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HDAC-selective Inhibitor Cay10603 Has Single Anti-tumour Effect in Burkitt's Lymphoma Cells by Impeding the Cell Cycle*

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Summary: Histone deacetylases (HDACs) inhibitors are novel in cancer therapy nowadays. HDAC6-selective inhibitors exert advantageous effects due to higher selectivity and less toxicity. We explored the anti-tumor effect and the molecular mechanism of cay10603, a potent HDAC6 inhibitor in Burkitt's lymphoma cells. Our study revealed cay10603 inhibited the proliferation of Burkitt's lymphoma cell lines, and induced caspase-dependent apoptosis. Cay10603 inhibited the expression of CDKs and cyclins to impede cell cycle progression in both Burkitt's lymphoma cell lines. Cay10603 also showed the additive effect with vp16 notably. Our data presented the promising anti-tumor effect of cay10603 in the Burkitt's lymphoma therapy.
Key words: Burkitt's lymphoma; HDAC6; cell cycle; apoptosis

Burkitt's lymphoma (BL) is a highly-invasive non-Hodgkin lymphoma which is the fastest growing human tumour, and manifested as acute leukaemia. BL can be categorized into Epstein-Barr-virus (EBV)-associated endemic subtype, sporadic subtype and immunodeficiency related subtype. Currently multidrug chemotherapy is the principal means for BL treatment, which is composed of doxorubicin, alkylators, vincristine, and vp-16, etc.[1]. The high toxicity of chemotherapy drugs in these regimens limited the application to the older patients, leading to the relapse and poor prognosis[2, 3]. Furthermore, the immune suppression associated with the intensive chemotherapeutic regimens also makes the administration difficult for the endemic and immunodeficiency related BL patients. Hence, less toxic but effective therapeutic strategies are necessary.

In recent years, histone deacetylases (HDACs) have been recognized as promising therapeutics for human tumors[4, 5], especially in hematological malignancy therapy[6–9]. HDACs regulate the dynamic balance of acetylation and deacetylation of both histone and non-histone to play critical role in the growth and transformation of tumours. Eighteen HDACs have been identified in humans, and are divided into four classes: class I (HDAC1, 2, 3 and 8), class II (HDAC4, 5, 6, 7, 9 and 10), class III (SIRT1-7) and class IV (HDAC11)[10]. In spite of two HDAC inhibitors (HDACi), Romidepsin (FK228) and SAHA (vorinostat, class I & II HDACi) being approved by the US Food and Drug Administration (FDA) for cutaneous T-cell lymphoma treatment, the cytotoxicity caused by the pan-HDACi off-target effects limits its application in research[11]. Inhibitors against specific HADC isomer offer an advantage due to less toxicity. HDAC6 is class IIb HDAC, which is located predominantly in cytoplasm. The over-expression of HDAC6 suggests poor prognosis and more aggressive course in B and T cell lymphomas[12]. HDAC6 modulates the acetylation of multiple non-histone substrates such as tubulin, HSP90, p53, which were involved in cancer development and progression[13, 14]. HDAC6 inhibitors influence the biological behaviour of tumour cells by regulating such key regulator. In many solid tumours, HDAC6 was reported to be highly expressed, and the anti-tumour effect of HDAC6 inhibitors were also discussed[15, 16]. Cay10603 is a novel potent and selective inhibitor of HDAC6, but not further explored to date. Cay10603 was reported to be of high selectivity and exert about 10-fold stronger effect than SAHA to

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block pancreatic cancer cell growth[17]. It also showed promising effect on inhibiting the proliferation in lung adenocarcinoma cells[18]. However, the anti-tumour effect of cay10603 in BL was not mentioned and the related molecular mechanism remained unclear.

Our present study investigated the anti-tumour effect of cay10603 on BL cells and the underlying mechanism by using CA46 (no EBV infection) and Raji cells (EBV infection). We demonstrated cay10603 inhibited the proliferation of both BL cell lines and induced apoptosis by activating caspase-dependent apoptotic pathways. Cay10603 inhibited the expression of CDK1/CDK2 and cyclins to impede cell cycle progression. Furthermore, we also observed cay10603 enhanced the chemo-sensitivity of vp16. Our data reported for the first time the anti-tumour effect of HDAC6-selective inhibitor cay10603 on BL cells, and it provided the encouraging evidence for cay10603 as a potential option for BL therapy.

1 MATERIALS AND METHODS

1.1 Cell Lines and Cell Culture

BL cell lines, CA46 and Raji cells were purchased from the Cell Culture Center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). CA46 cells were cultured in RPMI-1640 (Gibco, China) containing 20% fetal bovine serum (Corning, USA) and Raji cells were cultured in RPMI-1640 (Gibco, China) containing 10% fetal bovine serum (Corning, USA). Other culture conditions were the same for both cell lines, containing 1% penicillin and streptomycin (Hyclone, USA) at 37°C in 5% CO_2 incubator.

1.2 Reagents and Antibodies

Cay10603 was provided by Selleck (USA). Reagents were dissolved in DMSO (Sigma, USA), and stored at –20°C until use. The vp16 was provided by Hengrui Medicine Co., Ltd (China). Cell Titer 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) was purchased from Promega (USA). Apoptosis was quantified using the Annexin V-FITC and Propidium Iodide (PI) binding assay, purchased from Dojindo (Japan). Cell Cycle Detection Kit was purchased from Key GEN BioTECH (China). Antibodies against human Cyclin D1, Cyclin E, Cyclin B, CDK1, CDK2 were provided by Wenleibio Co., Ltd (China), and antibodies against human β-actin, cleaved caspase 3, PARP, caspase 8 were obtained from Cell Signaling Technology (USA). Antibodies against human α-tubulin, acetyl-α-tubulin, HDAC6 were obtained from ImmunoWay Biotechnology Company (USA).

1.3 MTS Assay

The MTS assay was performed according to the manufacturer's specification, with slight modifications on protocol described. BL cells were seeded in 96-well plates in normal medium (2.5×10^4 cells/well in 100 μL/well), then cay10603-containing medium was added to the wells (50 μL/well up to a total volume 150 μL/well) at concentrations ranging from 0.01 to 5 μmol/L for 24, 48 and 72 h. Subsequently, 20 μL of MTS reagents were added to each well, after 4 h of incubation, absorbance at 490 nm was measured. The percentage of cell proliferation was calculated by dividing the absorbance of treated BL cells by absorbance of control cells within each group. All experiments were conducted at 3 to 5 times.

1.4 Cell Cycle Assay

Cell cycle was quantified according to the manufacturer's specification with slight modifications, using the Cell Cycle Detection Kit. Cells were seeded in 24-well plates (1×10^6 cells/well in 3 mL/well). After incubated with 2 μmol/L cay10603 for 12, 24 and 48 h, cells were harvested, washed with PBS and added to 500 μL of 70% ethanol at –20°C overnight. Then, the cells were washed with PBS three times, and stained with 50 μL RNase A for 30 min at 37°C, subsequently stained with 450 μL PI for 30 min at 4°C. The stained cells were analyzed for DNA content by flow cytometry (FACS Calibur) and the percentage of cells in different stages of the cell cycle was quantified.

1.5 Apoptosis Assay

Apoptosis was quantified using the Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) binding assay, following the manufacturer's instructions (Dojindo, Japan), and analysed by flow cytometry (FACS Calibur). CA46 and Raji cells were exposed to 2 μmol/L cay10603 at a density of 3.3×10^5 cells/mL (1×10^6 cells/well in 3 mL/well) for 48 h. Cells were harvested, washed with PBS, and added with 300 μL 1×binding buffers, then incubated with Annexin V-FITC and PI in the dark at room temperature for 15 min. Annexin V+ cells were considered as apoptotic cells, in which Annexin V+/PI- and Annexin V+/PI+ indicated early and late apoptosis respectively.

1.6 Western Blotting Analysis

Proteins were detected by Western blotting with indicated antibodies. Briefly, BL cells were exposed to cay10603 (2 μmol/L) for 12, 24 and 48 h, respectively. Cells were harvested, washed and lysed with the RIPA lysis buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail (Sigma, USA), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Total cell lysates were denatured with 5× sample buffer. Protein lysates (30 μg) were separated by SDS-PAGE gel and transferred to PVDF membranes (Millipore, USA). Membranes were incubated with primary antibodies diluted according to manufacture's recommendations and horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (1:2000 dilutions). Signals were observed using enhanced chemiluminescence substrate reagents (Amersham Life Science, USA) and visualized by chemiluminescence imaging system. The density for each band was analyzed via Gel-Pro Analyser (version 4.0) software (Media Cybernetics, USA).

1.7 Statistical Analysis

Statistical analyses were performed using SPSS software (version 22.0). Data are presented as mean±standard deviation (SD). All the experiments were performed in triplicates and repeated at least three times. The unpaired Student's *t*-test was used for the comparison of two groups, and $P<0.05$ was considered to be significant.

2 RESULTS

2.1 Cay10603 Significantly Inhibited Proliferation of Both CA46 and Raji Cells

HDAC6 was endogenously expressed in two BL cell lines as shown in fig. 1A. Cay10603 acted as a potent deacetylase inhibitor demonstrated by high acetylation of target protein α-tubulin (fig. 1B). The anti-proliferative effect of cay10603 on BL cells was evaluated with escalating concentrations 0.01–5 μmol/L for 24–72 h. Cay10603 inhibited the growth of CA46 and Raji cells in a dose- and time-dependent manner (fig. 1C and 1D). Much stronger cell growth inhibition was observed after 48 and 72 h treatment in both cell lines. CA46 cells seemed more sensitive than Raji cells, as shown in fig. 1C and 1D at 48 h. IC50 for CA46 was 0.13 μmol/L, while 1.47 μmol/L for Raji cells.

2.2 Cay10603 Induced Cell Cycle Arrest in BL Cell Lines

Cay10603 (2 μmol/L) treatment impeded cell cycle progression of both BL cells but in different patterns. After cay10603 treatment, CA46 cells were arrested at G_2/M and cell cycle was blocked at G_0/G_1 phase rapidly, especially at 24 h after cay10603 treatment (fig. 2A). As shown in fig. 2B, cell population in G_2/M phase was increased by 12.2% [(41.1±3.2)% *vs.* (28.9±3.0)%, $P<0.01$] 12 h after cay10603 treatment, and increased by 42.6% [(69.7±5.0)% *vs.* (27.1±6.3)%], $P<0.01$) at 24 h. As a consequence, cell accumulation in sub-G_0/G_1 phase appeared in cay10603-treated CA46 cells, and the cells accumulated dramatically at 48 h. We observed the cell population in sub-G_0/G_1 phase reached 38.4 folds of DMSO-treated cells [(30.3±11.1)% *vs.* (0.8±0.2)%, $P<0.01$], indicating an increase of apoptosis.

While in Raji cells, the same dose of cay10603 exerted effects on cell cycle weakly and mildly. After cay10603 treatment for 48 h, cell cycle arrested in G_0/G_1 and a block in G_2/M phase were observed (fig. 2A). Cell proportion of Raji cells in G_0/G_1 phase was increased by 17.5% [(78.0±1.5)% *vs.* (60.5±4.92)%, $P<0.01$] while that in G_2/M phase was decreased by 10.7% [(11.0±1.2)% *vs.* (21.7±4.0)%, $P<0.05$] at 48 h after cay10603 exposure. At 48 h, percentage of cay10603-treated Raji cells in sub-G_0/G_1 phase was increased from (2.1±0.8)% to (6.6±1.2)% ($P<0.01$).

2.3 Cay10603 Decreased Protein Expression of Regulatory CDKs and Cyclins, Impairing Cell Cycle Progression in BL Cells

Western blotting results revealed that cay10603

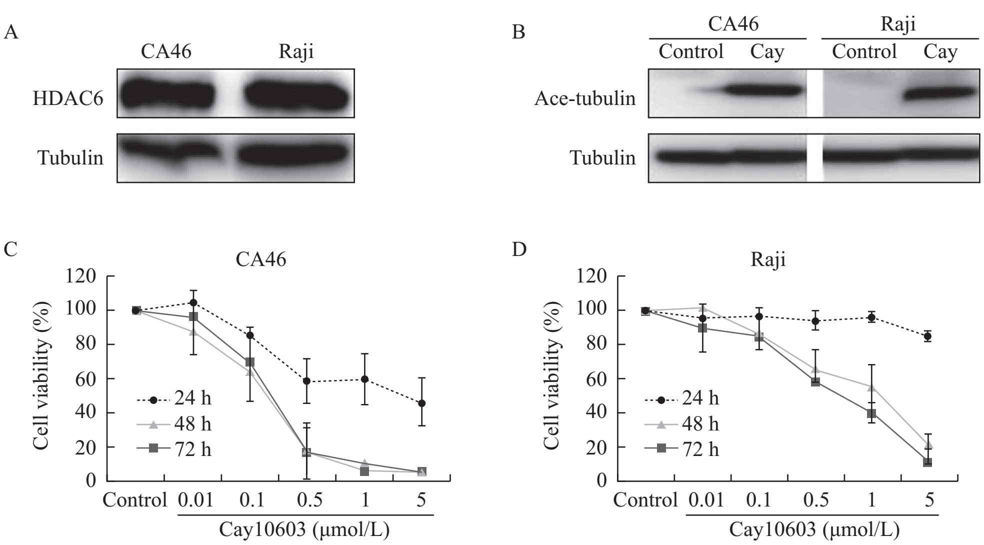

Fig. 1 HDAC6-selective inhibitor cay10603 inhibited growth of CA46 and Raji cells in a time- and dose-dependent manner
A: HDAC6 expressed in CA46 and Raji cells endogenously. B: The activity of HDAC6 was detected by Western blotting. C: Cay10603 treatment inhibited proliferation of CA46 cells. Different concentrations of cay10603 (0.01–5 μmol/L) induced growth inhibition in CA46 cells at 24, 48, and 72 h. D: Cay10603 treatment inhibited proliferation of Raji cells. Different concentrations of cay10603 (0.01–5 μmol/L) induced growth inhibition of Raji cell lines at 24, 48, and 72 h. Data showed that the mean±SD were representative of at least three independent experiments. Cay indicated cay10603.

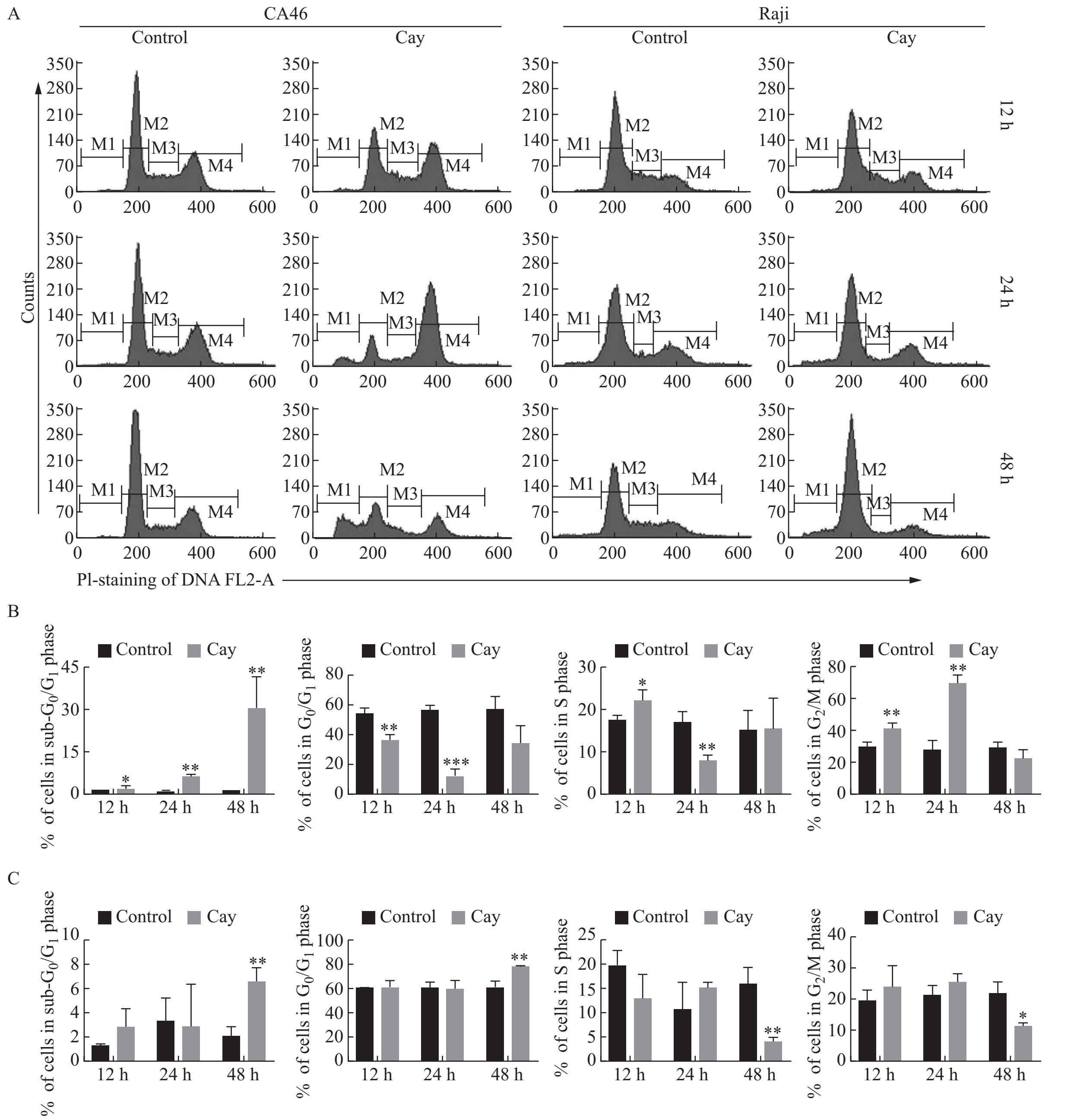

Fig. 2 Cay10603 induced cell cycle arrest in BL cells

A: Cell cycle profile of CA46 and Raji cell exposed to cay10603 (2 μmol/L) for 12, 24 and 48 h detected by flow cytometry. The bars of M1, M2, M3 and M4 indicate the sub-G_0/G_1, G_0/G_1, S and G_2/M phase, respectively. B: Cay10603 blocked cells cycle progression of CA46 cells in G_2/M phase. Quantification of the cell cycle distribution (%) of CA46 cells in different phases after cay10603 treatment for 12, 24 and 48 h. C: Cay10603 blocked cells cycle progression of Raji cells in G_0/G_1 phase. Quantification of the cell cycle distribution (%) of Raji cells in different phases after cay10603 treatment for 12, 24 and 48 h. Values represent the mean±SD from three independent experiments. $^{*}P<0.05$; $^{**}P<0.01$, $^{***}P<0.001$ *vs.* control group

altered the expression of various CDKs and cyclins in both cell lines. In CA46 cells, cay10603 resulted in down-regulation of CDK1 (decreased by about 34.0%) and cyclin B (decreased by about 33.0%) as early as 12 h and maintained in a low expression level with time (fig. 3A), which was responsible for the cell cycle arrest in G_2/M. Except the critical alterations in CA46 cells, we also observed reduced CDK2, cyclin E and cyclin D1 within various exposure time. As for Raji cells, we observed the down-regulation of both CDK2 (reduced by 27.0%) and CDK1 (reduced by 43.0%) 12 h after cay10603 treatment, and decreased gradually to the minimum level at 48 h (reduced by 60.0% and 81.0%, respectively). At the same time, expression of cyclin D1 (reduced by 43.0%) and cyclin E (13.0% reduced) also decreased, but expression of cyclin B was not significantly changed (fig. 3A), responsible for the cell cycle arrest in G_0/G_1.

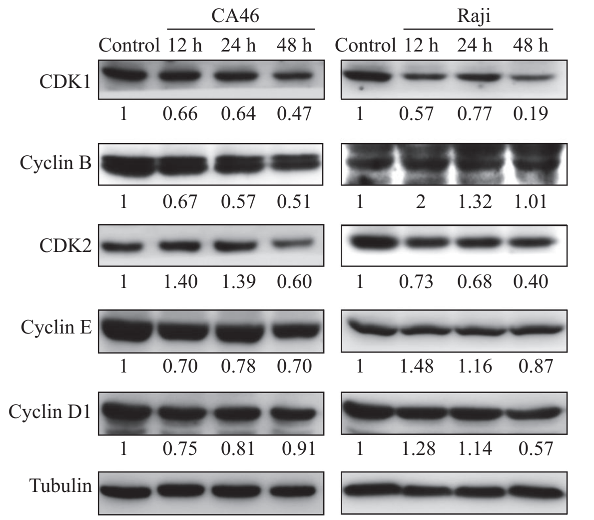

Fig. 3 Cay10603 reduced the expression of cell cycle regulatory CDKs, cyclins in CA46 and Raji cells

Expression of CDK1, CDK2, cyclin B, cyclin D1, cyclin E was detected with corresponding antibodies by Western blotting. Representative data are shown, and tubulin (α-tubulin) served as a loading control.

2.4 Cay10603 Induced Caspase-dependent Apoptosis in BL Cells

From the cell cycle analysis above, we noticed cay10603 exposure (48 h) induced cell apoptosis. In order to confirm the effect of cay10603 on inducing apoptosis, we treated two BL cell lines with cay10603 and DMSO as control. The cell apoptosis was analyzed by Annexin V/PI staining. Cay10603 increased apoptosis of both cell lines at 48 h (fig. 4A). As shown in fig. 4B, the apoptosis rate of CA46 cells increased by 23.9 folds [(66.9±7.6)% *vs.* (2.8±1.0)%, $P<0.001$] after cay10603 treatment. They appeared to be more sensitive to cay10603 than Raji cells, which only increased by 2.1 folds [(22.2±2.7)% *vs.* (10.8±2.2)%, $P<0.01$]. In both cell lines, caspase-8, caspase-3 and PARP were cleaved after cay10603 treatment, indicating the caspase cascade activation (fig. 4C). After the caspase cascade was blocked by Q-VD-Oph, a pan-caspase inhibitor, the activation of caspase-3, caspase-8 and PARP was also blocked although both cell lines were exposed to cay10603 (fig. 4C).

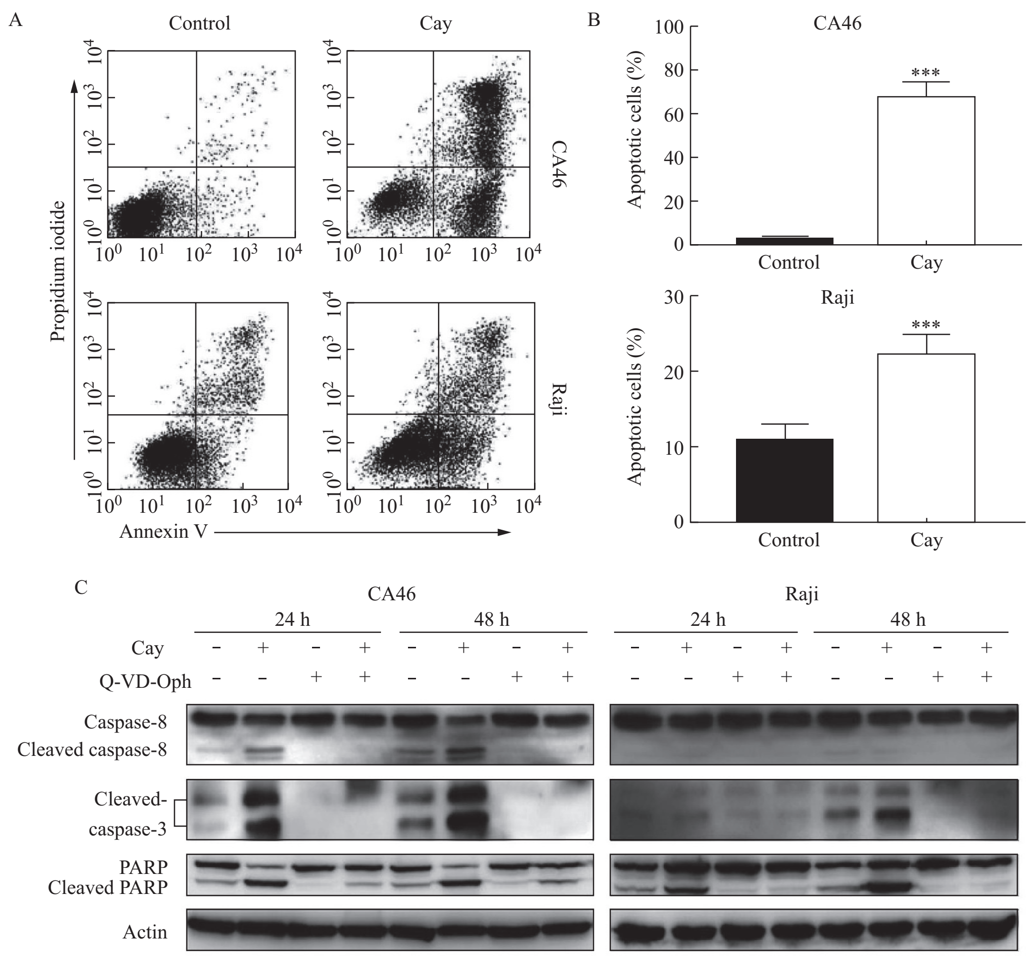

Fig. 4 Cay10603 activated caspase-dependent apoptosis in CA46 and Raji cells

A: Cay10603 induced apoptosis of CA46 and Raji cells detected by flow cytometry; B: Quantification of the apoptosis rate of both BL cells. $^{***}P<0.001$ *vs.* control group; C: The cleaved caspase-8, cleaved caspase-3, and cleaved PARP were activated after exposure to cay10603 (2 μmol/L) for 24 and 48 h. The expression levels of caspase-8, cleaved caspase-3, and PARP were detected with corresponding antibodies by Western blotting. Q-VD-Oph (10 μmol/L) acted as an inhibitor for pan-caspase. Cay indicated cay10603.

2.5 Cay10603 Enhanced the Chemo-sensitivity of Vp16

In both cells, combination of cay10603 with vp16 increased the apoptosis induced by vp16 alone (fig. 5A). As shown in fig. 5B, apoptosis caused by vp16 increased to 13.2 folds (71.1±2.8% *vs.* 5.4±1.0%, $P<0.001$) in CA46 cells, while 2.1 folds (54.3±9.0% *vs.* 26.0±0.42%, $P<0.05$) in Raji cells after combined treatment with cay10603. Furthermore, the apoptosis rate was also enhanced as compared with cay10603 alone [CA46, (71.1±2.8)% *vs.* (53.5±7.0)%; Raji, (54.25±9.0)% *vs.* (24.0±0.5)%]. We then investigated whether the caspase cascade was activated after treatment of cay10603 combined with vp16. Our results showed cay10603 promoted the caspase cascade activation reliably as compared with vp16 alone, especially at 48 h (fig. 5C). The pan-caspase inhibitor blocked the caspase-3/8 and PARP activation, which proved the apoptosis was induced in caspase-dependent manner (fig. 5D).

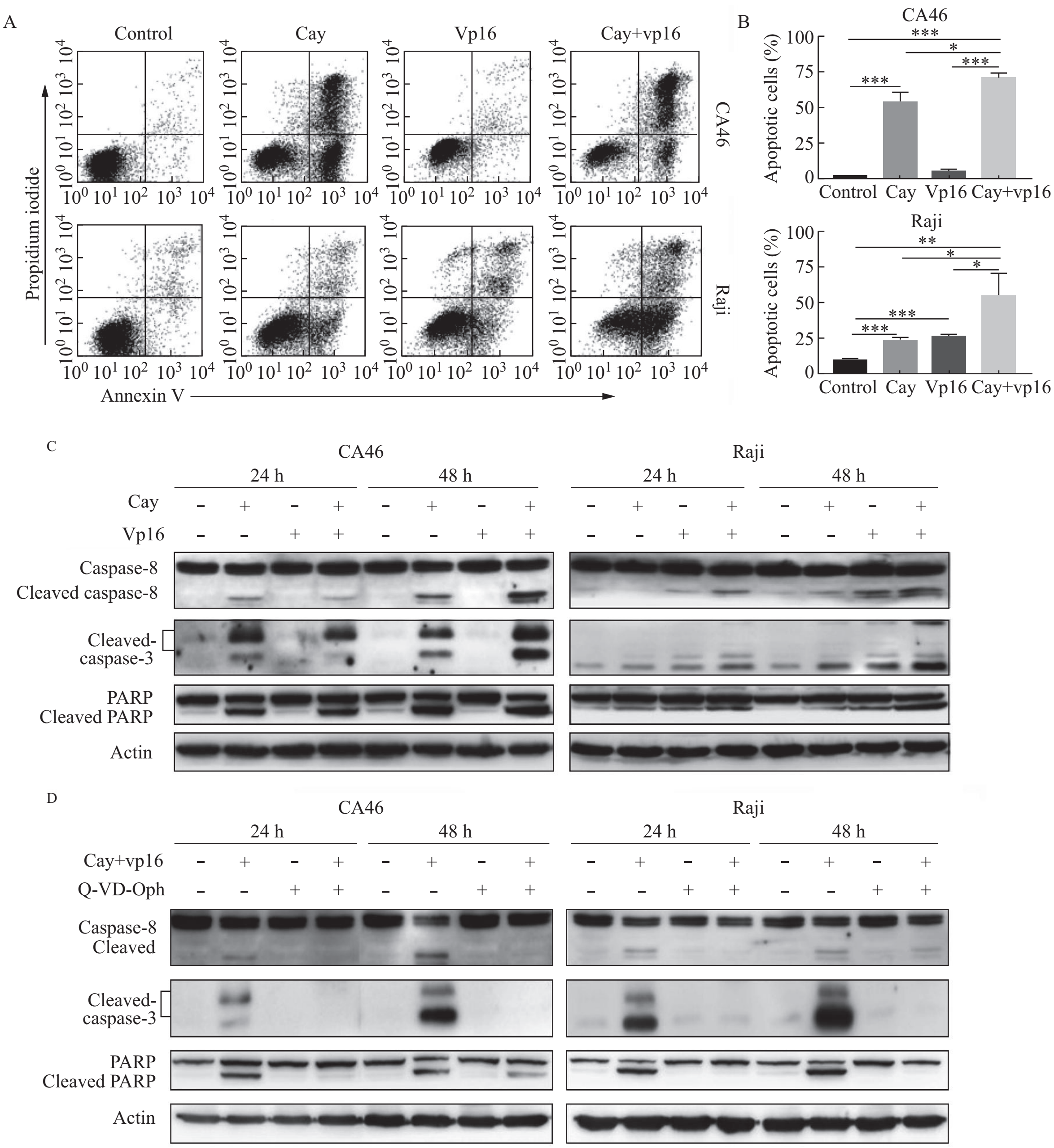

Fig. 5 Cay10603 increased the chemo-sensitivity of vp16 treatment in CA46 and Raji cells

A: Combination of cay10603 with vp16 increased the cell apoptosis compared with cay10603 or vp16 alone. B: Quantification of the apoptosis rate of both cell lines. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. C: The cay10603 (2 μmol/L) combined with vp16 (500 nmol/L) treatment for 24 and 48 h induced apoptosis by activating the caspase-dependent pathway. A panel of proteins with caspase-8, cleaved caspase-3 and PARP were analysed by Western blotting. D: The Q-VD-Oph blocked the activation of caspase cascade proteins. The expression of caspase-8, cleaved caspase-3 and PARP was detected after treatment with Q-VD-Oph (10 μmol/L) for 24 and 48 h, respectively. Representative data were shown. Cay indicated cay10603.

3 DISCUSSION

Considering the limitations of traditional chemotherapy drugs, the research of targeted drugs for treating BL is imperative. Many new targeted drugs under development are promising in the treatment of hematological tumors, such as the proteasome inhibitor Carfilzomib[19]. In addition, chemotherapy resistance is reversed when targeted drugs are combined with chemotherapy drugs. Carfilzomib combined with panobinostat (pan-HDACi) has entered Phase I clinical trials in relapsed and refractory multiple myeloma[20]. However, the pan-HDACi off-target effect produces severe cytotoxicity, which limits its application in tumor therapy. HDAC6 selective inhibitors become research hotspots due to their high efficiency and low toxicity.

HDACs played a critical role in carcinogenesis and tumour progression. HDAC6 influences the acetylation modification of many critical regulatory targets involved in cell fate[21–23]. Nowadays, HDAC6 selective inhibitors attract much attention in personalized cancer therapy because of the advantage of high selectivity to tumour cells rather than normal cells[24–26]. In this study, we elucidated for the first time the biological effects of HDAC6-selectivity inhibitor cay10603 on BL cell lines. For EBV infection is critical for BL, we chose EBV infection cells, Raji cells and EBV infection free cell line, CA46 to conduct our study. Raji cell is resistant to apoptosis[27, 28], CA46 cell is reported to be resistant to multiple death stimuli, for lacking of pro-apoptotic proteins like Bax, Bak and Bcl-Xs although free of EBV[29]. Our data indicated that cay10603 inhibited the growth of both BL cell lines. CA46 cell was more sensitive than Raji cell, for 48-h IC50 for CA46 cells was nearly 10% of that of Raji cells. Cay10603 induced apoptosis of both BL cell lines, especially for CA46 cell. We speculated the different sensitivity might be related to the EBV infection state of cells. The cell type of origin differed between EBV-positive and negative BL, where EBV-positive BL arises from memory cells, but EBV-negative disease might originate from an earlier germinal centre counterpart[30]. We considered the different response to cay10603 might result from the infection of EBV or not, but the specific mechanism needs further study.

We verified cay10603 induced apoptosis by activating caspase-cascade (figs. 4 and 5). Furthermore, we also revealed that cay10603 markedly increased apoptosis induced by vp16, a common agent in BL chemotherapeutic regimen, indicating that cay10603 is superior for BL chemotherapy, for improving anti-tumour effect as well as lessening the toxicity of chemotherapeutic agent. BL is a highly invasive non-Hodgkin lymphoma with malfunctional regulation on cell cycle control, which leads to rapid cell growth[31]. Thus focusing on the adjustment of cell cycle progression may be a breakthrough point in BL therapy. In present study, we proved that cay10603 induced cell cycle arrest in BL cells, although two cell lines differed in the phase of cell cycles. Cyclins activation and CDK-cyclin complexe formation run the cell cycle successfully and initiate mitosis[32–34]. In our data, cay10603 down-regulated the expression of CDK1 and cyclin B in CA46 cells, which induced the cell cycle block at G_2/M and hindered the cell cycle progression. While the reduced expression of CDK2 and cyclin E in Raji cells also interpreted the cell cycle arrest in G_0/G_1 phase. For there are diverse compensatory regulation among CDKs-cyclins[35], we also observed several other alterations on CDKs-cyclins in both cell lines (fig. 3). Our result is consistent with the study of Pai *et al*, which reported HDAC6 inhibitor blocked the cell cycle in G_0/G_1 phase in lung cancer cells by promoting the degradation of CDK2[36]. The altered expression of CDKs-cyclins induced the cell cycle arrest, which might be the major mechanism underlying the anti-proliferation effect of cay10603 on BL. The detail mechanisms by which cay10603 acts on CDKs-cyclins in hematological malignancies requires further study.

Among the diverse key proteins HADC6 target, p53 is a critical molecule regulating cell cycle and apoptosis[37]. At least 30% of BL patients have mutant p53 detected by biopsy, as have also been found in CA46 and Raji cells[38]. It's well known p53 acetylation was associated with cell cycle progression and apoptosis[39–41]. We observed p53 of both BL cell lines acetylated at lysine 381 (data not shown). Furthermore, in CA46 cells, cay10603 also inhibited the total expression as well as phosphorylated p53 at Ser15 (data not shown). Above observations suggested p53 might exert the anti-tumour effect on cay10603 by regulating the cell cycle and the detailed mechanism demands further study.

In present study, we verified for the first time the anti-tumour effect of cay10603 on BL cells by impeding the cell cycle. Our data provided encouraging evidence for HDAC6-selective inhibitor cay10603 as a potential option for BL therapy.

Conflict of Interest Statement

We declare no financial and personal relationships with other people or organizations that can influence our work inappropriately. There is no professional or other personal interest in any product, service and/or company that could be construed as influencing the position presented in the manuscript entitled. All other authors have no conflict of interest.

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