xenografted mice ($P < 0.05$). 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 phosphopetide 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 synthesised 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

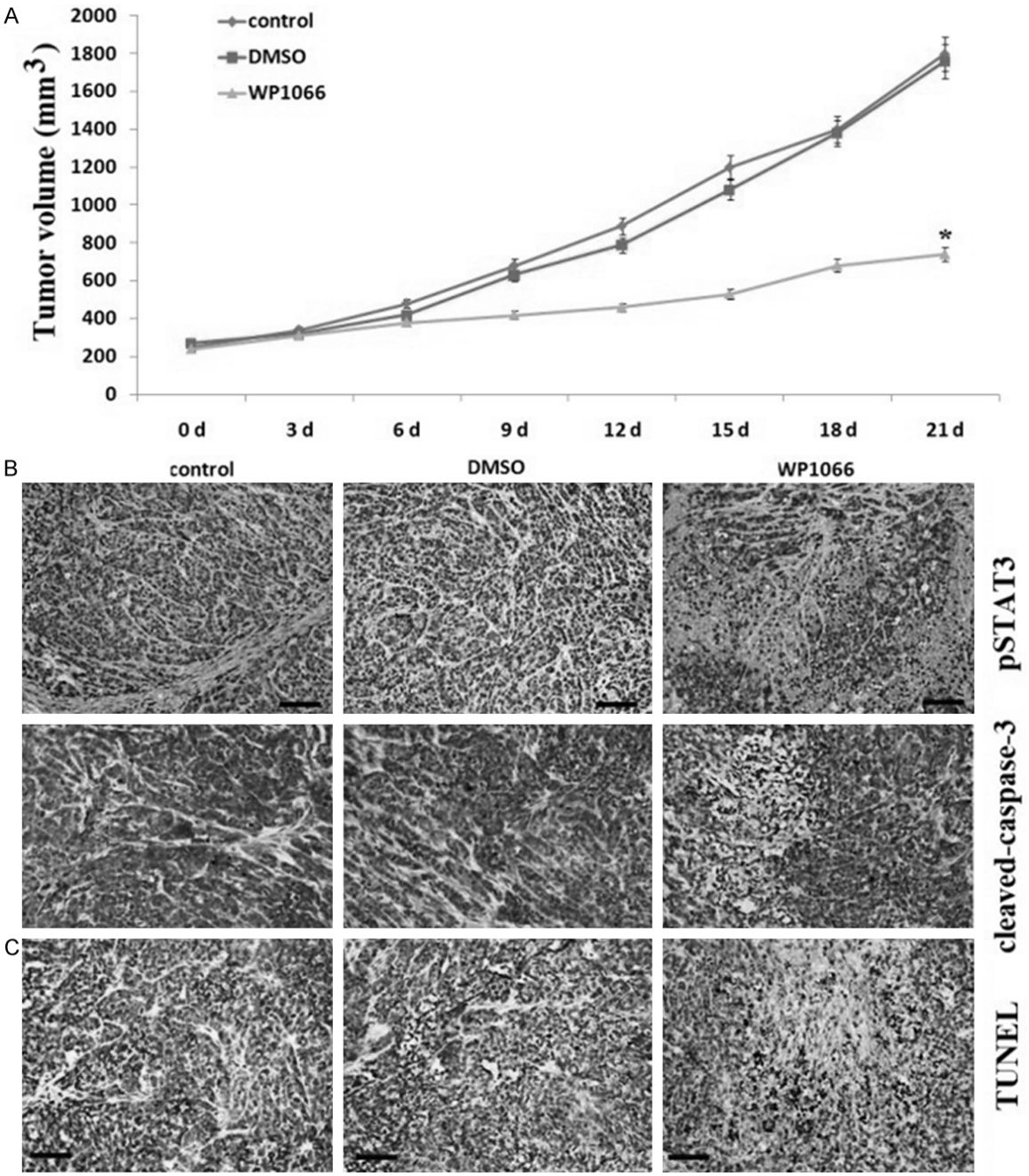

Figure 4. WP1066 inhibited tumor growth in mouse xenografts with Y79 cells. A. WP1066 decreased tumor volumes compared with vehicle group. B. WP1066 inhibited STAT3 phosphorylation and induced caspase-3 cleavage in mouse xenografts *in vivo by* immunohistochemical assay. C. WP1066 induced apoptosis by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

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^{757FF} 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

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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References

[1] Villegas VM, Hess DJ, Wildner A, Gold AS, Murray TG. Retinoblastoma. Curr Opin Ophthalmol 2013; 24: 581-588.

[2] Chintagumpala M, Chevez-Barrios P, Paysse EA, Plon SE, Hurwitz R. Retinoblastoma: review of current management. Oncologist 2007; 12: 1237-1246.

[3] Shields CL, Shields JA. Retinoblastoma management: advances in enucleation, intravenous chemoreduction, and intra-arterial chemotherapy. Curr Opin Ophthalmol 2014; 21: 203-212.

[4] François J, Matton MT, De Bie S, Tanaka Y, Vandenbulcke D. Genesis and genetics of retinoblastoma. Ophthalmologica 1975; 170: 405-425.

[5] Zhang J, Benavente CA, McEvoy J, Flores-Otero J, Ding L, Chen X, Ulyanov A, Wu G, Wilson M, Wang J, Brennan R, Rusch M, Manning AL, Ma J, Easton J, Shurtleff S, Mullighan C, Pounds S, Mukatira S, Gupta P, Neale G, Zhao D, Lu C, Fulton RS, Fulton LL, Hong X, Dooling DJ, Ochoa K, Naeve C, Dyson NJ, Mardis ER, Bahrami A, Ellison D, Wilson RK, Downing JR, Dyer MA. A novel retinoblastoma therapy from genomic and epigenetic analyses. Nature 2012; 481: 329-34.

[6] Wegenka UM, Buschmann J, Lütticken C, Heinrich PC, Horn F. Acute-phase response factor, a nuclear factor binding to acute-phase response elements, is rapidly activated by interleukin-6 at the posttranslational level. Mol Cell Biol 1993; 13: 276-288.

[7] Tong M, Wang J, Jiang N, Pan H, Li D. Correlation between p-STAT3 overexpression and prognosis in lung cancer: a systematic review and meta-analysis. PLoS One 2017; 12: e0182282.

[8] Yang XW, Li L, Hou GJ, Yan XZ, Xu QG, Chen L, Zhang BH, Shen F. STAT3 overexpression promotes metastasis in intrahepatic cholangiocarcinoma and correlates negatively with surgical outcome. Oncotarget 2017; 8: 7710-7721.

[9] Ji K, Zhang M, Chu Q, Gan Y, Ren H, Zhang L, Wang L, Li X, Wang W. The role of p-STAT3 as a prognostic and clinicopathological marker in colorectal cancer: a systematic review and meta-analysis. PLoS One 2016; 11: e0160125

[10] Wu P, Wu D, Zhao L, Huang L, Shen G, Huang J, Chai Y. Prognostic role of STAT3 in solid tumors: a systematic review and meta-analysis. Oncotarget 2016; 7: 19863-19883.

[11] Cao HH, Chu JH, Kwan HY, Su T, Yu H, Cheng CY, Fu XQ, Guo H, Li T, Tse AK, Chou GX, Mo HB, Yu ZL. Inhibition of the STAT3 signaling pathway contributes to apigenin-mediated anti-metastatic effect in melanoma. Sci Rep 2016; 6: 21731.

[12] Venturutti L, Romero LV, Urtreger AJ, Chervo MF, Cordo Russo RI, Mercogliano MF, Inurrigarro G, Pereyra MG, Proietti CJ, Izzo F, Díaz Flaqué MC, Sundblad V, Roa JC, Guzmán P, Bal de Kier Joffé ED, Charreau EH, Schillaci R, Elizalde PV. Stat3 regulates ErbB-2 expression and co-opts ErbB-2 nuclear function to induce miR-21 expression, PDCD4 downregulation and breast cancer metastasis. Oncogene 2016; 35: 2208-2222.

[13] Aoki Y, Feldman GM, Tosato G. Inhibition of STAT3 signaling induces apoptosis and decreases survivin expression in primary effusion lymphoma. Blood 2003; 101: 1535-1542.

[14] Xu Q, Briggs J, Park S, Niu G, Kortylewski M, Zhang S, Gritsko T, Turkson J, Kay H, Semenza GL, Cheng JQ, Jove R, Yu H. Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways. Oncogene 2015; 24: 5552-5560.

[15] Amin HM, McDonnell TJ, Ma Y, Lin Q, Fujio Y, Kunisada K, Leventaki V, Das P, Rassidakis GZ, Cutler C, Medeiros LJ, Lai R. Selective inhibition of STAT3 induces apoptosis and G_1 cell cycle arrest in ALK-positive anaplastic large cell lymphoma. Oncogene 2004; 23: 5426-5434.

[16] Bollrath J, Phesse TJ, von Burstin VA, Putoczki T, Bennecke M, Bateman T, Nebelsiek T, Lund-

gren-May T, Canli O, Schwitalla S, Matthews V, Schmid RM, Kirchner T, Arkan MC, Ernst M, Greten FR. gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. Cancer Cell 2009; 15: 91-102.

[17] Turkson J, Bowman T, Garcia R, Caldenhoven E, De Groot RP, Jove R. Stat3 activation by Src induces specific gene regulation and is required for cell transformation. Mol Cell Biol 1998; 18: 2545-2552.

[18] Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. Oncogene 2000; 19: 2474-2488.

[19] Yu CL, Meyer DJ, Campbell GS, Larner AC, Carter-Su C, Schwartz J, Jove R. Enhanced DNA-binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein. Science 1995; 269: 81-83.

[20] Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE Jr. Stat3 as an oncogene. Cell 1999; 98: 295-303.

[21] Ling X, Arlinghaus RB. Knockdown of STAT3 expression by RNA interference inhibits the induction of breast tumors in immunocompetent mice. Cancer Res 2005; 65: 2532-2536.

[22] Yu H, Jove R. The STATs of cancer--new molecular targets come of age. Nat Rev Cancer 2004; 4: 97-105.

[23] Jo DH, Kim JH, Cho CS, Cho YL, Jun HO, Yu YS, Min JK, Kim JH. STAT3 inhibition suppresses proliferation of retinoblastoma through down-regulation of positive feedback loop of STAT3/miR-17-92 clusters. Oncotarget 2014; 5: 11513-11525.

[24] Ferrajoli A, Faderl S, Van Q, Koch P, Harris D, Liu Z, Hazan-Halevy I, Wang Y, Kantarjian HM, Priebe W, Estrov Z. WP1066 disrupts Janus kinase-2 and induces caspase-dependent apoptosis in acute myelogenous leukemia cells. Cancer Res 2007; 67: 11291-11299.

[25] Iwamaru A, Szymanski S, Iwado E, Aoki H, Yokoyama T, Fokt I, Hess K, Conrad C, Madden T, Sawaya R, Kondo S, Priebe W, Kondo Y. A novel inhibitor of the STAT3 pathway induces apoptosis in malignant glioma cells both in vitro and in vivo. Oncogene 2007; 26: 2435-2444.

[26] Horiguchi A, Asano T, Kuroda K, Sato A, Asakuma J, Ito K, Hayakawa M, Sumitomo M, Asano T. STAT3 inhibitor WP1066 as a novel therapeutic agent for renal cell carcinoma. Br J Cancer 2010; 102: 1592-1599.

[27] Lu K, Chen N, Zhou XX, Ge XL, Feng LL, Li PP, Li XY, Geng LY, Wang X. The STAT3 inhibitor WP1066 synergizes with vorinostat to induce apoptosis of mantle cell lymphoma cells. Biochem Biophys Res Commun 2015; 464: 292-298.

[28] Honda S, Sadatomi D, Yamamura Y, Nakashioya K, Tanimura S, Takeda K. WP1066 suppresses macrophage cell death induced by inflammasome agonists independently of its inhibitory effect on STAT3. Cancer Sci 2017; 108: 520-527.

[29] Liu T, Ma H, Shi W, Duan J, Wang Y, Zhang C, Li C, Lin J, Li S, Lv J, Lin L. Inhibition of STAT3 signaling pathway by ursolic acid suppresses growth of hepatocellular carcinoma. Int J Oncol 2017; 51: 555-562.

[30] Tian J, Xiao H, Wu R, Cao Y, Li C, Xu R, Pierson CR, Finlay JL, Yang F, Gu N, Lin J. The antiproliferative and colony-suppressive activities of STAT3 inhibitors in human cancer cells is compromised under hypoxic conditions. Anticancer Res 2017; 37: 547-553.

[31] Lee HT, Xue J, Chou PC, Zhou A, Yang P, Conrad CA, Aldape KD, Priebe W, Patterson C, Sawaya R, Xie K, Huang S. Stat3 orchestrates interaction between endothelial and tumor cells and inhibition of Stat3 suppresses brain metastasis of breast cancer cells. Oncotarget 2015; 6: 10016-10029.

[32] Turkson J, Ryan D, Kim JS, Zhang Y, Chen Z, Haura E, Laudano A, Sebti S, Hamilton AD, Jove R. Phosphotyrosyl peptides block Stat3-mediated DNA binding activity, gene regulation, and cell transformation. J Biol Chem 2001; 276: 45443-45455

[33] Judd LM, Menheniott TR, Ling H, Jackson CB, Howlett M, Kalantzis A, Priebe W, Giraud AS. Inhibition of the JAK2/STAT3 pathway reduces gastric cancer growth in vitroand in vivo. PLoS One 2014; 9: e95993.

[34] Sau S, Mondal SK, Kashaw SK, Iyer AK, Banerjee R. Combination of cationic dexamethasone derivative and STAT3 inhibitor (WP1066) for aggressive melanoma: a strategy for repurposing a phase I clinical trial drug. Mol Cell Biochem 2017; 12: 209-218.

[35] Horiguchi A, Asano T, Kuroda K, Sato A, Asakuma J, Ito K, Hayakawa M, Sumitomo M, Asano T. STAT3 inhibitor WP1066 as a novel therapeutic agent for renal cell carcinoma. Br J Cancer 2010; 102: 1592-1599.

[36] Zhou X, Ren Y, Liu A, Jin R, Jiang Q, Huang Y, Kong L, Wang X, Zhang L. WP1066 sensitizes oral squamous cell carcinoma cells to cisplatin by targeting STAT3/miR-21 axis. Sci Rep 2014; 4: 7461.