

Histone deacetylase inhibitor and PD-1 blockade synergistically inhibit B-cell lymphoma progression in mice model by promoting T-cell infiltration and apoptosis

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Received April 22, 2024; Accepted July 26, 2024

DOI: 10.3892/or.2024.8792

Abstract. B-cell lymphoma is difficult to cure because of its biological and clinical heterogeneity, and due to native chemoresistance. Immunotherapies that overcome cancer-induced immune evasion have been the center of recent developments in oncology. This is emphasized by the accomplishment of various agents that disrupt programmed cell death protein 1 (PD-1)-mediated immune suppression in diverse tumors. However, while PD-1 blockade has been effective in numerous malignancies, a significant proportion of cancers, including B-cell lymphoma, show certain rates of primary resistance to these therapeutic strategies. Histone deacetylase inhibitors (HDACi) have exhibited anticancer activity through suppressing cell proliferation, inducing differentiation and triggering apoptosis. The present study aimed to explore a therapeutic strategy combining a HDACi (romidepsin) and PD-1 blockade (BMS-1) in B-cell lymphoma, utilizing a constructed mouse model of B-cell lymphoma. The IC₅₀ of the two inhibitors was confirmed by MTT assay, and their inhibitory effects were revealed to be dose- and time-dependent. The data demonstrated that the combined treatment of romidepsin and BMS-1 synergistically inhibited the growth of B-cell lymphoma. Furthermore, it was revealed that romidepsin and BMS-1 synergistically triggered apoptosis in mouse B-cell lymphoma. The synergistic effect of these agents was capable of activating tumor-infiltrating lymphocytes, particularly CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells. The results of the present study underscore the potential of

HDAC inhibition in conjunction with PD-1 blockade as a novel therapeutic approach for B-cell lymphoma, highlighting the synergistic effects of these two mechanisms in enhancing antitumor immunity.

Introduction

Lymphoma, a malignant hematological tumor that originates from the lymph nodes or other lymphoid tissues, is mainly divided into Hodgkin lymphoma and non-Hodgkin lymphoma (NHL) subgroups. Collectively, B-cell lymphoma is the most important subgroup of NHL, which causes significant clinical challenges because of heterogeneity and frequent recurrence (1). The programmed cell death protein 1 (PD-1) receptor and its ligand, PD-L1, play a pivotal role in the tumor immune escape mechanism. Immune checkpoint therapy targeting PD-1 and PD-L1 has received regulatory approval for the treatment of specific malignancies, including hematological malignancies (2). PD-1 blockade has demonstrated notable efficacy in recovering T-cell activation in various malignancies, including melanoma, gastric carcinoma, lung cancer and Hodgkin lymphoma (3-9). This immunotherapeutic approach capitalizes on the body's own immune system to combat cancer cells; however, most patients with B-cell lymphoma have no response to PD-1 blockade therapy. The reasons for the poor clinical efficacy of PD-1 blockade in B-cell lymphoma remain indistinct; therefore, drug screening that improves the response to PD-1 blocking therapies is a challenge for B-cell lymphoma.

Histone acetylation is regulated by the homeostasis of histone acetyltransferases and histone deacetylases (HDACs). HDAC expression is frequently dysregulated in numerous types of cancer, including B-cell lymphoma (10-12). HDACs are categorized into four classes based on their structure, mechanism and cellular localization (13): Class I (HDAC1, HDAC2, HDAC3, HDAC8), Class IIa (HDAC4, HDAC5, HDAC7, HDAC9), Class IIb (HDAC6, HDAC10) and Class IV (HDAC11). Altered HDAC activity is associated with neurodegenerative disorders, genetic diseases and cancer (14-16). In cancer, HDAC overexpression is correlated with poor outcomes, and can contribute to the development and progression of hematological malignancies, such as acute lymphoblastic leukemia,

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Key words: histone deacetylase inhibitor, programmed cell death protein 1, apoptosis, lymphocyte, lymphoma

acute myeloid leukemia and chronic myeloid leukemia (13). A total of four HDAC inhibitors (HDACis), belinostat, vorinostat, panobinostat and romidepsin, have been applied in hematological cancers (17,18). Previous studies have illustrated that HDACis can heighten tumor immunogenicity (19,20). In recent years, the pursuit of novel HDACis has been unwavering, leading to significant progress. Wu *et al* (21) examined chidamide, an innovative HDACi, to ascertain its therapeutic efficacy in diffuse large B-cell lymphoma (DLBCL), offering a scientific basis for targeting HDACs in such patients. Bioinformatics analyses coupled with experimental data have revealed notably elevated expression levels of several HDACs (HDAC1, 2, 3, 4, 6, 7, 8 and 9) in DLBCL lymph node samples relative to whole blood cell controls. Furthermore, the mutation frequency of HDACs in DLBCL tissues has been shown to be amplified. Targeting HDACs with selective inhibitors, such as chidamide, presents a prospective therapeutic strategy for patients with DLBCL (21). HDACi drugs are a novel class of antitumor drugs that regulate gene expression and cellular function by inhibiting the activity of HDACs (22). Although no HDACi drugs are currently available specifically for the treatment of B-cell lymphoma in the clinic, available research has suggested that this class of drugs shows promising efficacy and potential in the treatment of other types of tumors. In 2006, the first HDACi was approved by the Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphoma, namely SAHA (vorinostat) (23). After SAHA, three other HDACis were approved by the FDA (24). Studying HDACi drugs for B-cell lymphoma treatment is significant as it allows initial exploration of their mechanism, assessing their therapeutic potential and safety. Understanding their impact on tumor cell processes, such as proliferation, differentiation and apoptosis, provides a scientific basis for new therapeutic strategies.

Based on the current research, the following hypothesis was proposed: The synergistic effects of HDACis with PD-1 blockade therapies may improve efficacy in B-cell lymphoma. Romidepsin is an oral selective HDACi that primarily inhibits HDAC1 and HDAC2 activity. It is widely used to treat specific types of lymphoma and other blood cancers, such as cutaneous T-cell lymphoma and peripheral T-cell lymphoma (25). BMS-1 is a monoclonal antibody that targets the PD-1 receptor, blocking its interaction with PD-L1 and PD-L2; this disruption renews the T cell-mediated immune response, particularly against tumor cells expressing PD-L1 (26). However, their role in B-cell lymphoma cells is not yet fully understood. Therefore, the present study investigated the combined therapeutic potential of romidepsin and BMS-1 in B-cell lymphoma and its preliminary mechanism of action through *in vivo* experiments, aiming to improve the treatment of this type of lymphoma, and to provide a theoretical basis and a new therapeutic strategy for future clinical application.

Materials and methods

Cell culture. Mouse B-cell lymphoma cell lines are often considered a valid model to study human lymphomas because they have similar biological properties. As there are currently no HDACi drugs specifically approved for the treatment of B-cell lymphomas, the present study aimed to use this relatively

simple and controlled model for initial testing to explore new drug candidates. The mouse B-cell lymphoma A20 cell line (cat. no. TIB-208) was obtained from the American Type Culture Collection, and was cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 11875093) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 10099141C) and penicillin/streptomycin (Biosharp Life Sciences; cat. no. BL505A) in a humidified incubator containing 5% CO₂ at 37°C.

MTT assay. To analyze the effects of individual inhibitors and their combination on the proliferation of A20 cells, the MTT assay was employed. Briefly, A20 cells were seeded into 96-well plates at a suitable density of 5x10³ cells/well, along with appropriate controls. Different concentrations of romidepsin (0, 1, 2, 5, 10 and 20 μM) or BMS-1 (0, 2.5, 5, 7.5, 10 and 15 nM) (purchased from Shanghai Aladdin Biochemical Technology Co., Ltd.) were added for treatment, with at least three replicates for each concentration. After incubation at 37°C for 48 h, 50 μl/well MTT (1 mg/ml; Sigma Aldrich; Merck KGaA) was added. Following a 3-h incubation, DMSO (150 μl/well; Beyotime Institute of Biotechnology) was added and the absorbance was measured at 570 nm using a solution mixture. Dose-response curves were plotted based on the results. IC₅₀ values were calculated using GraphPad Prism.

Isograft mouse model. A total of 32 Balb/c mice (female; age, 4 weeks; weight, 20 g), which are widely used in animal experiments in immunology and physiology, were purchased from Charles River Bio-company, and were adaptively maintained in the indicated environment (pathogen-free, 12-h light/dark cycle, 22°C). Mouse B-cell lymphoma A20 isografts were established by the intradermal injection of logarithmic growth phase A20 cells (1x10⁷ cells/ml, 200 μl, 2x10⁶/per mice) into the right flank. A total of 1 week after the injection, by which time the mice were tumor-bound, the mice were randomly divided into four groups (n=8/group), and were administered saline (control), BMS-1 (500 μg/ml; 100 μl/each), romidepsin (1 mg/kg body weight), or a combination of both drugs daily for 19 days. Tumor size was measured using a micrometer caliper. Tumor volume (V, mm³) was calculated every 2 days using the following formula: V=(a x b²)/2, where a refers to the longest diameter and b to the shortest diameter. When the volume of the tumor reached 1,500 mm³, the mice were euthanized by cervical dislocation. Once it was confirmed that the mice showed no signs of life (that is, there was no chest movement, and the eyelids were pale with no visual response), tumors were collected to measure the volume. Data are expressed as the mean ± SEM. In addition, the body weight of each mouse was measured to evaluate the toxicity of the treatment on the mice. Before sacrifice, peripheral blood from each mouse was collected to obtain serum and to detect IFN-γ levels using an ELISA kit (cat. no. MM-0182M1; <http://www.mmbio.cn/goods.php?id=989>), according to the manufacturer's instructions. In addition, the collected tumor tissues were fixed with formalin and embedded in paraffin. All animal studies were approved by the Ethics Committee of Hangzhou Medical College (approval no. 2021-246; Hangzhou, China), and were conducted according to the AAALAC and the IACUC guidelines.

Immunofluorescence analysis of mouse tissue. Mouse tissues (5 μm) were deparaffinized by xylene (Sinopharm Chemical Reagent Co., Ltd.; cat. no. 100234192) and rehydrated. Slices were placed in anhydrous ethanol (Sinopharm Chemical Reagent Co., Ltd.; cat. no. 100092680) for 5 min (repeated 3 times). Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol. Heat-induced antigen retrieval was carried out for all sections by incubating them in a steamer with 0.01 M citrate buffer (pH 6.0) at 95°C for 30 min. Secondly, CD3-FITC + CD4-PE antibodies and CD3-FITC + CD8-PE antibodies (all obtained from Abcam; CD3 antibody, cat. no. ab33429; 1:100; CD4 antibody, cat. no. ab288724; 1:100; and CD8 antibody, cat. no. ab316778; 1:200) were diluted with BSA (cat. no. A8020; Beijing Solarbio Science & Technology Co., Ltd.) to a concentration of 1:100, and were used to incubate the sections at 4°C overnight. Finally, 10 $\mu\text{g}/\text{ml}$ DAPI (Beyotime Institute of Biotechnology) was applied for 5 min and the sections were visualized under a fluorescence microscope (Olympus Corporation). The number of positive cells was examined using ImageJ software (National Institutes of Health).

TUNEL assay for cell apoptosis. After deparaffinization, tumor tissue sections were subjected to TUNEL staining assay for apoptotic cell detection. Briefly, after gently washing with phosphate-buffered saline, the sections were incubated in 70% ethanol for 30 min at 4°C. The subsequent analysis was performed using a commercially available TUNEL assay kit (cat. no. ab206386; Abcam), according to the manufacturer's protocol. The data were further visualized under a fluorescence microscope (Olympus Corporation) and were analyzed using ImageJ software.

Statistical analysis. All data are expressed as the mean \pm SEM. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc.; Dotmatics) and SPSS 13.0 (SPSS, Inc.) software packages. Statistical significance was determined using two-way ANOVA or one-way ANOVA for multiple groups, and Tukey's Honestly Significant Difference was used for the post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Combined use of romidepsin and BMS-1 produces a synergistic effect in inhibiting lymphoma growth. To investigate the effects of a HDACi (romidepsin) and PD-1 blockade (BMS-1) on lymphoma, the mouse B-cell lymphoma A20 isografts were established. First, the MTT assay confirmed that both single-drug treatments impacted the proliferative capacity of A20 cells in a time- and dose-dependent manner. The IC_{50} values for BMS-1 and romidepsin were 5.045 μM and 11.16 nM, respectively (Fig. 1A). In the preliminary experiment, the inhibitory effect of the two inhibitors on tumor growth was time- and dose-dependent (Fig. S1). As revealed in Fig. 1B and C, both romidepsin and BMS-1, when administered individually, could exert a certain impact on tumor growth and tumor volume compared with the control group. Furthermore, the combination of romidepsin and PD-1 blockade more significantly reduced tumor growth and tumor

volume than that in the groups treated with romidepsin and PD-1 blockade alone, with more than a 5-fold decrease in their tumor volume after 19 days compared with in the control group. In addition, the body weights of the combination group were lighter than those in the other groups as time progressed (Fig. 1D). Furthermore, the tissues treated with BMS-1 or in combination with romidepsin significantly elevated the levels of IFN- γ , a T-cell biomarker (27), while romidepsin alone had no significant effect on serum IFN- γ levels detected by ELISA at the end of the experiment (Fig. 1E). The results suggested that romidepsin and BMS-1 treatment synergistically inhibited the growth of A20 isograft lymphomas, which might be associated with the action of romidepsin on the activation of T-cell infiltration.

Combination treatment of romidepsin and BMS-1 increases apoptosis in A20-derived lymphoma. To determine whether apoptosis was required for the efficacy of the combination treatment, the TUNEL assay was performed to detect apoptosis in A20 lymphoma mouse tissues. As shown in Fig. 2A and B, the groups treated with romidepsin and BMS-1 independently exhibited a significantly higher induction of apoptosis compared with the control group. Furthermore, the combination of romidepsin and BMS-1 resulted in a significantly greater enhancement of tumor apoptosis than when either romidepsin or BMS-1 was used alone. These data indicated that romidepsin and BMS-1 synergistically triggered apoptosis in murine B-cell lymphoma.

Combination treatment of romidepsin and BMS-1 activates the CD4⁺ and CD8⁺ tumor-infiltrating lymphocytes (TILs) in A20-derived lymphoma. To determine whether CD4⁺ and CD8⁺ TILs were involved in the efficacy of combination treatment, an immunofluorescence assay was performed to assess the A20 lymphoma mouse tissues. As demonstrated in Fig. 3A and B, the groups treated only with romidepsin or BMS-1 showed a significant increase in CD4⁺ TILs compared with those in the control group. In addition, the combination of romidepsin and BMS-1 led to a significantly greater activation of CD4⁺ TILs compared with the groups treated with romidepsin or BMS-1 alone. Additionally, as revealed in Fig. 4A and B, the groups treated with romidepsin and BMS-1 alone exhibited increased activation of CD8⁺ TILs compared with that in the control group. Furthermore, the combination of romidepsin and BMS-1 more significantly activated CD8⁺ TILs than that in the groups treated with romidepsin and BMS-1 alone. These data suggested that romidepsin and BMS-1 combination accelerated CD4⁺ and CD8⁺ TILs activation.

Discussion

Patients with relapsed/refractory B-cell lymphoma frequently have a poor prognosis (28); therefore, it is essential to explore combination therapeutic strategies for B-cell lymphoma. One mechanism facilitating PD-1 blockade resistance may be reduction of MHC class I and II expression in cancer (29). MHC molecules present peptides to CD4⁺ and CD8⁺ T cells; thus, MHC is indispensable for the identification of cancer cells by antigen-specific CD4⁺ and CD8⁺ T cells. Furthermore, HDACis

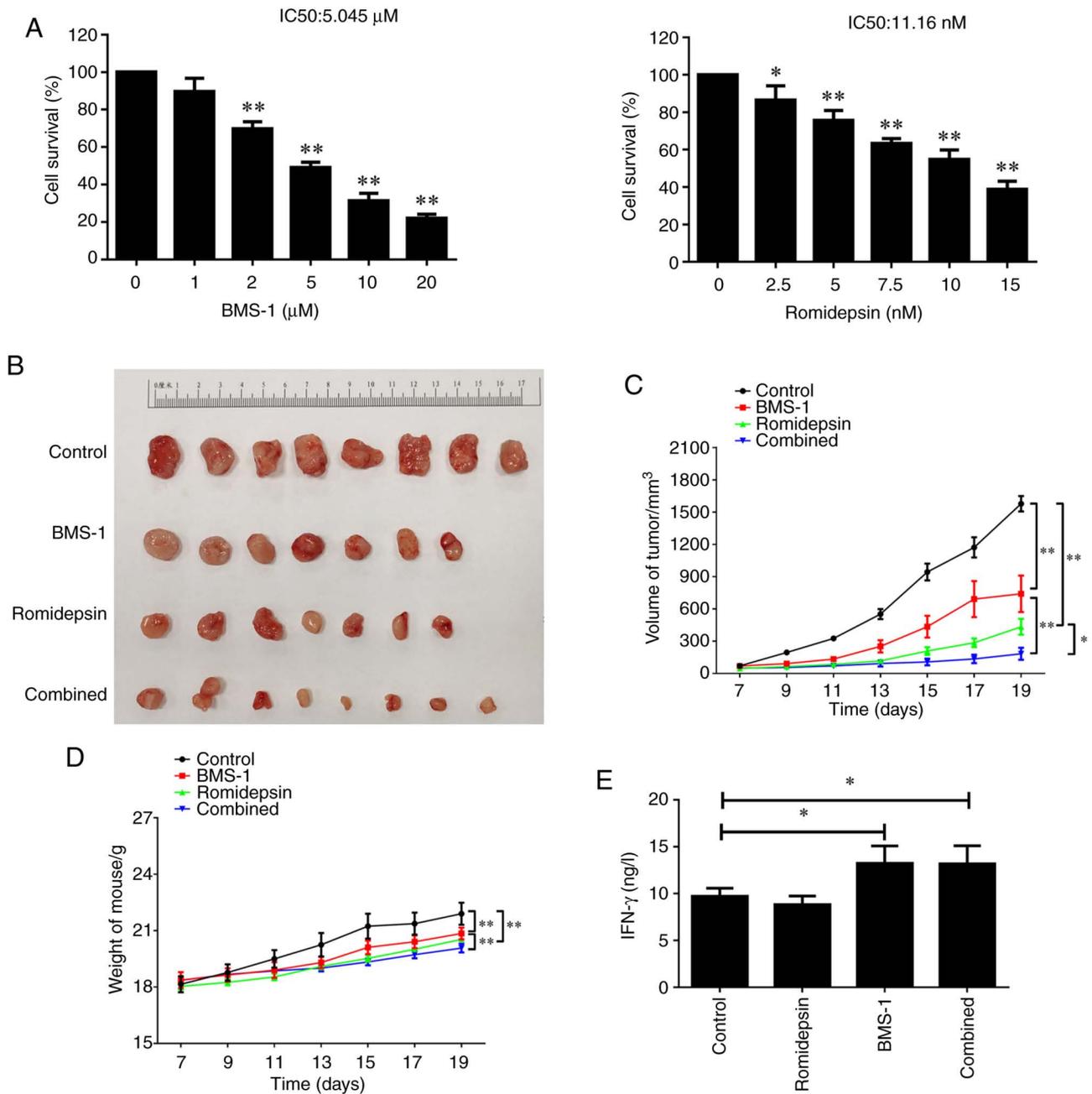


Figure 1. Combined application of romidepsin and BMS-1 produces a synergistic effect on the suppression of lymphoma development. (A) MTT assay was used to detect the proliferative capacity of A20 cells after treatment with different concentrations of individual drugs and their combination. One-way ANOVA was used to evaluate the comparisons among multiple groups. (B) A20 cells were injected into BALB/c mice (2×10^6 cells/mouse). Tumor-bearing mice were then treated with saline, different concentrations of BMS-1 (500 μ g/ml, 100 μ l/mouse) or romidepsin, (1 mg/kg body weight), and their combination every day for 19 days. Images are shown of the tumors from the four groups at the end of the experiment. (C) Tumor size or (D) body weight was measured every 2 days starting at day 7, respectively. Two-way ANOVA was used to evaluate the comparisons among multiple groups. (E) Serum IFN- γ levels were detected by ELISA at the end of administration, and one-way ANOVA was used to evaluate the comparisons among multiple groups. All experiments included appropriate controls and were repeated at least three times to ensure reproducibility. Tukey's Honestly Significant Difference was used for the post-hoc test. * $P < 0.05$ and ** $P < 0.01$.

can upregulate the expression of chemokines that attract T cells to the tumor microenvironment (TME). By enhancing the migration of T cells into the TME, HDACis can synergistically enhance the efficacy of PD-1 blockade, ensuring a sufficient number of activated T cells are available to interact with the tumor following inhibition of the PD-1 pathway (30,31). In the present study, using an intradermal mouse tumor model, it was found that HDACi and PD1-blockade treatment synergistically activated CD4⁺ and CD8⁺ TILs.

Studies have demonstrated that a portion of HDAC family members are abnormally expressed in tumors, such as pancreatic cancer, breast cancer, lung cancer and ovarian cancer (32-35). It has been reported that several HDACis exert potential roles in anticancer immunity (36). For instance, the antitumor effects of vorinostat are associated with the immune system (37,38). Inhibitors have also shown promising anticancer outcomes when used in combination with traditional chemotherapy drugs in tumors (39,40);

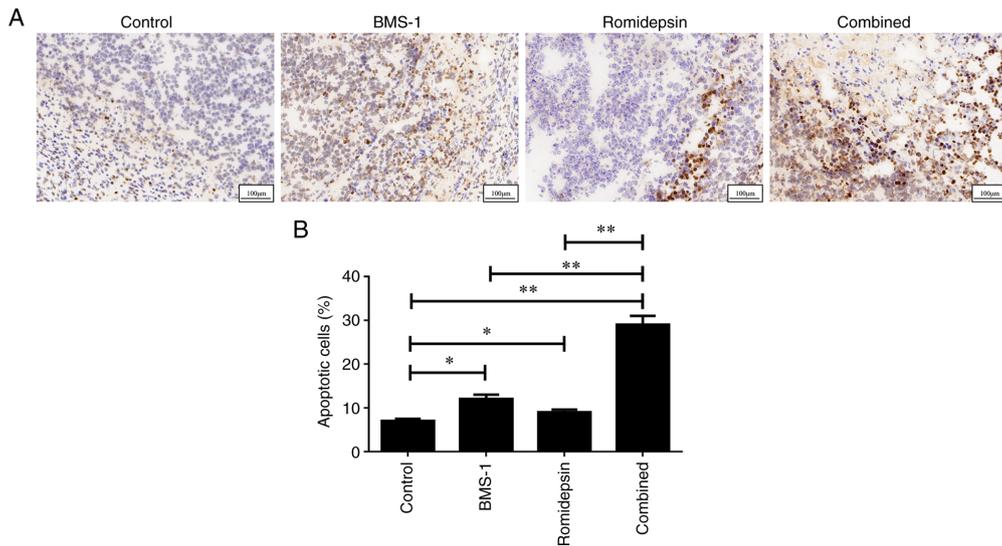


Figure 2. Combined treatment of BMS-1 and romidepsin increases apoptosis in A20-derived lymphoma. (A) Representative immunohistochemistry-TUNEL images of the four groups (Ctrl, BMS-1, romidepsin and combination). (B) Quantification of apoptotic cells in the four groups (Ctrl, BMS-1, romidepsin and combination). All experiments included appropriate controls and were repeated at least three times to ensure reproducibility. One-way ANOVA was used to evaluate the comparisons among multiple groups. *P<0.05 and **P<0.01.

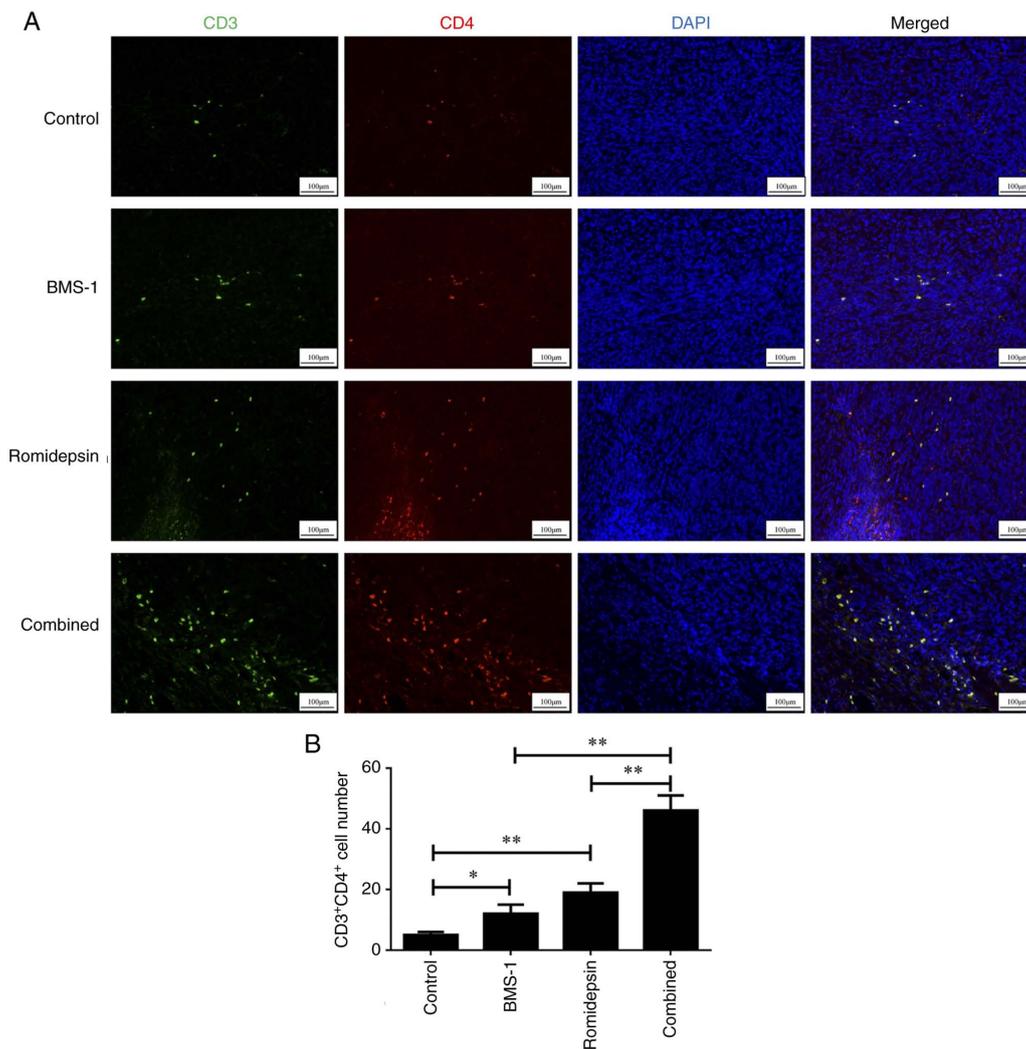


Figure 3. Combined treatment of BMS-1 and romidepsin activates CD4⁺ TILs in A20-derived lymphoma. (A) Representative images of CD3 and CD4 under a fluorescence microscope in the Ctrl, BMS-1, romidepsin and combination groups. (B) Quantification of CD4⁺ TILs in Ctrl, BMS-1, romidepsin and combination groups. All experiments included appropriate controls and were repeated at least three times to ensure reproducibility. One-way ANOVA was used to evaluate the comparisons among multiple groups. *P<0.05 and **P<0.01. TILs, tumor-infiltrating lymphocytes.

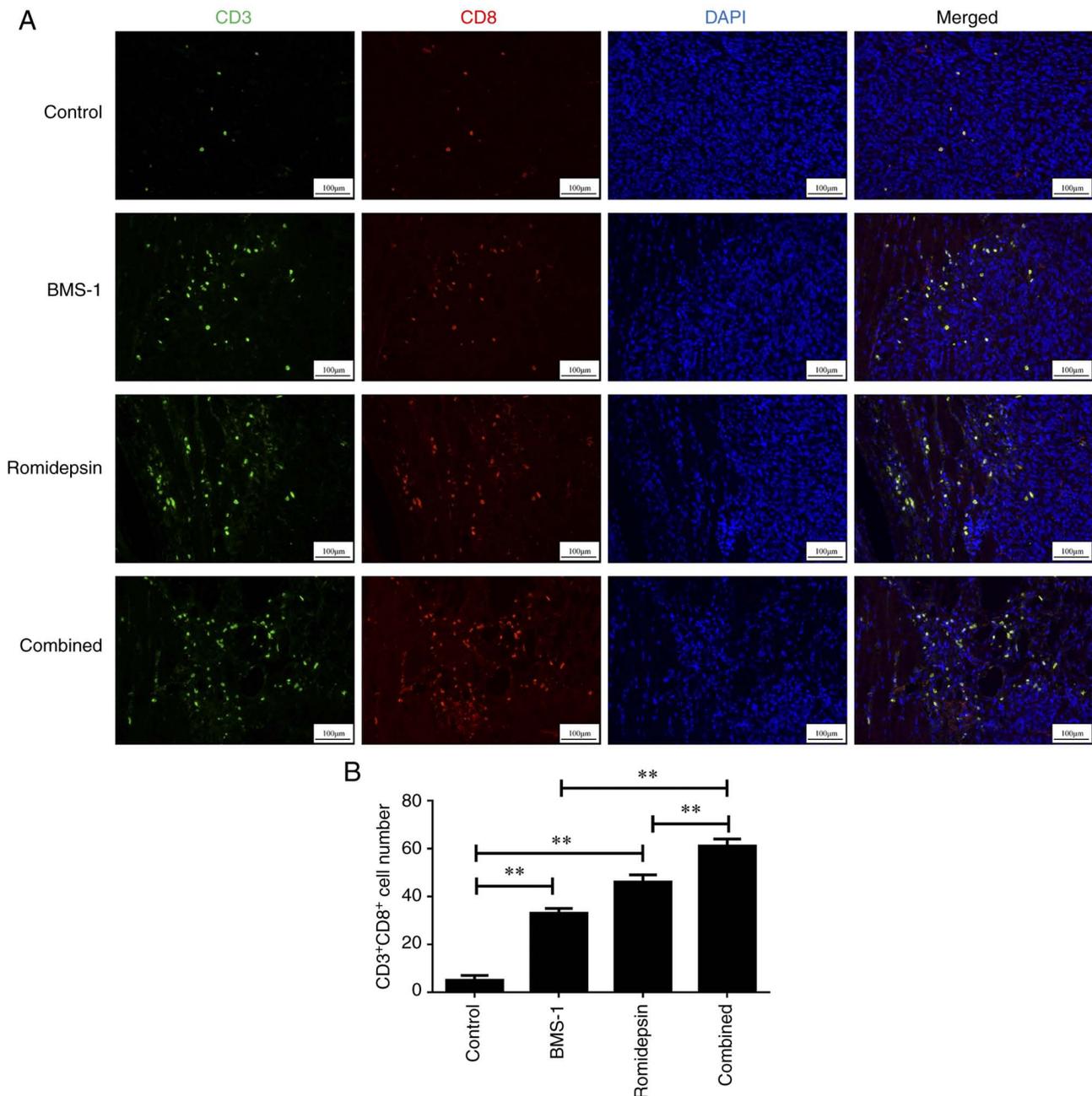


Figure 4. Combined treatment of BMS-1 and romidepsin activate CD8⁺ TILs in A20-derived lymphoma. (A) Representative images of CD3 and CD8 under a fluorescence microscope in the Ctrl, BMS-1, romidepsin and combination groups. (B) Quantification of CD8⁺ TILs in Ctrl, BMS-1, romidepsin and combination groups. All experiments included appropriate controls and were repeated at least three times to ensure reproducibility. One-way ANOVA was used to evaluate the comparisons among multiple groups. **P<0.01. TILs, tumor-infiltrating lymphocytes.

however, their role in B-cell lymphoma cells is not yet fully understood.

The present study examined the therapeutic efficacy of a combination of a HDACi (romidepsin) and PD-L1 inhibitor (BMS-1) in the treatment of an A20-induced B-cell lymphoma model. The findings revealed that both romidepsin and BMS-1 independently inhibited tumor growth in a dose- and time-dependent manner, and induced the apoptosis of tumor cells. Furthermore, when used in combination, there was a significant reduction in tumor growth and increase in apoptosis, suggesting a synergistic effect. On the one hand, romidepsin works by inhibiting HDACs, which leads to hyperacetylation of histones and modulation of gene expression; this results in

cell cycle arrest and the induction of tumor cell apoptosis (41). However, romidepsin not only affects gene expression and survival of tumor cells, but also regulates the immune system. Its ability to enhance the immune response in the TME may be related to increased expression of tumor-associated antigens and the modulation of immune checkpoint molecules. This dual action makes romidepsin a promising agent in anticancer therapy and immunotherapy (2). On the other hand, BMS-1 functions as a PD-L1 inhibitor, blocking its interaction with the PD-1 receptor on T cells. This interference relieves the immune suppression imposed on T cells by tumor cells expressing PD-L1, thereby enhancing the immune response against the tumor (26). This is consistent with the present

findings, which demonstrated that the individual administration of romidepsin and BMS-1 could stimulate CD4⁺ and CD8⁺ TILs in an intradermal mouse tumor model, but that combining these treatments was more effective.

While the present study shows promising synergy between romidepsin and BMS-1 in B-cell lymphoma treatment, providing insights into their combined use in the clinic, several limitations must be acknowledged. Firstly, the present study noted an increase in IFN- γ levels only upon romidepsin administration; however, the combination of romidepsin and BMS-1 did not substantially enhance IFN- γ production, which may be due to the regulation of IFN- γ production by multiple cytokines and signaling pathways, as well as the existence of complex feedback mechanisms in the immune system. This will be the direction of our future research. Secondly, the research focused solely on A20 lymphoma cells, leaving other B-cell lymphoma subtypes unexplored; varying responses across subtypes could impact treatment generalizability. Finally, the longer-term effects and analyses of tumors observed at various time points after treatment and at different doses of this combination have not been explored, requiring extensive clinical trials to confirm chronic outcomes and safety. In conclusion, the present study highlighted the synergistic effects of a HDACi and PD-1 blockade on anticancer response, and recommended this combination therapeutic strategy for the treatment of B-cell lymphoma.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Natural Science Foundation of China (grant no. 82174018), the Zhejiang Medical and Health Science and Technology Project (grant nos. 2022KY470 and 2021KY009) and the Zhejiang TCM Science and Technology Project (grant no. 2022ZB005).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HJ and YG designed the study. TW and XY performed experiments and analyzed the data. TW wrote the draft of the manuscript. TW, XY, HJ and YG confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All animal studies were performed in compliance with the regulations and guidelines of Zhejiang Hospital animal care and conducted according to the AAALAC and the IACUC guidelines. The present study was approved by the Ethics Committee of Hangzhou Medical College (approval no. 2021-246; Hangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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