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The STAT3 inhibitor WP1066 synergizes with vorinostat to induce apoptosis of mantle cell lymphoma cells

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ABSTRACT

Mantle cell lymphoma (MCL) is an aggressive B-cell non-Hodgkin lymphoma (NHL) characterized by the translocation t (11; 14) (q13; q32). Drug resistance remains a formidable obstacle to treatment and the median survival for MCL patients is between 3 and 5 years. Thus, there is an urgent need to discover novel approaches to MCL therapy. The signal transducer and activation of transcription 3 (STAT3) has been found to be constitutively activated in several subtypes of MCL cell lines and MCL tumors. WP1066, a small-molecule inhibitor of STAT3, exerted antitumor activity in hematological and solid malignancies by inhibiting key survival and growth signaling pathways. In the present study, we evaluated the antiproliferative and proapoptotic activity of WP1066 combined with pan-histone deacetylase (HDAC) inhibitor vorinostat (SAHA) in a panel of MCL cell lines. In addition, potential mechanisms involved were also explored. The outcome showed that combination of WP1066 with SAHA resulted in synergistic growth inhibition and apoptosis induction in MCL cell lines *in vitro*. Furthermore, combination of WP1066 with SAHA inhibited the constitutive STAT3 activation and modulated mRNA expressions of anti- and pro-apoptotic genes. Our findings suggest that agents targeting the STAT3 pathway such as WP1066 may be useful therapeutic drugs for MCL when combined with SAHA.

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1. Introduction

Mantle cell lymphoma (MCL), a morphologically distinct subtype of aggressive B-cell non-Hodgkin lymphoma (NHL), is characterized by the cytogenetic abnormality t (11; 14) (q13; q32) and overexpression of cyclin D1 [1]. As a clinically heterogeneous disease, MCL comprises approximately 3–10% of NHL and is frequently resistant to conventional chemotherapy. Despite the introduction of novel therapeutic agents, such as rituximab, drug resistance remains a formidable obstacle to treatment and the median survival for MCL patients is between 3 and 5 years [2]. Thus, novel approaches to MCL therapy are urgently needed.

Histone deacetylases (HDACs) are enzymes that regulate the structural conformation of chromatin and the posttranslational modification of numerous key proteins [3]. HDAC inhibitors represent an emerging class of therapeutic drugs that induce tumor apoptosis through damage to the mitochondria and modulating the acetylation status of a wide range of protein targets [4]. Vorinostat (suberoylanilide hydroxamic acid, SAHA) is one of pan-HDAC inhibitors demonstrated to be effective in treating hematologic malignancies, including B-cell NHL [5]. SAHA monotherapy has been approved in the United States for the treatment of cutaneous T-cell lymphoma. However, SAHA monotherapy is rarely effective against diseases in MCL patients [5,6]. Hence, combinations of this HDAC inhibitor and other novel agents with synergistic effects are necessary.

WP1066, a small-molecule inhibitor of signal transducer and activator of transcription 3 (STAT3), has a marked antitumor effect on acute myelogenous leukemia, polycythemia vera, melanoma, malignant glioma cells and renal cell carcinoma [7–11]. It suppresses the Janus kinases (JAKs)/STAT3 signaling pathway by inhibiting activation of STAT3 and downregulating the expressions of JAKs upstream from STAT3. Our previous studies demonstrated that by disrupting antiapoptotic signaling of the microenvironment, WP1066 increased sensitivity of chronic lymphocytic leukemia cells to HDAC inhibitor-induced cytotoxicity [12]. However,

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the combination of WP1066 and SAHA has not been evaluated in treating MCL. In this study, we expanded our previous work by investigating the antiproliferative and proapoptotic activity of WP1066 combined with SAHA in a panel of MCL cell lines, as well as the potential mechanisms involved.

2. Materials and methods

2.1. Antibodies and regents

Primary rabbit monoclonal antibodies against phosphorylated STAT3 (p-STAT3) (Tyr705, D3A7), STAT3 (D3Z2G), caspase-3 (8G10) and poly(ADP ribose) polymerase (PARP) [specific to the full-length (116 kDa) and the cleaved form (89 kDa) of PARP, 46D11] were obtained from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-GAPDH monoclonal antibody was from Zhongshan Goldenbridge (ZSGB-BIO; Beijing, China). SAHA was purchased from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO, USA). WP1066 was from EMD Chemicals (San Diego, CA, USA). SAHA and WP1066 were dissolved in 100% dimethyl sulfoxide (DMSO; Solarbio, Beijing, China) to a stock concentration of 10^{-2} M and stored at −80 °C.

2.2. Cell lines and cell culture

Four previously described Human MCL cell lines, Jeko-1, SP53, Mino, and Granta 519 were used in this study [13]. Jeko-1 was purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). SP53, Mino, and Granta 519 were kind gifts from Dr. Michael Wang (Department of Lymphoma and Myeloma, the University of Texas MD Anderson Cancer Center, Houston). All of these MCL cell lines were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 incubator.

2.3. Cytotoxicity assays

Effect of drugs on cell survival was assessed by carrying out triplicate assays with the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). MCL cells (1×10^4 cells/100μl/well, respectively) were seeded into 96-well plates and treated simultaneously with seven serial dilutions of drugs for 48 h. Thereafter, the cells were incubated with 10 μL of CCK-8 for 4 h according to the manufacturer's recommendations. The absorbance at 450 nm was measured using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Half-maximal inhibitory concentration (IC_{50}) values were calculated using CalcuSyn software (version 2.0, Biosoft, Cambridge, UK). The interaction between drugs was analyzed according to the Chou and Talalay method by using CalcuSyn software. Combination index (CI) values < 1, $= 1$, and >1 indicated synergism, additive effect and antagonism, respectively [14].

2.4. Analyses of cell apoptosis

Effects of drugs on apoptosis of MCL cells were evaluated by annexin V/7-AAD assay and western blot analysis of caspase-3 and the cleavage of PARP, which is a substrate of caspase-3. For flow cytometric analysis, 1×10^6 of MCL cells were seeded in 6-well plates incubated with single drug or drug combination for 48 h. After culture, cells were harvested and labeled with annexin V-phycoerythrin (PE) and 7-aminoactinomycin D (7-AAD) according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). The rates of cellular apoptosis were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo Version 7.6 software (Tree Star Inc., Ashland, OR, USA).

2.5. Real-time quantitative polymerase chain reaction (PCR)

Total RNA was extracted by TRIzol reagent (Takara, Dalian, China) from cells following incubation with single drug or drug combination for 48 h. Reverse transcription to complementary DNA (cDNA) was conducted using PrimeScript RT reagent kit with gDNA eraser (Takara). Amplification reactions were performed using a SYBR Premix Ex Taq II kit (Takara) on a LightCycler 480 real-time PCR system (Roche Diagnostics, Mannheim, Germany). Specific primers for real-time PCR were obtained from Invitrogen (Beijing, China), and the primer sequences are listed in Table 1. Actin was used as an internal control. Isolation of RNA, reverse transcription and real-time PCR were performed following the manufacturer's instructions. Real-time PCR for each gene of each cDNA sample was assayed in triplicate. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method with LightCycler 480 Gene Scanning Version 1.5 Software (Roche Diagnostics).

2.6. Protein extraction and western blot analyses

After designated treatments, MCL cells were harvested and lysed in lysis buffer (Shenergy Biocolor, Shanghai, China) with 1 × final concentration of phosphatase inhibitor cocktail (PhosSTOP; Roche). After incubation on ice for 30 min, the cell lysate was centrifugated at 12000 g for 15 min at 4 °C. The total protein concentration of the samples was determined by the BCA assay (Shenergy Biocolor). 30 μg of total protein was loaded to 8% SDS/PAGE gel and subjected to electrophoresis. Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), which was incubated with blocking solution (5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20) for 1 h and incubated with the indicated antibodies overnight at 4 °C. All primary antibodies were used at 1:1000 dilution. Anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (HRP; ZSGB-BIO) were used at 1:10000 dilution. After incubation with chemiluminescent HRP substrate (Millipore), the membrane was finally observed by system LAS4000 mini (Fuji film, Tokyo, Japan) and analyzed by Multi Gauge Version 3.0 software (Fuji film).

2.7. Statistical analysis

SPSS for Windows (version 17.0, SPSS, Chicago, IL, USA) was used for statistical analysis. For all results, values represent the mean from at least three independent experiments. Data which complied with normal distribution were expressed as mean ± standard

Table 1
Primers sequences.

Gene	Primer sequence
Bcl-2	5′-ATGTGTGTGGAGAGCGTCAA-3′
	5′-ACAGTTCCACAAAGGCATCC-3′
Bax	5′-CCCGAGAGGTCTTTTTCCGAG-3′
	5′-CCAGCCCATGATGGTTCTGAT-3′
c-Myc	5′-GGCTCCTGGCAAAAGGTCA-3′
	5′-AGTTGTGCTGATGTGTGGAGA-3′
$p21^{Cip1}$	5′-CGATGGAACTTCGACTTTGTCA-3′
	5′-GCACAAGGGTACAAGACAGTG-3′
cyclin D1	5′-CAAATGGAGCTGCTCCTGGTG-3′
	5′-CTTCGATCTGCTCCTGGCAGG-3′
β-actin	5′-TGACGTGGACATCCGCAAAG-3′
	5′-CTGGAAGGTGGACAGCGAGG-3′

deviation (SD). One-way ANOVA followed by LSD post-hoc tests was used for comparison between groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of sensitivity of MCL cell lines to WP1066 and SAHA

MCL cell lines were treated with different concentrations of WP1066 or SAHA for 48 h. Dose-response curves were shown in Fig. 1A. The IC_{50} values for WP1066 and SAHA single treatment in four MCL cell lines were shown in Table 2. A wide diversity of cell responses to the WP1066 treatment was observed. For example, the IC_{50} for WP1066 single treatment was 4.71 μM in Mino cells, whereas Jeko-1 cells needed a WP1066 concentration higher than 20 μM to induce 50% growth inhibition. Western blot analysis revealed high levels of p-STAT3 in untreated Granta 519 and SP53 cells, significantly lower levels in Mino cells and almost no detectable expression in Jeko-1 cells (Fig. 1B). To gain insight into inhibition of the STAT3 pathway in Granta 519 and SP53 cells, we measured levels of p-STAT3 at 24 h after treatment with different concentrations of WP1066. Both cell types showed dose-dependent inhibition of phosphorylation after 24 h incubation with WP1066 (Fig. 1C).

3.2. WP1066 synergistically interacted with SAHA to inhibit growth of MCL cell lines

To test whether targeting STAT3 activation can enhance the effect of SAHA in Granta 519 and SP53 cells, cells were simultaneously treated with WP1066. Significant decreases in the cell growth of Granta 519 and SP53 cells were observed in response to treatment with combined 5 μM WP1066 and 5 μM SAHA than with either agent alone, in a time-dependent manner up to 72 h (Fig. 2A). To formally examine an interaction of WP1066/SAHA combination, the cells were treated with varying concentrations of SAHA and WP1066 at the ratio of 1:1. Granta 519 cells were treated with 0.5–10 μM of SAHA and WP1066. SP53 cells were treated with 0.25–8 μM of SAHA and WP1066. Factional effect-CI curves were constructed according to the Chou-Talalay method (Fig. 2B). Table 3 showed the CI values of WP1066/SAHA combination in four MCL cell lines. These data revealed that WP1066/SAHA combination provided highly synergistic anti-MCL activity with CIs ≤0.5 in all four representative MCL cell lines.

Table 2
50% inhibitory concentration (IC_{50}) values.

	Granta 519	SP53	Mino	Jeko-1
WP1066 (μM)	11.86	6.31	4.71	>20
SAHA (μM)	10.49	7.73	2.64	2.25

3.3. Combination of WP1066 with SAHA inhibited STAT3 activation and induced caspase-dependent apoptosis

Annexin V/7-AAD assay showed that both WP1066 and SAHA single treatment induced cell apoptosis, and combination of the two drugs resulted in a significant increase ($P < 0.01$) in percentage of apoptotic cells in Granta 519 and SP53 cell lines (Fig. 3A–D). Moreover, western blot analysis showed that WP1066 in combination with SAHA significantly inhibited STAT3 activation and induced downregulation of pro-caspase-3 expression. Concomitantly, cleavage of PARP was further increased, whereas SAHA single treatment had only a moderate effect on its expression (Fig. 3E).

3.4. WP1066/SAHA combination modulated multiple mRNA expressions of apoptosis regulation genes

To further investigate whether WP1066/SAHA combination-induced apoptosis was dependent on the mitochondrial apoptosis pathway, the effect of the two drugs on the mRNA levels of Bcl-2, Bax, c-Myc, cyclin D1 and $p21^{Cip1}$ genes was measured by real-time PCR (Fig. 4). Following treatment with 5 μM SAHA alone or in combination with 5 μM WP1066 for 24 h, mRNA expressions of $p21^{Cip1}$ and Bax were significantly upregulated in Granta 519 cells, while mRNA expressions of c-Myc and cyclin D1 were reduced. However, no significant difference in the expression of Bcl-2 mRNA was observed in the WP1066/SAHA combination-treated and untreated Granta 519 cells. The similar results were also observed in SP53 cell line.

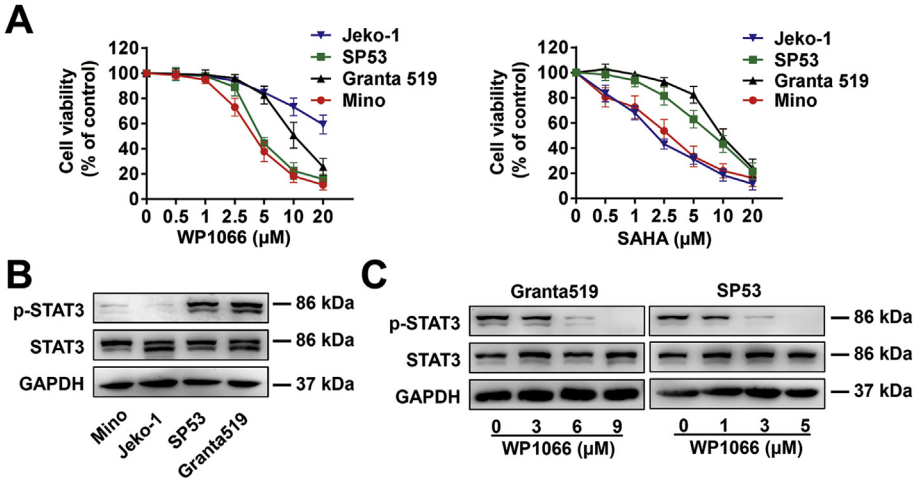

Fig. 1. Characterization of WP1066 and SAHA response in human MCL cell lines. (A) Effects of WP1066 and SAHA on the viabilities of MCL cells. Cells were incubated with increasing concentrations of WP1066 or SAHA for 48 h before cell viability was determined by the CCK-8 assay. (B) Assessment of STAT3 activity in untreated MCL cell lines using western blot analysis. (C) WP1066 inhibited STAT3 phosphorylation in Granta 519 and SP53 cells. Cells were treated with the indicated concentration of WP1066 for 24 h and cell lysates were subjected to western blot analysis.

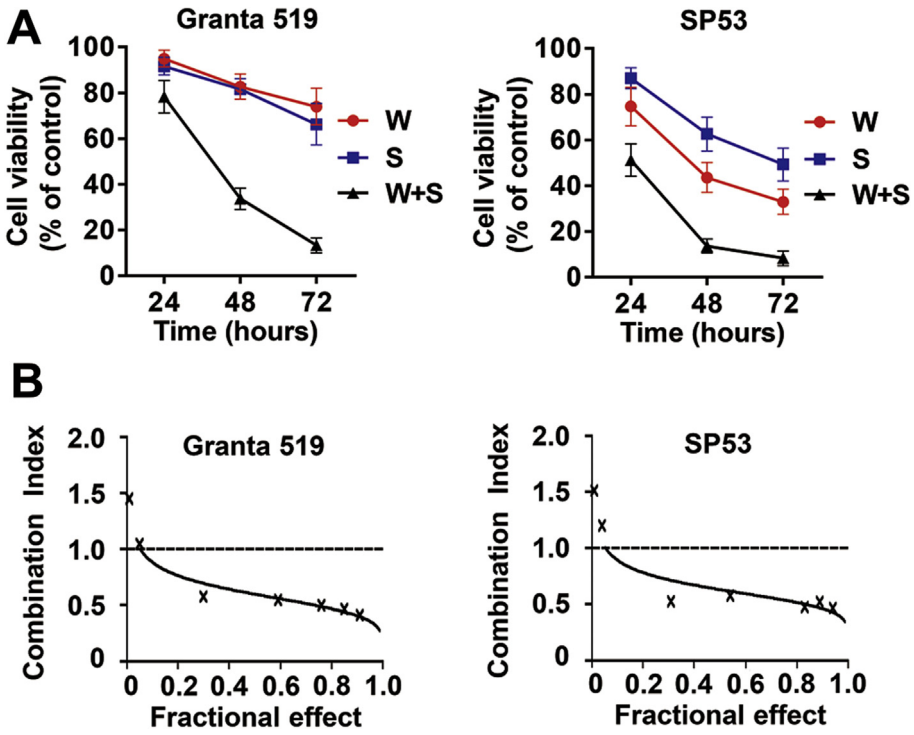

Fig. 2. WP1066 synergistically interacted with SAHA to inhibit growth of Granta 519 and SP53 cells. (A) Granta 519 and SP53 cells were incubated with WP1066 (W, 5 μM), SAHA (S, 5 μM), or the combination of both for 24, 48 and 72 h before cell viability was determined by the CCK-8 assay. (B) Fractional effect/combination index curves were generated according to the Chou-Talalay method. Cells were treated for 48 h with increasing doses of WP1066 and SAHA at the ratio of 1:1.

Table 3
Combination index.

SAHA (μM)	WP1066 (μM)		
Granta 519	1	3	10
1	0.95	0.81	0.87
3	0.70	0.67	0.56
10	0.78	0.64	0.55
SP53	1	3	10
1	0.48	0.74	0.93
3	0.57	0.51	0.73
10	0.89	0.52	0.54
Mino	1	2.5	5
1	0.39	0.54	0.78
2.5	0.43	0.52	0.63
5	0.43	0.44	0.52
Jeko-1	1	5	20
1	1.07	0.93	1.19
2.5	0.93	0.60	0.74
5	1.13	0.73	0.42

4. Discussion

Several clinical studies have demonstrated that SAHA could offer sustained antitumor activity in patients with advanced solid and hematologic malignancies such as follicular lymphoma (FL) and marginal zone lymphoma (MZL) [15–17]. In most of these studies, the mean maximum steady-state concentrations ($C_{ss.max}$) of oral SAHA in patients with the maximum tolerated dose (MTD) were no more than 2.5 μM. Major toxicities were manageable grade 3/4 thrombocytopenia and neutropenia. However, SAHA did not produce formal responses with an acceptable safety profile in previously treated patients with MCL [5,6,18]. In the present study, we assessed the IC_{50} values for SAHA single treatment and the activation status of STAT3 in a series of MCL cell lines. The results showed that the p-STAT3 positive cell lines Granta 519 and SP53 were resistant to the SAHA single treatment. Furthermore, it is suggested that constitutive STAT3 activation might contribute to a higher resistance of SAHA treatment.

Early studies revealed STAT3 protein as a pro-tumorigenic factor in various human malignant tumors [19]. The occurrence and development of MCL are associated with disturbances of multiple cell survival and apoptosis pathways, such as those related to STAT3, nuclear factor-kappa B (NF-κB), Akt/protein kinase B (PKB) and extracellular signal-regulating kinase 1/2 (ERK1/2). STAT3 has been found to be constitutively activated in several subtypes of MCL cell lines and MCL tumors [13,20,21]. Human MCL cells secreted IL-6, which activated the JAK2/STAT3 pathways in MCL. The inhibition of IL-6/JAK2/STAT3 pathway could completely or partially inhibit cell growth, enhance spontaneous cell apoptosis and increase sensitivity to antitumor drugs in MCL cells [13]. Pham et al. demonstrated that WP1130, a novel inhibitor of both JAK2 and STAT3, increased sensitivity of MCL cells to bortezomib-induced cytotoxicity [21]. With these findings, combining HDAC inhibitors with STAT3 inhibitors is rational.

Our studies identified that WP1066, a potent STAT3 inhibitor, was effective in blocking constitutively activated STAT3 in MCL cell lines. In contrast, a relative loss of STAT3 activity in Jeko-1 cell line may explain its lack of sensitivity to the WP1066 treatment. WP1066 targeted STAT3 signaling pathway in a dose- and time-dependent manner, and synergistically interacted with SAHA to inhibit growth of MCL cell lines. Our results were consistent with previous studies [22,23], which showed that activation of STAT3 pathway was associated with resistance to HDAC inhibitors and interruption of this signaling pathway could be a potential target for therapeutic intervention in cutaneous T-cell lymphoma and diffuse large B-cell lymphoma. Moreover, the results of western blot analysis indicated that a decreased level of p-STAT3,

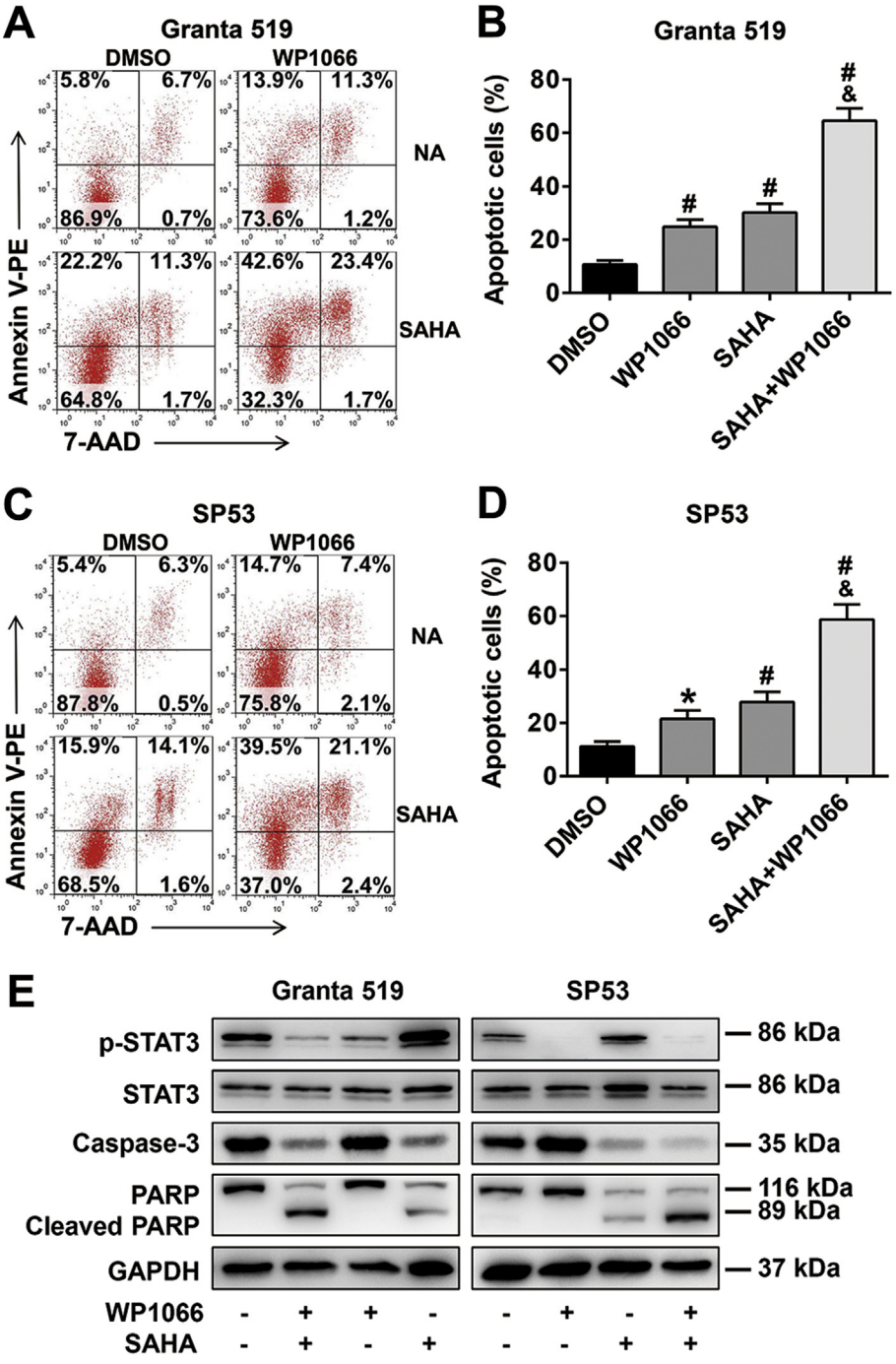

Fig. 3. Effects of WP1066 and SAHA on the apoptosis of Granta 519 and SP53 cells. Cells were incubated with WP1066 (5 μM for Granta 519 and 2.5 μM for SP53 cells), SAHA (5 μM for Granta 519 and 2.5 μM for SP53 cells), or the combination of both for 48 h. (A) Apoptosis was quantified by flow cytometric analysis of annexin V-PE/7-AAD dual staining to Granta 519 cells. Annexin V-positive cells were considered as being apoptotic. (B) Statistical analysis of A. $^{\#}P < 0.01$ vs. group treated with DMSO; $^{\&}P < 0.01$ vs. group treated with SAHA. (C) Apoptosis was determined by flow cytometric analysis in SP53 cells. (D) Statistical analysis of C. $^{*}P < 0.05$ and $^{\#}P < 0.01$ vs. group treated with DMSO; $^{\&}P < 0.01$ vs. group treated with SAHA. (E) Effects of WP1066 and SAHA on the constitutive STAT3 activation and caspase-dependent apoptosis in MCL cells. Cell lysates were subjected to western blotting analysis.

concomitant pro-caspase-3 reduction and PARP cleavage may be important indicators of efficacy.

STAT3 has been documented to upregulate the expressions of a number of genes, including Bcl-2, c-Myc and cyclin D1 [23]. All of these genes are frequently overexpressed in MCL and implicated in drug resistance [24]. Proteins encoded by these genes exert cellular survival and proliferative effects, including cell cycle progression, apoptosis inhibition and cell transformation [25]. In p-STAT3 positive cell lines Granta 519 and SP53, treatment with WP1066/SAHA combination resulted in downregulation of c-Myc and cyclin D1 mRNA, with no significant difference in the expression of Bcl-2 mRNA. Pro-apoptotic gene Bax belongs to the Bcl-2 family and is an important member of the mitochondrial apoptosis pathway [26]. Cyclin-dependent kinase (CDK) inhibitor $p21^{Cip1}$ is a critical

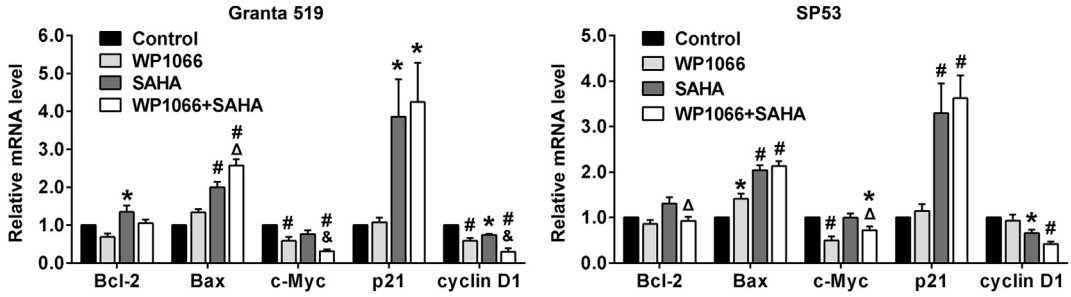

Fig. 4. Effects of WP1066 and SAHA on the mRNA expression levels of Bcl-2, Bax, c-Myc, $p21^{Cip1}$ and cyclin D1 in Granta 519 and SP53 cells. Cells were treated with WP1066 (5 μM for Granta 519 and 2.5 μM for SP53 cells), SAHA (5 μM for Granta 519 and 2.5 μM for SP53 cells), or the combination of both. After 24 h, RNA from cells was purified and real-time PCR was performed. $^{*}P < 0.05$ and $^{\#}P < 0.01$ vs. control group; $^{\Delta}P < 0.05$ and $^{\&}P < 0.01$ vs. group treated with SAHA.

cell-cycle regulatory protein which interacts with cyclin-CDK2 and -CDK4 to inhibit cell cycle progression at G_1 phase. SAHA single treatment triggered mRNA expressions of $p21^{Cip1}$ and Bax, and WP1066/SAHA combination could further increase Bax mRNA level in Granta 519 cells. Although we do not completely understand the mechanisms that underlie the observed synergism, our results suggest that changes in mRNA levels of these anti- and pro-apoptotic genes may play critical roles in the WP1066/SAHA combination-induced apoptosis of MCL cells.

In summary, our study indicated that combining STAT3 inhibitor WP1066 with HDAC inhibitor SAHA resulted in synergistic anti-tumor activity in MCL cell lines *in vitro*. The mechanism involved in this drug synergism was associated with downregulation of constitutive STAT3 activation, modulation of anti- and pro-apoptotic genes expression and caspase-dependent apotosis induction. Our results suggest that agents such as WP1066 and other STAT3-selective inhibitors may be candidate drugs for future use in the treatment of MCL as adjuvants, which could overcome the drug resistance induced by the aberrant activation of STAT3 signaling pathway.

Conflicts of interest statement

The authors declare no conflict of interest.

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Transparency document

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