

MGCD0103 induces apoptosis and simultaneously increases the expression of NF- κ B and PD-L1 in classical Hodgkin's lymphoma

RENHONG HUANG¹, XIAOWEI ZHANG², ZHIJUN MIN¹,
ABDELBARI SOPHIA SHADIA², SHUN'E YANG³ and XIAOJIAN LIU²

¹Department of Gastrointestinal Surgery, Shanghai Pudong Hospital, Fudan University Pudong Medical Center, Shanghai 201399; ²Department of Medical Oncology, Shanghai Cancer Center, Fudan University, Shanghai 200031;

³Department of Lymphoma, The Affiliated Tumor Hospital of Xinjiang Medical University, Urumqi, Xinjiang 830011, P.R. China

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Abstract. At present there is no consensus on the treatment of classical Hodgkin's lymphoma (CHL) following relapse. The aim of the present study was to access the class I-selective histone deacetylase (HDAC) inhibitor (HDACI) MGCD0103 on the expression levels of Bcl-2, nuclear factor (NF)- κ B and programmed death-ligand 1 (PD-L1) in CHL, to explore the possible therapeutic value of MGCD0103 in combined relative target drugs for patients with CHL. In L1236 and L428 cell lines, apoptosis and cell cycle stage were identified using flow cytometry, and the effects of HDACI on CHL were assessed in terms of Bcl-2, NF- κ B and PD-L1 expression levels, which were detected by western blotting and co-focusing experiments. The results demonstrated that MGCD0103 could induce cell apoptosis and cell cycle arrest, down-regulate Bcl-2 and increase NF- κ B and PD-L1 expression levels in L1236 and L428 cell lines. MGCD0103 decreases Bcl-2 levels and upregulates PD-L1, which indicates that the combined use of HDACIs and a PD-L1 inhibitor in theory may improve treatment outcomes in patients with CHL. MGCD0103 may also up-regulate NF- κ B, which seems to induce resistance towards anti-apoptotic drugs. Clinical trials combining HDACIs with NF- κ B and/or PD-L1 inhibitors should be designed to further improve treatment outcomes for patients with CHL.

Introduction

Hodgkin's lymphoma (HL), a B cell-derived lymphoma, is a potentially curable lymphoid malignancy (1). Classical HL (CHL), which accounts for 95% of all HL, is a lymphatic hematopoietic systemic disease characterized by the occurrence of Hodgkin-Reed-Sternberg (HRS) cells in affected reactive lymphadenopathy (2). CHL is classified into 4 types, including nodular sclerosis HL, lymphocyte-depleted classical HL, lymphocyte-rich HL and mixed cellularity HL (3). At present, the most popular therapeutic regimen consists of adriamycin, bleomycin, vinblastine and dacarbazine, and remains the first-line therapy for patients with CHL (4). CHL is curable in the majority of cases (70-80% all stages) treated by conventional chemotherapy and/or combined radiotherapy; peripheral stem-cell transplantation can improve the outcome of patients with relapse following first-line chemotherapy, but with an increased rate of inevitable risks of lung and heart disease, or even other secondary cancers (5). Therefore, novel therapeutics are required for patients with CHL.

Histone deacetylase (HDAC) is a protein deacetylase that causes genetic changes, altering chromatin structure and modulating transcriptional and translational processes (6,7). HDACs are expressed in various malignant tumors, down-regulating relevant tumor suppressor genes (8). HDACs serve an important role in regulating carcinogenesis in various tumors, including CHL (9,10). HDAC inhibitors (HDACIs), a class of therapeutic anticancer drugs, have been widely investigated (7,11). HDACIs induce a series of changes, including chromatin remodeling, regulation of transcription factors, cell cycle arrest and apoptosis induction (12-14). The class I HDAC-selective inhibitor MGCD0103, a specific benzamide histone deacetylase inhibitor, has been effective in controlling a number of cancers such as follicular lymphoma, myelogenous leukemia and Hodgkin's lymphoma in clinical trials (15-17). Previous studies suggested that HDACIs are a target for specific epigenetic changes associated with cancer and other diseases, and many HDACIs have entered clinical studies (18). A better understanding of gene expression and phenotype, homeostasis and neoplastic development that is altered by HDACs would help gain more knowledge about

Correspondence to: Professor Xiaojian Liu, Department of Medical Oncology, Shanghai Cancer Center, Fudan University, 270 Dong'an Road, Shanghai 200031, P.R. China
E-mail: liuxiaojian3000@hotmail.com

Mr. Shun'e Yang, Department of Lymphoma, The Affiliated Tumor Hospital of Xinjiang Medical University, 789 Suzhou Road, Urumqi, Xinjiang 830011, P.R. China
E-mail: yangshune@medmail.com.cn

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CHL, and may represent efficient tools for enhancing treatment in patients with CHL.

Nuclear factor (NF)- κ B has been recently investigated and demonstrated to have an important role in CHL (19,20). Programmed death-ligand 1 (PD-L1) inhibitors are used in treating patients with relapsed CHL (21,22). Academic researches indicated that PD-L1 inhibitors, including nivolumab and pembrolizumab demonstrate remarkable activity in relapsed CHL (23,24). MGCD0103 effects on B cell lymphoma-2 (Bcl-2), NF- κ B and PD-L1 levels require further study. In the present study, the expression levels of HDAC1, 2, 3 and 11 in CHL tissues were examined, and the effects of MGCD0103 on NF- κ B and PD-L1 levels in CHL were assessed, to explore the potential therapeutic value of the class-I HDAC inhibitor MGCD0103 in combined relative target drugs for patients with CHL.

Materials and methods

Reagents. The anchorage-dependent cell line L1236 and the suspension-cultured cell line L428 used in the present study were obtained from Chinese Academy of Sciences (Shanghai, China). The HDACI MGCD0103 was supplied from MethylGene, Inc. (Toronto, Canada). Antibodies against Bcl-2 (cat. no. ab32124) were provided by the Department of Pathology, Shanghai Cancer Center, Fudan University (Shanghai, China). Antibody against α -tubulin (cat. no. ab52866) was obtained from Abcam (Cambridge, MA, USA). Antibodies against NF- κ B (cat. no. 8242) and PD-L1 (cat. nos. 13684 and 25048) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (IgG) antibody (cat. no. 10285-1-AP) was acquired from ProteinTech Group, Inc. (Chicago, IL, USA). Annexin V FITC Apoptosis Detection kit was acquired from BD Biosciences (Franklin Lakes, NJ, USA). Fluorescent-dye conjugated secondary antibodies (Alexa Fluor[®] 488-conjugated; cat. no. ab150077) were obtained from Abcam (Cambridge, UK).

Cell culture and group design. The L1236 and L428 cell lines, maintained at an atmosphere of 5% CO₂ and 37°C, were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplied with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Cells in the exponential phase were harvested and used in subsequent experiments. Cells in the MGCD0103 0.5, 1 and 2 μ M groups were treated for 24 h with HDACI MGCD0103 at 0.5, 1 and 2 μ M, respectively. An equal volume of dimethyl sulfoxide (DMSO) was added in the control group.

Protein extraction and western blot analysis. Radioimmunoprecipitation assay extraction reagents with 1% phenylmethanesulfonyl fluoride and 1% DL-dithiothreitol were applied to extract the total protein of the L236 cells. L428 cells were harvested and dissolved in 9.8 M urea (S1961; Beyotime Institute of Biotechnology, Haimen, China), 15 mM EDTA (P1045; Beyotime Institute of Biotechnology) and 30 mM Tris medium (ST774; Beyotime Institute of Biotechnology), and treated with a cell disruption step using the ultrasonic technique. Disrupted cells were then centrifuged (1,000 \times g; 5 min; 4°C),

insoluble compounds were removed and the supernatant was collected. A bicinchoninic protein assay (Pierce; Thermo Fisher Scientific, Inc.) was employed to measure the concentrations of the lysate protein of the two cell types. Equal amounts of protein (20 μ g) in each group were separated by 12% SDS-PAGE, and then the proteins were transferred onto polyvinylidene difluoride membranes (PVDF). Subsequently, 5% non-fat dry milk dissolved in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20; pH 7.40) was used to block non-specific antigens on the PVDF membranes at room temperature for 1 h. Subsequently, the membranes were incubated with primary antibodies at 4°C overnight (anti-Bcl-2, 1:1,000; anti-NF- κ B, 1:1,000; anti-PD-L1, 1:1,000). Subsequently, the membranes were washed with TBST three times for 5 min and incubated with goat anti-rabbit IgG secondary antibody (1:1,000) at room temperature for 1 h. α -tubulin was used as a loading control (α -tubulin antibody, 1:1,000). The images of western blotting were captured using an Omega Lum G imaging system (Gel Company, Inc., San Francisco, CA, USA) and the intensity of bands was determined using AlphaEase FC software 4.1.0 (Alpha Innotech Corporation; ProteinSimple, San Jose, CA, USA).

Cell apoptosis and cycle analyzed by flow cytometry. According to the manufacturer's protocol, cell apoptosis and cycle analysis were measured using propidium iodide and Annexin-V staining. Initially, L1236 and L428 cells were treated with MGCD0103 or DMSO for 24 h at 37°C as described above. L1236 cells were seeded onto a 6-well plate with RPMI 1640 medium at a density of 1 \times 10⁶ cells/ml and L428 cells were seeded onto a 6-well plate and suspended at a density of 1 \times 10⁶ cells/ml in RPMI-1640 medium per well 4°C, following treatment. Subsequently, L1236 and L428 cells were harvested and washed with PBS twice. For the analysis of the cell cycle, cells were treated with RNase (Invitrogen; Thermo Fisher Scientific, Inc.) at a final concentration of 0.2 mg/ml, and stained with propidium iodide (FITC Annexin V Apoptosis Detection kit I; BD Biosciences, Franklin Lakes, NJ, USA) at a final concentration of 10 μ g/ml in the dark at 4°C for 20 min, subsequently. Finally, cells were detected by Immunocytometer Systems (FACSCalibur; BD Biosciences) and data was analyzed using Flowing software (version 2.5.1; <http://flowingsoftware.btk.fi/>). For the analysis of cell apoptosis, both types of cells were suspended in binding buffer from the kit at a density of 1 \times 10⁵/well, respectively. Subsequently, the cells were stained with 5 μ l propidium iodide and 5 μ l Annexin V-fluorescein isothiocyanate for 30 min in darkness at 4°C, and then detected using Immunocytometer Systems.

Fluorescence staining and confocal laser scanning techniques. Coverslips were kept flat on the bottom of a 6-well plate following cleaning, disinfection and 24-h ultraviolet irradiation. Subsequently, the L1236 cells were seeded on coverslips at a density of 1 \times 10⁵/well and cultured in an incubator at 37°C for 12 h. Cells were treated with MGCD0103 (0.5, 1 and 2 μ M) or with DMSO in the control cells for 24 h at 37°C. L1236 and L428 cells were rinsed with PBS three times for 5 min and fixed with 4% paraformaldehyde at 25°C for 15 min, followed by permeabilization of the cells in 0.2% Triton X-100 at 25°C for a further 20 min. Subsequently, the coverslips were rinsed with PBS again three times for 5 min

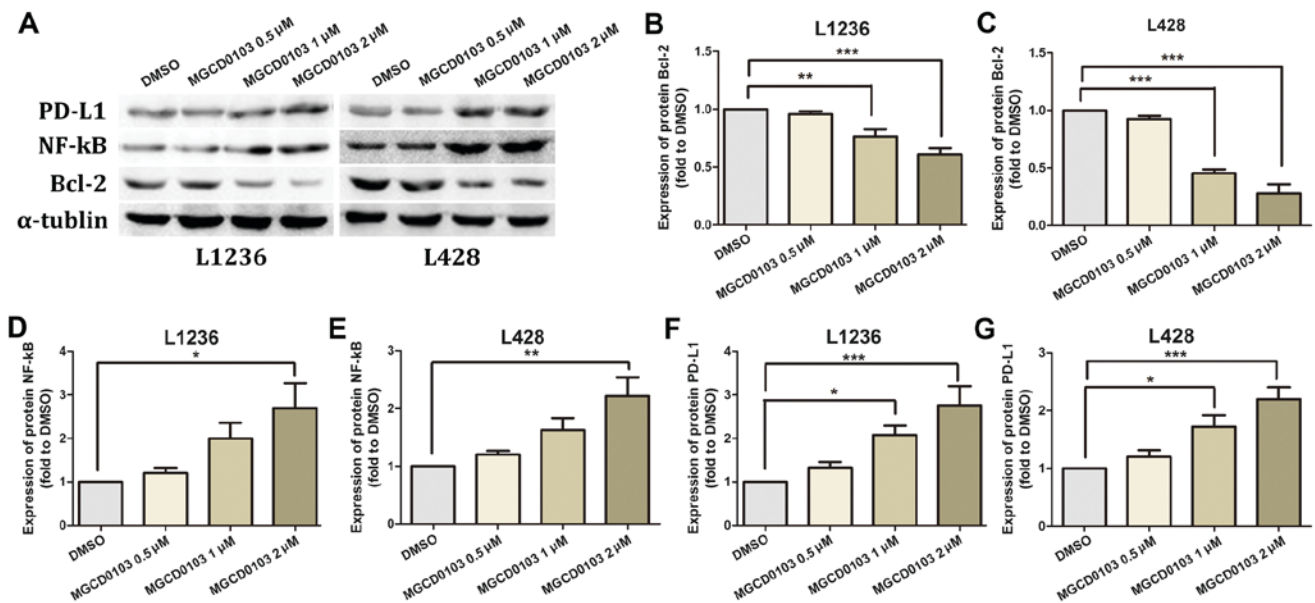


Figure 1. (A) Western blotting was employed to assess Bcl-2, NF- κ B and PD-L1 protein levels, with α -tubulin as a loading control. In MGCD0103 groups, cells were treated for 24 h with MGCD0103 at 0.5, 1 and 2 μ M, with the DMSO group considered as control cells. (B and C) Bcl-2 levels decreased in a dose-dependent manner in the MGCD0103 groups compared with the control group. (D and E) NF- κ B expression was higher in the MGCD0103 groups than in controls. (F and G) Similarly, PD-L1 expression was higher in the MGCD0103 group compared with control cells. Data are presented as the mean + the standard error of the mean. * P <0.05, ** P <0.01, and *** P <0.001. Bcl-2, B cell lymphoma-2; NF, nuclear factor; PD-L1, programmed death-ligand 1; DMSO, dimethyl sulfoxide.

and blocked by incubating the L1236-attached cells in 5% bovine serum albumin (BSA; A8010; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at 25°C for 60 min. L428 cells were suspended and blocked in 5% BSA at 25°C for 60 min. Cells were then incubated with rabbit anti-human Bcl-2 (1:100), NF- κ B (1:400) and PD-L1 (1:50) antibodies for 12 h at 4°C. Following washing with PBS three times for 5 min, the cells were incubated with Alexa 488-coupled goat anti-rabbit IgG secondary antibodies (1:1,000) for 1 h at 4°C in the dark. Finally, DAPI was used as a counterstain to label the nuclei at 25°C for 15 min. The stained L1236-attached cells and L428-suspension cells were the acquired and images were captured under fluorescent and laser confocal microscopy (magnification, x600; Lexel Laser, Fremont, CA, USA).

Statistical analysis. Data are presented as the mean + standard error of the mean, and experiments were performed and repeated three times independently. Statistical analysis data of the total Annexin-V positive cells (% DMSO), data of the cell cycle distribution (% of DMSO), and the data of expressions of Bcl-2, NF- κ B and PD-L1 (integrated optical density at the wavelength of 520 nm/area; compared with DMSO) were analyzed for significant differences using Student's t-test. Bcl-2, NF- κ B and PD-L1 protein expression (relevant to DMSO) were analyzed for significant differences using one-way analysis of variance and post hoc Turkey's tests. SPSS 20.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. P <0.05 was considered to indicate a statistically significant difference.

Results

MGCD0103 downregulates Bcl-2, and increases NF- κ B and PD-L1 expression levels. Two HL L1236 and L428 cell lines

were treated with varying concentrations of MGCD0103 (0, 0.5, 1 or 2 μ M) for 24 h, and protein levels of Bcl-2, NF- κ B and PD-L1 were measured by western blotting (Fig. 1A). No statistically significant differences were identified in protein expression levels following treatment of the 2 cell lines with MGCD0103 at a concentration of 0.5 μ M (Fig. 1B-G). In the L1236 cell line, MGCD0103 significantly inhibited Bcl-2 expression at a concentration of 1 (P <0.01) and 2 μ M (P <0.001; Fig. 1B), and upregulated NF- κ B at 2 μ M (P <0.05, Fig. 1D) and PD-L1 at 1 (P <0.05) and 2 μ M (P <0.001; Fig. 1F). Similarly, in the L428 cell line, MGCD0103 inhibited Bcl-2 expression at 1 (P <0.001) and 2 μ M (P <0.001; Fig. 1C), and upregulated NF- κ B at 2 μ M (P <0.01; Fig. 1E) and PD-L1 at 1 (P <0.05) and 2 μ M (P <0.001; Fig. 1G).

Laser confocal microscopy was also applied to examine the protein expression levels following treatment with MGCD0103 at 2 μ M (Figs. 2 and 3). The expression levels of various proteins were assessed using the IOD/area ratio. These findings suggested that Bcl-2 expression was decreased whereas NF- κ B and PD-L1 were upregulated in the MGCD0103 2 μ M group compared with the DMSO group in the L1236 cell line; all differences were statistically significant (P <0.05; Fig. 3A, C and E). In the L428 cell line, Bcl-2 was also downregulated and NF- κ B upregulated following treatment with 2 μ M MGCD0103 (P <0.05; Fig. 3B and D); and although there was no significant difference, PD-L1 was markedly increased in the MGCD0103 2 μ M group compared with the DMSO group (Fig. 3F).

MGCD0103 induces cell apoptosis and cell cycle arrest in L1236 and L428 cells. To explore the role of MGCD0103 on cell apoptosis, flow cytometry was applied (Fig. 4). Following treatment with MGCD0103 at a final concentration of 2 μ M

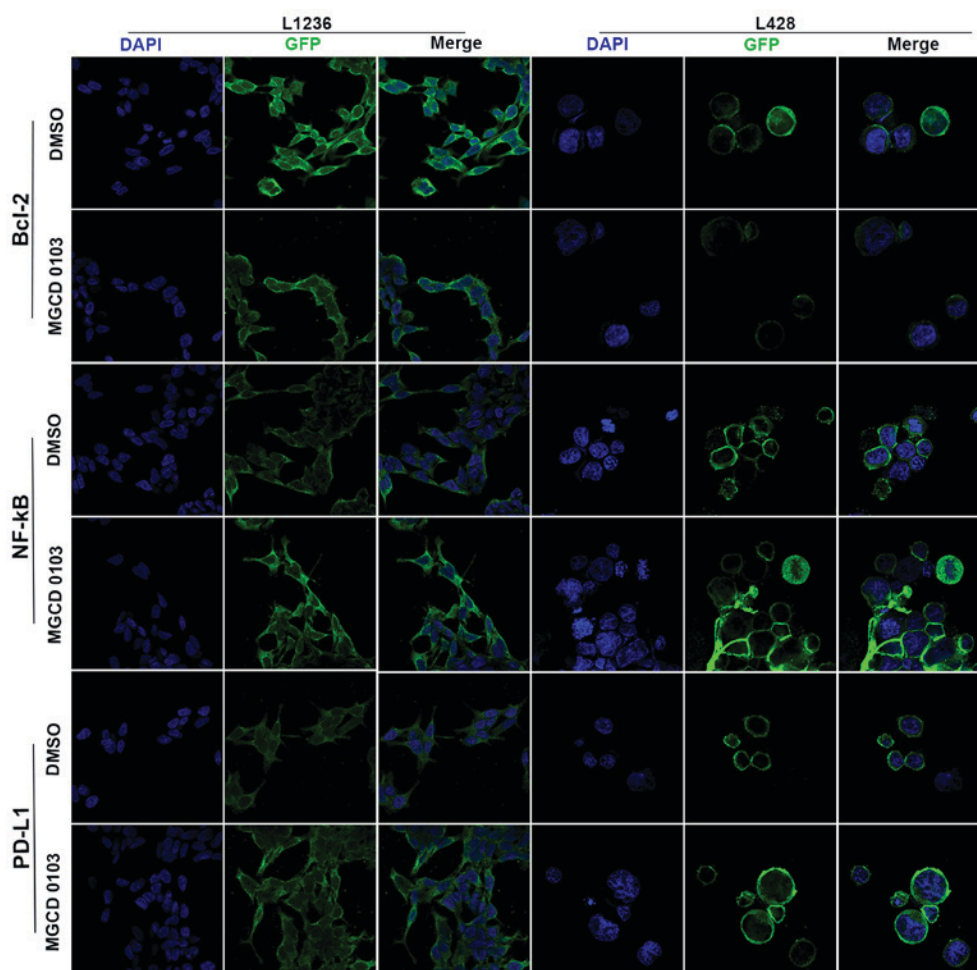


Figure 2. Bcl-2, NF- κ B and PD-L1 protein levels were measured by representative confocal microscopic images (magnification, x600). In the MGCD0103 2 μ M group, cells were treated for 24 h with MGCD0103 at 2 μ M, with DMSO considered as a control. Bcl-2, B cell lymphoma-2; NF, nuclear factor; PD-L1, programmed death-ligand 1; DMSO, dimethyl sulfoxide; GFP, green fluorescent protein.

for 24 h, the apoptosis rate was significantly increased in the MGCD0103 group compared with the DMSO group, as revealed by the increased proportion of total Annexin-V positive-stained cells (Fig. 4A and C). Quantitative analysis of total Annexin-V positive-stained cells was performed, and total rates of Annexin-V positive cells were significantly increased following treatment with MGCD0103 in the L1236 ($P < 0.01$; Fig. 4B) and L428 ($P < 0.05$; Fig. 4D) cell lines. Furthermore, flow cytometry was employed to assess the effects of MGCD0103 on cell cycle in L1234 and L428 cells (Fig. 5). The results demonstrated that MGCD0103 treatment resulted in significantly decreased numbers of cells in the G1 phase in L1236 ($P < 0.05$; Fig. 5A and B) and L428 ($P < 0.001$; Fig. 5C and D) cells, with significantly increased cells in the G2 phase in L1236 ($P < 0.05$; Fig. 5B) and L428 ($P < 0.01$; Fig. 5D) cells compared with control groups. These findings demonstrated that MGCD0103 could induce cell apoptosis and cell cycle arrest.

Discussion

No consensus is currently available regarding the treatment of CHL following relapse. Immune checkpoint inhibitors are a potential avenue for such patients; however, the complete remission rate

is ~17-21% (25). A previous study demonstrated that expression levels of certain HDACs are associated with clinicopathological characteristics in CHL (26). The results suggested that HDAC1, 3 and 11 are expressed at increased levels in CHL, whereas HDAC2 is decreased (26). In addition, increased expression of HDAC1 predicts shorter progression-free and overall survival (OS), while an increased expression of HDAC11 predicts lower OS (26). The current findings provided insights into the effects on Bcl-2, NF- κ B and PD-L1 levels by the treatment of the class I HDAC1 MGCD0103 in an experimental system; namely, that MGCD0103 enhanced the expression levels of PD-L1 and NF- κ B, and reduced the expression of Bcl-2 in CHL.

Bcl-2, a regulatory protein of the Bcl-2 family, serves an important role in promoting cell survival and inhibiting pro-apoptotic proteins (27). It has been demonstrated that Bcl-2 overstimulation and overexpression, and upregulation of the oncogene myc may induce aggressive B-cell malignancies (28). The present findings demonstrated that MGCD0103 had a direct dose-dependent effect in inducing Bcl-2 expression, an apoptosis-related protein, and arresting cell cycle in CHL cell lines.

NF- κ B is a protein complex associated with DNA transcription, cytokine regulation and cell survival in multiple cell types (29). As a nuclear transcription factor, NF- κ B promotes cell proliferation in acute myelogenous leukemia cells (30).

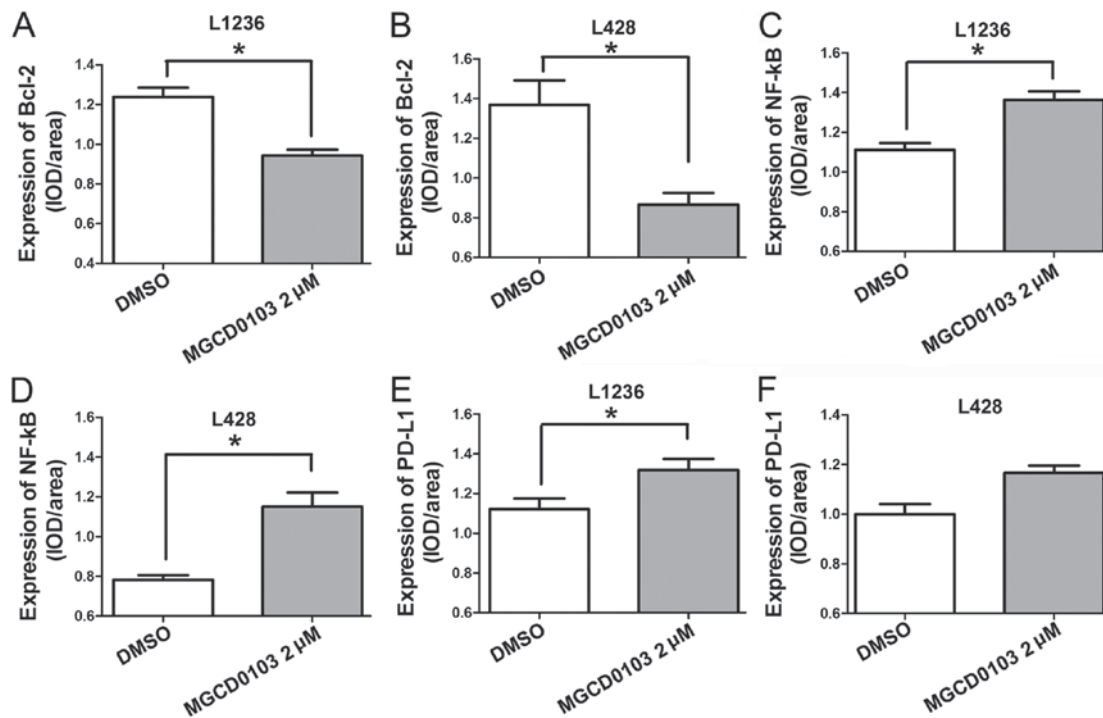


Figure 3. Representative confocal microscopic data for IOD/area detection. (A and B) The IOD/area ratio for Bcl-2 was lower in the MGCD0103 group than in control cells, in both cell lines. IOD/area ratios for (C and D) NF-κB and (E and F) PD-L1 were higher in the MGCD0103 group than in controls, in both cell lines. Data are presented as the mean + the standard error of the mean. *P<0.05. IOD, integrated optical density; Bcl-2, B cell lymphoma-2; NF, nuclear factor; PD-L1, programmed death-ligand 1; DMSO, dimethyl sulfoxide.

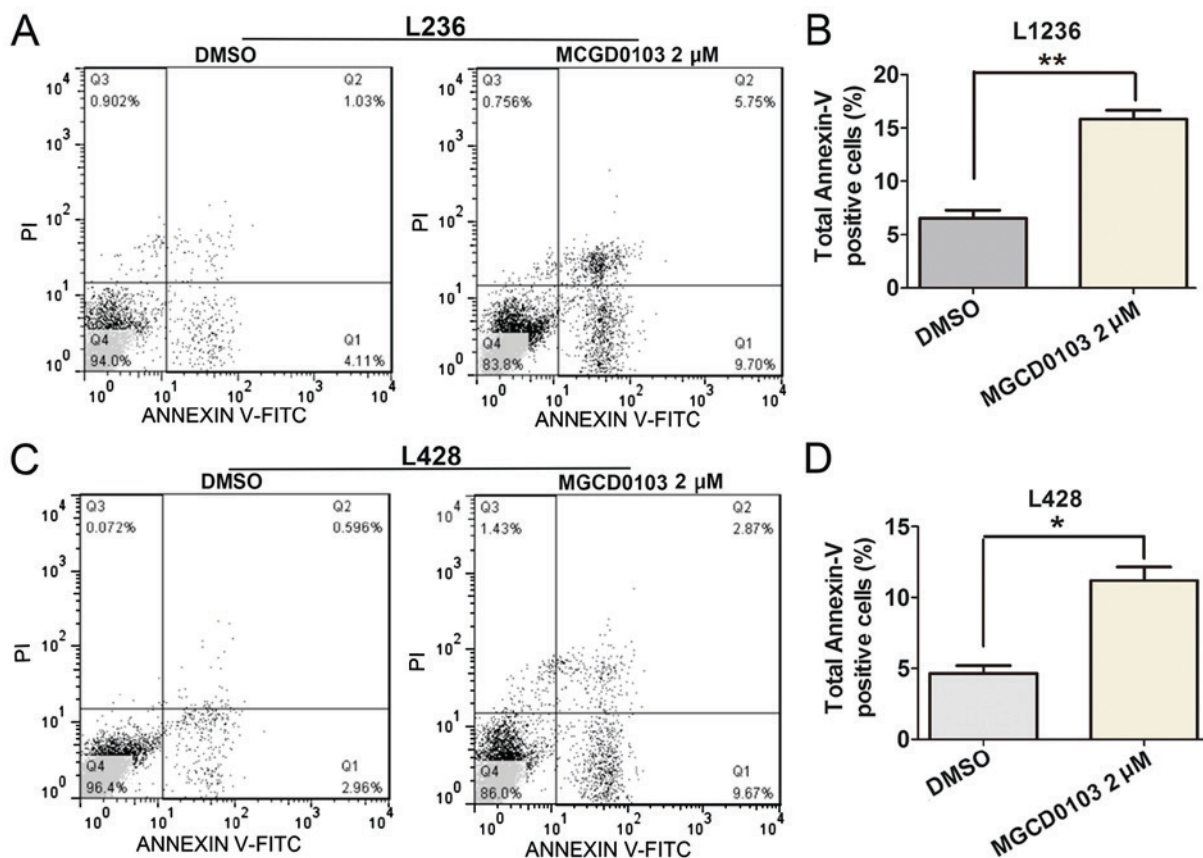


Figure 4. Apoptotic rates were determined by flow cytometry with Annexin-V/PI double-staining. In the MGCD0103 2 μM group, cells were treated with MGCD0103 at a concentration of 2 μM, with the DMSO group considered the control group; cells were treated for 24 h. Total percentages of Annexin-V-positive cells in DMSO and MGCD0103 groups were measured in (A and B) L1236 and (C and D) L428 cells. The percentage of Annexin-V-positive cells in the DMSO groups was lower than that of the MGCD0103 group, in both cell lines. Data are presented as the mean + the standard error of the mean. *P<0.05 and **P<0.01. PI, propidium iodide; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate.

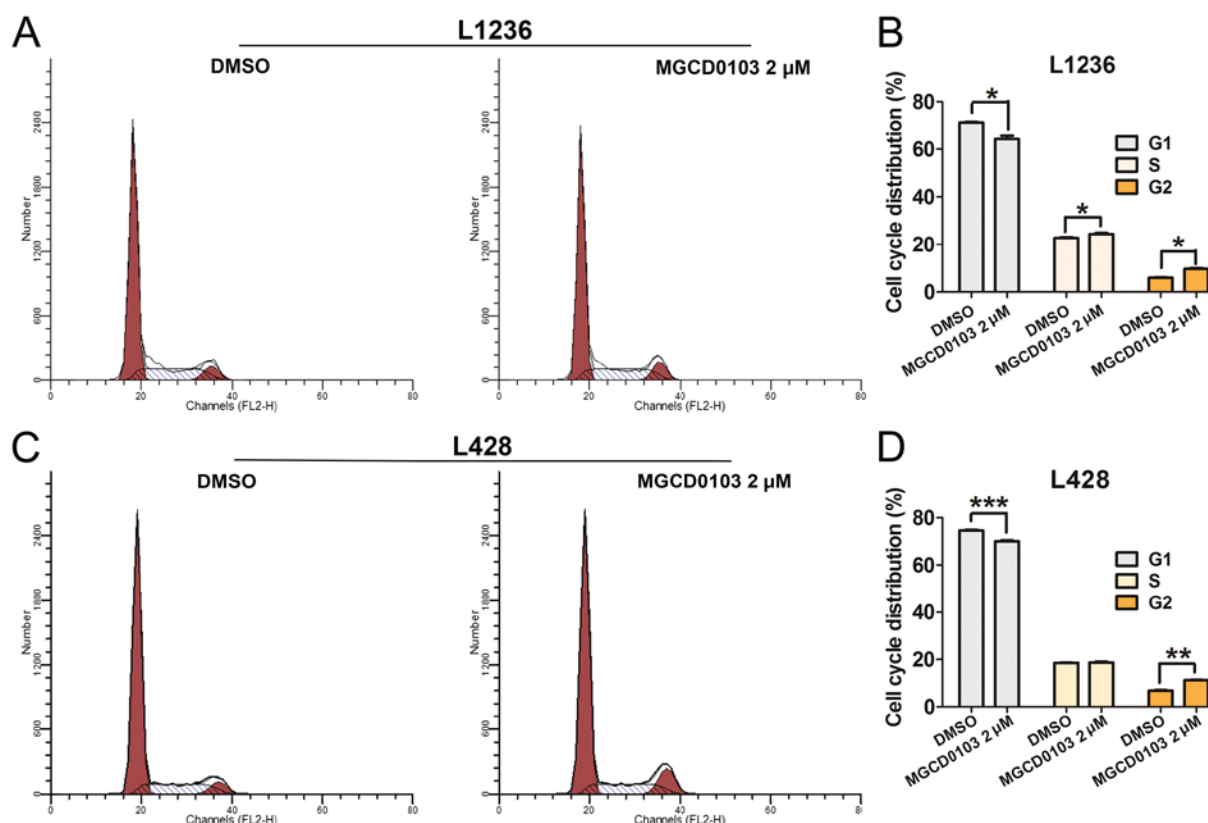


Figure 5. Cell cycle distribution was detected by flow cytometry, and quantitative analysis of the different cell cycle phases was performed in (A and B) L1236 and (C and D) L428 cells. In the MGCD0103 2 μ M group, cells were treated with MGCD0103 at a concentration of 2 μ M for 24 h, with DMSO as control. Fewer cells in the G1 phase were found in the MGCD0103 group compared with the DMSO group, both in L1236 and L428 cells; whereas more cells in the G2 phase were detected in the MGCD0103 group compared with DMSO treated cells, both in L1236 and L428 cells. Cells in the S phase were significantly increased in the MGCD0103 group of L1236 cells. Data are presented as the mean + the standard error of the mean. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. DMSO, dimethyl sulfoxide.

VvpE, an elastase mediated by NF- κ B, is associated with cell death and the inflammatory response in human intestinal epithelial cells (31). It was demonstrated that NF- κ B is widely expressed in malignant lymphoma, and activation of NF- κ B subunits may be associated with the biological functions of HL (32). Buglio *et al* (33) identified that MGCD0103 is able to induce tumor necrosis factor- α expression and secretion, in association with NF- κ B activation. They demonstrated that MGCD0103 may synergize with proteasome inhibitors by HDAC6-independent mechanisms, providing mechanistic rationale for exploring this potentially less-toxic combination for the treatment of lymphoma. Thus, HDACi combined with NF- κ B inhibitor may yield synergistic anti-tumor effects, in accordance with the present findings.

PD-L1, also known as B7 homolog 1 or cluster of differentiation 274 (CD274), is a transmembrane protein encoded by the CD274 gene. PD-L1 has been demonstrated to serve an important role in suppressing the immune system in multiple processes, including pregnancy, inflammation and autoimmune diseases (34–36). Notably, antibodies specifically targeting PD-L1 ligands have provided novel treatments of multiple types of cancer (37). In metastatic renal cell carcinoma, McDermott *et al* (38) demonstrated that immune-oncology monotherapy can be regarded as ideal second-in-line treatment option. Increased expression of PD-L1 predicts a poor prognosis in colon carcinoma and PD-L1 may describe a future treatment target (39). Previous studies further demonstrated the efficacy

of PD-1-targeted therapy in patients with metastatic gastric cancer (40). Previous studies have indicated that PD-1 is associated with inducing T cell tolerance, and can limit T cell responses that may prevent immune-mediated tissue damage (41–43). PD-L1 is correlated with antitumor immunity (44). PD-L1 expressed on the cell surface may help identify immune checkpoint blockade therapies for patients with non-Hodgkin's lymphoma (45). It has been suggested that MGCD0103 may directly inhibit CHL cell growth and survival (46). The present study demonstrated that MGCD0103 may enhance the protein expression levels of NF- κ B and PD-L1; these findings indicated that MGCD0103 may regulate cell-mediated immunity of CHL. To a certain extent, this effect of MCD0103 is detrimental to anti-tumor immune function in the microenvironment in which HRS cells reside. Therefore, whether MGCD0103 and PD-1 inhibitors have synergistic effects in the treatment of CHL requires further investigation.

Previous studies have indicated that HDACi may regulate PD-L1 expression; however these findings have been inconsistent. Booth *et al* (47) recently demonstrated that HDACi are capable of reducing HDAC protein expression levels as well as PD-L1 amounts in melanoma cells; meanwhile, Woods *et al* (48) revealed that class I HDACi upregulate PD-L1 in melanoma. Therefore, these studies indicated that HDACs have dual-regulation functions and mechanisms in regulating multiple physiological and biochemical processes. The present findings indicated that HDACi may upregulate PD-L1. This

may depend on tumor type and specific molecular biological characteristics in the specific tumor microenvironment.

Briere *et al* (49) demonstrated that MGCD0103 upregulated PD-L1 and antigen presentation genes including class I and II human leukocyte antigen family members in a panel of non-small cell lung cancer cell lines *in vitro*. It was concluded that the combination of MGCD0103 and PD-L1 inhibitor demonstrated increased anti-tumor activity compared with either therapy alone in two syngeneic tumor models. In addition, MGCD0103 decreased T-regulatory cell numbers in the tumor microenvironment.

The present results demonstrate that the type I HDACi MGCD0103 decreases Bcl-2 levels and upregulates PD-L1, which indicates the decreased immune ability of CD4+ in the microenvironment of CHL. The combined use of HDACis and a PD-L1 inhibitor theoretically may improve treatment outcome in patients with CHL. Furthermore, the type I HDACi MGCD0103 may also upregulate NF- κ B, which seems to induce resistance towards anti-apoptotic drugs. It seems, therefore, necessary to use anti-NF- κ B drugs in combination with HDACis. Clinical trials combining HDACis with NF- κ B and/or PD-L1 inhibitors should be designed to further improve treatment outcomes for patients with CHL.

The present study had some limitations. The molecular mechanisms by which HDACis affect CHL have not been deeply investigated in this primary study. A previous study demonstrated that blockage of PD-L1/PD-L2 on 9p24.1 may prolong progression-free survival in patients with CHL (50). However, the effects of HDACis on 9p24.1 amplification in CHL have not yet been reported. Based on the present data, the effects of HDACis on 9p24.1 amplification deserve further assessment. The current study focused on exploring the possibility of combining HDACis and other targeted drugs such as NF- κ B and/or PD-L1 inhibitors. Therefore, the effects of HDACis on CHL were assessed in terms of Bcl-2, NF- κ B and PD-L1 expression levels.

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Availability of data and materials

All data generated or analyzed during the study are included in this article.

Authors' contributions

XL, SY, ASS and ZM conceived and designed the present study. RH and XZ performed the experiments. RH, XZ, ASS, ZM and XL collected the data. RH, XZ, SY, XL, ASS and ZM performed the data analysis and interpretation. RH, XL, SY,

ASS and ZM were responsible for literature search. RH was involved in the preparation of manuscript. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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