Chidamide induces EBV lytic infection and synergistically with tenofovir eliminate EBV-positive Burkitt lymphoma and inhibit EBV DNA replication

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Research Article

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Abstract

Purpose

The prognosis of patients with EBV- associated refractory/ relapsed Burkitt lymphoma (BL) is poor and there is no effective way to completely eliminate latent EBV infection. EBV was shown to transform from latency to lytic phase by histone deacetylase (HDAC) inhibitors, and EBV lytic activation could be inhibited by tenofovir, a potent inhibitor of EBV lytic DNA replication. Herein, we explored the anti-tumor effect and EBV clearance ability of novel HDAC inhibitor chidamide combined with tenofovir in EBV positive BL.

Methods

Raji, Namalwa and CA46 cells were used to evaluate the effects of chidamide combined with tenofovir on their cell viability, cell cycle and apoptosis induction. The EBV DNA copies and mRNA level of EBV related genes were detected by qPCR. Western blotting was performed to detect the cell cycle and apoptosis related pathways in BL cells. BL cell tumor-bearing NSG mice model was used to detect the anti-tumor effects of chidamide combined with tenofovir in vivo.

Results

In the study, chidamide exhibited HDAC inhibitory activity and downregulated the mRNA levels of HDAC1, 2, 3. Further, chidamide inhibited BL cell proliferation, arrested cell cycle progression, and induced BL cell apoptosis mainly through regulating MAPK pathway. Additionally, chidamide upregulated EBV DNA load and promoted the transcription of lytic genes including BZLF1, BMRF1, BMRF2 and BMLF1. Compared with chidamide alone, the addition of tenofovir further induced growth arrest and apoptosis, inhibited EBV lytic genes transcription and lytic DNA replication induced by chidamide in EBV positive BL cells. Furthermore, our in vivo data showed enhancive tumor-suppression effects of chidamide combined with tenofovir in BL cell tumor-bearing mice model.

Conclusions

The above data confirmed a synergistic effect of chidamide combined with tenofovir in inducing growth inhibition and apoptosis in EBV-positive BL cells.

1. Introduction

As a highly aggressive non-Hodgkin lymphoma, Burkitt lymphoma (BL) is closely related to Epstein-Barr Virus (EBV) infection [1]. Despite multi-drugs intensive chemotherapy has improved the prognosis of patients with BL to some extent, patients with refractory/relapsed BL still have poor prognosis, with long-
term survival rate of only 10%-20% [2, 3]. Moreover, lymphoma patients with high EBV DNA load were shown to have poor prognosis [4–5]. Unfortunately, the current chemotherapy regimens could not eliminate EBV. Further, there is no effective anti-EBV treatment at present, which limits the treatment of EBV associated lymphomas including BL.

EBV, a gamma herpes virus, was shown to promote the clonal formation, occurrence and development of EBV associated lymphoma through multiple latent genes including $EBNA1$, $LMP-1$, etc [6–9], which are the key factors mediate self-replication of EBV. In addition, EBV encoded lytic genes such as $BZLF1$, $BMRF1$, $BMLF1$, $BMRF2$, etc, play a central role in the development of EBV-driven BL [10]. EBV lytic infection could be divided into three states: immediate-early lytic state, early lytic state and late lytic state [11]. EBV in each lytic stage could produce multiple gene coding products such as immediate-early protein $BZLF1$ and $BRLF1$. These gene products activate each other and trigger the expression of other early lytic proteins ($BMRF1$, $BALF1$, $BHRF1$, etc.) [12]. The resulting viral DNA replicates are then packaged into viral progeny and released to infect the neighboring cells, thus facilitating EBV lytic infection [13]. Although latency programs predominate in EBV-driven tumors, recent evidence suggests that lytic EBV replication may also be of pathogenic importance, at least in the early phases of cell transformation.

EBV usually persists in latent form [14]. Antiviral drugs against EBV DNA polymerase such as Acyclovir (ACV) and Ganciclovir (GCV) could not eliminate the latent EBV at present [15]. Fortunately, the anti-virus treatment is expected to be effective in EBV lytic state. Viral lytic reactivation from latency could be induced by various drugs or external stimuli [16], including histone deacetylase (HDAC) inhibitors [17]. Intentional switch the latent form of EBV infection to the lytic cycle represents a potentially effective therapeutic intervention for patients [18, 19]. Chidamide, a novel benzamide-type HDAC inhibitor, has been approved for the treatment of recurrent/refractory peripheral T-cell lymphoma (PTCL) in China [20]. The evidence of inducing the expression of $BZLF1$, $BRLF1$ and $BMRF1$ suggested that chidamide could promote the lytic cycle of EBV in NK/T cell lymphoma [21]. In addition, chidamide was found to have a significant anti-tumor effects in colon cancer and gastric cancer [22, 23], but its role in BL needed to be further explored.

Most of current antiviral drugs such as ACV and GCV are based on a broad spectrum antiviral characteristics and are problematic in treating EBV infection due to their low potency or high toxicit [15, 24]. Tenofovir is an acyclic nucleoside/ nucleotide analog and mainly used in the treatment of HIV and hepatitis B at present [25]. Intriguingly, tenofovir bisphosphate, an active component of tenofovir, was found to inhibit viral DNA polymerase by directly and competitively binding to natural deoxyribose substrates [26, 27]. It has been reported that tenofovir dipivoxil fumarate inhibited EBV lytic DNA replication efficiently in vitro and had better efficacy and safety against EBV than other agents [24]. The above data raising the question whether tenofovir could be used in treating EBV associated lymphoma such as BL?

In this study, we observed the effect of HDAC inhibitor chidamide on EBV lytic infection, further investigated the anti-tumor effect of chidamide combined with tenofovir on EBV positive BL and explored
their effects on the EBV DNA and related lytic genes.

2. Materials And Methods

2.1 Cell lines and culture media

The Raji, Namalwa and CA46 cell lines were provided by The American Type Culture Collection (Manassas, VA, USA). All cell lines were grown in Iscove's Modified Dulbecco's Medium containing 10% heat-inactivated fetal bovine serum (HyClone, Logan, Utah, USA).

2.2 Antibody and reagents

Chidamide powder was obtained from MedChemExpress (Monmouth Junction, NJ, USA) and dissolved in DMSO (Sigma Aldrich; Merck KGaA) before being stored at −80°C in the dark. Tenofovir was provided by Zhengda Tianqing Co. Ltd. (Nanjing, Jiangsu, China). The antibodies against death receptor 5 (DR5, 8074), Caspase8(9746), Caspase9(9502), Caspase3(9664), PARP(9532), Bax(2772), Bcl-2(15071), Mcl-1(39242), XIAP(2042), c-Myc(18583), p-JNK(9255), JNK(9252), p-ERK(4370), ERK(4695), p-P38(4511), P38(8690) and GAPDH(5174) were provided by Cell Signaling Technology (Danvers, MA, USA). The antibodies against cyclin D1(26939-1-AP), cyclin E1(11554-1-AP), p21(10355-1-AP) and p27(25614-1-AP) were provided by Proteintech (Chicago, USA).

2.3 Cell viability assay

Raji, Namalwa, and CA46 cells (2×10^4 cells per well) were inoculated into a 96-well plate and treated with incremental concentrations of chidamide (2.4, 8, 16, 32 µM) alone or in combination with tenofovir (5, 10, 20, 40, 80, 160 µM). Following the reaction time of 24 and 48 hours, 10 µL CCK-8 (Dojindo Laboratories, Kumamoto, Japan) was added to each hole and incubated at 37°C for 2 hours as directed by the manufacturer. The final absorbance [OD value] of cultured cells was determined using the SynergyHTX Biotek Instruments at 450 nm (Inc.). The drug interaction composite index (CI) was calculated using Compusyn; CI < 1 indicates a synergistic effect.

2.4 RNA extraction and real-time quantitative polymerase chain reaction

Chidamide (0, 4 and 8 µM) was applied to Raji, Namalwa and CA46 cells for 48 hours, and total RNA was extracted using Trizol (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. NanoDrop was used to measure RNA concentrations, which were then reverse-transcribed into cDNA using M-MLV reverse transcriptase (Life Technologies, Gaithersburg, USA). The LightCycler480 was used to perform quantitative polymerase chain reaction (qPCR) with SYBR® Green Supermix (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Table 1 shows all the primers for polymerase chain reaction (PCR) assay.
Table 1
Real-time polymerase chain reaction primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>HDAC1</td>
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</tr>
<tr>
<td></td>
<td>5’-TCATTCTGTTCTGGTTAGTC-3’(reverse)</td>
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<tr>
<td>HDAC2</td>
<td>5’-AGTTGCCCTTGATTGAGA-3’(forward)</td>
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<td></td>
<td>5’-CCACTGGTTGCTCTGGATTAT-3’(reverse)</td>
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<tr>
<td>HDAC3</td>
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<td>5’-GACTCTTGGTGAAAGCCTTG-3’(reverse)</td>
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<td>EBNA1</td>
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<td></td>
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<tr>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>5’-AATGCCGGCTGCAAGTCAA-3’(reverse)</td>
</tr>
<tr>
<td>BMRF1</td>
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<tr>
<td></td>
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<tr>
<td>ACTB</td>
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</tr>
<tr>
<td></td>
<td>5’-GCTGTCACCTTTACGTTCC-3’(reverse)</td>
</tr>
</tbody>
</table>

2.5 Western blot analysis

Raji, Namalwa and CA46 cells were treated with 0.4 or 8 µM of chidamide or in combination with tenofovir (40 µM). After 48 hours, cells were collected and lysed with cell lysis buffer containing protease inhibitors (Roche, Indianapolis, IN, USA). The bicinchoninic acid protein analysis reagent was used for quantitative analysis. Proteins were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to the polyvinylidene fluoride membrane, further sealed
with 5% skimmed milk, and incubated overnight with indicated antibodies at 4°C. The conjugated antibodies were detected by enhanced chemiluminescence (ECL) kit (BD Biosciences, San Jose, CA, USA) after the membrane cleaning with phosphoric acid buffered saline and Tween 20 (PBST) and incubation with the Horseradish peroxidase antibodies. The image was taken using GE Image Quant LAS4000 Mini.

2.6 Apoptosis analysis

BL cell lines were seeded with \(1 \times 10^6\) cells/per well in a 12-well plate and treated with chidamide (0, 4 and 8\(\mu\)M) alone or combined with tenofovir (40 \(\mu\)M) for 48 hours. Cells were collected and incubated with 5 \(\mu\)L Annexin V-FITC and 10 \(\mu\)L PI at room temperature in darkness for 10 min. Further, the detection was carried out by using flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed by FlowJo to determine Annexin V+/PI-(early apoptosis) and Annexin V+/PI+ (late apoptosis) cells percentages.

2.7 Cell cycle analysis

In 6-well plates (2\(\times\)10^6 cells per well), BL cell lines were inoculated and treated with chidamide (0, 4 and 8\(\mu\)M) for 48 hours. Cells were then collected and immobilized at \(-20°C\) in 70% precooled ethanol. Cells were washed with PBS the next day and resuspended in 0.25 mL staining solution (50 \(\mu\)g/mL RNase A and 50 \(\mu\)g/mL propidium iodide) protected from light for 30 min. Furthermore, the detection was carried out with the FACS Calibur system and the data were analyzed with the ModFit LT 3.3 software (Verity Software House Inc., Topsham, ME, USA).

2.8 Tumorigenicity assay in NSG mice

NOD-SCID L2Rynull (NSG) mice (female, 4-6w, \(~\)20 g) were purchased from Beijing Vitalstar Biotechnology Co. Ltd. (Beijing, China) and bred in a pathogen-free (SPF) facility. This study was approved by Xuzhou Medical University Institutional Ethics Committee. NSG mice of 6–7 weeks having similar weight were injected subcutaneously with \(1 \times 10^6\) Raji-GFP-Luc cells per mouse in the right armpit. The mice were randomly divided into 4 groups \((n = 3\) in each group): mice that received vehicle phosphate buffered saline intraperitoneally were defined as the control group. Mice that received chidamide, tenofovir, chidamide combined with tenofovir were defined as the chidamide group, tenofovir group and chidamide + tenofovir group, respectively. The bodyweight of mice and the long (a) and short diameter (b) of the tumor were measured every other day. From the 8th day after tumor cells injection, chidamide was given to the mice intraperitoneally at a dose of 30 mg/kg per day for 2 weeks, and tenofovir was given to the mice orally at a dose of 75 mg/kg per day for 2 weeks. On the 21st day, D-fluorescein was injected into the mice intraperitoneally, and IVIS imaging was performed to measure the tumor load. Then the mice were sacrificed and xenograft tumors were dissected to measure the weight, length and diameter, and fixed with formaldehyde solution for pathological examination.

2.9 Statistical analysis

Data are represented as the mean \(\pm\) standard deviation (SD). One-way ANOVA and Two-way ANOVA was used to analyze the statistical significance of multiple treatment groups. Statistical significance was
established at *P < 0.05, **P < 0.01, and ***P < 0.001. All statistical analyses were performed using the GraphPad Prism software ver. 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1 Chidamide inhibits proliferation and arrests cell cycle progression in BL cells

As a HDAC inhibitor, chidamide downregulated the mRNA expression levels of HDAC1, 2, 3 and upregulated the acetylation of histone H3 expression level in a dose-dependent manner in Raji, Namalwa and CA46 cell lines (Fig S1a and b). We first examined the effect of chidamide on the proliferation of BL cells. As shown in Fig S1c, chidamide existed antiproliferative effect and significantly inhibited the viability of BL cells in a dose and time-dependent manner. To further explore the action mode of chidamide on BL cells, cell cycle distribution was analyzed by flow cytometry. Higher percentage of G1 phase cells and lower percentage of G2/M and S phase cells were found in chidamide treated BL cells (Fig. 1a and 1b). Moreover, chidamide showed dose-dependent upregulation expression of p21, p27 and downregulation expression of Cyclin D1, Cyclin E1 in BL cells by western blotting (Fig. 1c). These results indicate that chidamide arrest cell cycle progression via upregulating p21 and p27 expression and downregulating Cyclin D1 and Cyclin E1 expression in BL cells.

3.2 Chidamide induces apoptosis and activates the apoptotic pathway in BL cells

We next examined whether chidamide induce apoptosis in BL cells. As shown in Fig. 2a and 2b, chidamide treatment induced apoptosis of Raji, Namalwa and CA46 cells in a dose-dependent manner. Western blotting analysis showed that the cleavage of Caspase 3 and PARP expression were upregulated in chidamide treated BL cells (Fig. 2c). Furthermore, the apoptotic pathway investigation showed that DR5, cleaved form of caspase 8 were upregulated in chidamide treated BL cells (Fig. 2c), suggesting an exogenous apoptotic pathway activation by chidamide. Meanwhile, chidamide also induced endogenous apoptotic pathway activation and upregulated pro-apoptotic protein Bax expression and downregulated anti-apoptotic proteins Mcl-1 and XIAP expression in a dose-dependent manner (Fig. 2d).

3.3 Chidamide inhibits c-Myc expression and regulates MAPK pathway in BL cells

Previous studies have shown that c-Myc are activated during the development of BL and are associated with poor prognosis. In our study, chidamide significantly inhibited c-Myc expression in BL cells (Fig. 3a). Further, chidamide affected MAPK pathway and upregulated p-JNK, p-P38 and downregulated p-ERK expressions in BL cells (Fig. 3b). To further verify whether JNK pathway was regulated by chidamide, JNK inhibitor SP600125 was used. Upregulation of p-JNK induced by chidamide was blocked in the presence of SP600125 (Fig. 3c). Moreover, upregulation of the Cleaved Caspase3 and PARP induced by chidamide
were also blocked in the presence of SP600125, suggesting a MAPK pathway dependent manner in chidamide induced apoptosis in BL cells.

3.4 Chidamide promotes EBV lytic gene expression and enters the lytic replication stage in EBV-positive BL cells

In order to evaluate the effect of chidamide on the EBV gene and EBV DNA level, EBV-positive Raji and Namalaw cells were treated with different concentrations of chidamide for 48 hours. As shown in Fig. 4a, EBV DNA level upregulated significantly in Raji and Namalaw cells after 4 µM chidamide treatment. The mRNA levels of latent genes (EBNA1 and RPSM1) decreased in a dose-dependent manner after receiving chidamide. Contrarily, levels of lytic viral transcripts including BZLF1, BMRF1, BMRF2 and BMLF1 increased significantly after chidamide treatment. The mRNA expression level of thymidine kinase (TK) gene, which is closely related to EBV lytic infection, increased significantly in the presence of chidamide (Fig. 4b). The above results suggest that chidamide promotes EBV to enter the lytic stage in EBV-positive BL cells.

3.5 Tenofovir synergistically with chidamide induce growth arrest of EBV positive BL cells

To determine whether tenofovir enhance the growth inhibitory effect of chidamide in EBV-positive BL cells, the CCK-8 assay was used to measure the proliferation of BL cells. The synergy between chidamide and tenofovir was demonstrated using CompuSyn, and CI was obtained for each tested cell line. As shown in Fig. 5, tenofovir combined with chidamide had synergistic effects on EBV positive Raji and Namalwa cells, but not on EBV negative CA46 cell.

3.6 Tenofovir synergistically with chidamide induce apoptosis of EBV positive BL cells through regulating MAPK pathway

Furthermore, we examined the apoptosis of Raji, Namalwa and CA46 cells induced by chidamide combined with tenofovir. Interestingly, the addition of tenofovir significantly enhanced apoptosis induced by chidamide and yielded the synergistic proapoptotic effect in Raji and Namalwa cells (Fig. 6A and 6B). By contrast, no synergistic effect was found in EBV-negative CA46 cell. We further investigated the effect of tenofovir on changes of apoptosis pathway induced by chidamide in EBV-positive Raji and Namalwa cells. Western blotting analysis showed that levels of Caspase9, Caspase3, PARP and pro-apoptotic protein Bax were significantly upregulated in the presence of both chidamide and tenofovir. Meanwhile, the expression of anti-apoptotic protein Bcl-2 was downregulated synchronously (Fig. 6C). Furthermore, western blotting analysis with different antibodies against intracellular signal pathways showed that combination of chidamide and tenofovir significantly increased the levels of phospho (p)-JNK, p-P38 and decreased the level of p-ERK (Fig. 6D).
3.7 Tenofovir blocks EBV DNA replication and lytic genes transcription induced by chidamide in EBV-positive BL cells

Further experiments were carried out to test the effect of tenofovir in EBV DNA replication and lytic genes transcription induced by chidamide. As shown in Fig. 7A, after 48 hours of chidamide treatment, the number of EBV viral copies increased significantly. Further addition of tenofovir effectively blocked the replication of EBV lytic DNA induced by chidamide and decreased EBV DNA load. In addition, transcription of the EBV lytic genes (\(BZLF1, BMRF1, BMRF2\) and \(BMLF1\)) and \(TK\) induced by chidamide were significantly inhibited by the addition of tenofovir (Fig. 7B). Collectively, our data suggest that chidamide is sufficient to induce EBV lytic cycles and sensitize EBV-positive cells to tenofovir therapy.

3.8 Tenofovir synergistically with chidamide exert enhancive tumor-suppression effect in BL cell tumor-bearing mice model

To observe the anti-tumor effects of chidamide combined with tenofovir \textit{in vivo}, BL cell tumor-bearing NSG mice model was used in this study. Tenofovir was insufficient to induce tumor remission as a single drug, and chidamide alone showed slight anti-tumor activity. Strikingly, tumor inhibitory effect induced by chidamide was significantly enhanced by the addition of tenofovir, reflected by significantly decrease of luminescence in tumor as well as reduced tumor size, weight and volume (Fig. 8A, B and C). Further pathological examination showed tumor tissue structure with nearly full field of tumor cells and a few necrotic cells in the control group and tenofovir group; while the tumor grafts in the chidamide group showed partially damaged tumor tissue structure with some necrotic tumor cells. In particular, enhancive tumor-suppression effect was observed in chidamide + tenofovir group with severely damaged tumor tissue structure accompanied by a large number of necrotic cells (Fig. 8D). We additionally detected apoptotic pathway and MAPK pathway by western blotting in extracted tumor tissue. Consistent with \textit{in vitro} study, our \textit{in vivo} study showed that levels of Caspase9, Caspase3, PARP and Bax were upregulated and the anti-apoptotic protein Mcl-1 was significantly downregulated in chidamide + tenofovir group. Meanwhile, chidamide combined with tenofovir significantly increased the levels of p-JNK and p-P38 and decreased the level of p-ERK in BL cell tumor-bearing mice model (Fig. 8E).

Discussion

EBV infection is closely associated with BL, which leads to the difficulties in treating refractory/relapsed BL patients. In addition, high EBV DNA load is associate with poor prognosis in many EBV positive lymphoma subtypes [1, 4, 5]. However, current treatment options ignored the EBV-positive status of BL due to difficulty in eliminating the latent EBV [28]. In this study, we confirmed that novel HDAC inhibitor chidamide effectively induced EBV lytic reactivation from latency, and found a synergistic effect of chidamide combined with tenofovir in inducing growth inhibition, G1 cell cycle arrest, apoptosis and change of related signaling pathway in EBV positive BL cells. In addition, we demonstrated that tenofovir significantly inhibited chidamide induced EBV lytic DNA replication and transcription of the EBV lytic
genes. Furthermore, a synergistic anti-tumor effect of tenofovir combined with chidamide in vivo was verified in BL cell tumor-bearing mice model, providing a potent new therapeutic strategy in the treatment of EBV-positive BL.

HDAC inhibitor is a class of anti-lymphoma therapeutics approved by FDA in the treatment of T cell lymphoma [20, 29]. In recent years, HDAC inhibitors were shown to be effective in diffuse large B-cell lymphoma, follicular and Hodgkin lymphoma, etc [17 30]. Vorinostat, the first approved HDAC inhibitor, combined with rituximab or R-CHOP showed enhanced effect in DLBCL patients [31,32]. Belinostat, another HDAC inhibitor, was well tolerated but resulted in only 10.5% overall response rate in relapsed or refractory aggressive B-NHL patients [33]. Novel HDAC inhibitor chidamide, was shown to induce growth inhibition, apoptosis, DNA damage and cell cycle arrest in NK/T lymphoma cells [21, 34]. In the present study, chidamide showed effects in inhibiting HDAC1,2,3 and inducing both exogenous and endogenous apoptosis in BL cells. To gain insight into the mechanism of chidamide-induced apoptosis, we detected MAPK, JAK-STAT and PI3K/AKT/mTOR molecules by western blotting. Amazingly, only MAPK pathway participated in mediating BL cells apoptosis (Fig 3, data not shown about JAK-STAT and PI3K/AKT/mTOR).

Lytic infection is a unique state of EBV, which represents the invasion to the host. EBV lytic genes BZLF1, BMRF1, BMRF2 and BMLF1 could be detected in a variety of tumors and the positive of them reflect the cleavage and replication of EBV [35]. BZLF1 is an EBV-encoded immune early lysis gene product initiating reactivation of the EBV lytic cycle [36]. BMRF1 encodes the EA-D protein, which is essential for viral DNA synthesis [37]. BMRF2 acts as a regulator of the EBV gene promoter in lymphocytes [38]. The BMLF1 protein is a multifunctional RNA-binding protein that may increase the viral or cellular gene expression [39]. EBV DNA load is closely related to the prognosis of lymphoma, irrespective of the latent or lytic status [4]. Elimination of EBV DNA has become an essential part that we must consider in the treatment of EBV related lymphoma. Lytic state of EBV is expected to be inhibited by antiviral drug nucleoside analogue [40, 41]. HDAC inhibitors such as romidepsin and sodium valproate were shown to trigger the switch of EBV from latent to lytic cycle in the previous studies [42-45]. In our study, novel HDAC inhibitor chidamide was found to promote EBV lytic gene expression and lytic DNA replication and induce EBV into the lytic cycle. Our data suggest that chidamide is particularly effective in inducing EBV into lytic reactivation, which might render EBV-positive BL cells more susceptible to anti-virus therapy.

EBV lytic DNA replication depends on DNA polymerase, but the clinical application of current antiviral nucleoside/ nucleotide analog ACV or GCV in EBV infection is limited due to their low potency or high toxicity [15]. Tenofovir, a new nucleoside compound primarily used for the treatment of HIV and hepatitis B, has been shown to be highly effective against EBV lytic infection in vitro [24]. In the present study, we showed that tenofovir synergistically with chidamide mediated enhance co-lethal effect of BL cells. Significantly changes of MAPK signal pathway further confirmed the synergistic effect of these two drugs. Notably, tenofovir significantly inhibited EBV DNA replication induced by chidamide in EBV-positive BL cells. Intriguingly, we also found that tenofovir significantly inhibited transcription of the EBV lytic genes induced by chidamide. As a complex double-stranded DNA virus, the DNA polymerase of EBV is
mainly responsible for the replication of DNA in lytic infection, which is different from that in latent infection. Tenofovir was shown to inhibit EBV lytic DNA replication mainly through targeting virus DNA polymerase system. Tenofovir may prevent the release of EBV infectious virus particles from cells, and therefore block the transcription of lytic genes and synergistically with chidamide eliminate EBV positive tumor cells.

To further verify the synergistic effect of chidamide combined with tenofovir, we used a BL cell tumor-bearing mice model. Consistent with our results in vitro, tumor inhibitory effect induced by chidamide was significantly enhanced by the addition of tenofovir in vivo. Additional changes of apoptotic and MAPK pathway by western blotting further confirmed the in vivo synergistic anti-apoptotic effects of this two-drug combination scheme (Fig 8e). In this study, we are curious about the failure of tenofovir alone to exert anti-tumor effect. In addition, due to the limitation of observation days, we did not observe the effect of chidamide combined with tenofovir on the survival of mice. More experiments need to be done to further explore the anti-tumor effects and mechanism of this two-drugs in vivo.

Overall, we for the first time demonstrated that novel HDAC inhibitor chidamide in combination with tenofovir exerted synergistic anti-tumor effects in EBV-positive BL. Most strikingly, we showed that the addition of tenofovir significantly inhibited chidamide induced EBV lytic reactivation, which will be an effective strategy for the elimination of EBV and related tumors. Due to the complexity of molecular pathogenesis in EBV related tumors, more research works need to be done to allow this two-drug combination regimen emerge as a first-line therapy to treat relapsed/refractory EBV-positive lymphomas.

**Abbreviations**

BL, Burkitt lymphoma; CCK-8, Cell Counting Kit-8; CHI, Chidamide; CI, combination index; EBNA1, Epstein–Barr nuclear antigen 1; EBV, Epstein Barr virus; LMP1, latent membrane protein 1; NHL, non-Hodgkin's lymphoma; PBS, phosphate buffered saline; PBST, phosphate buffered saline with Tween 20; qPCR, Quantitative polymerase chain reaction; SD, standard deviation; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TFV, Tenofovir; TK, Thymidine kinase.

**Declarations**

**Statement of Ethics**

Not applicable.

**Conflict of Interest Statement**

The authors do not have any actual or potential conflicts of interest with other people or organizations.

**Acknowledgments**
We thank numerous individuals participated in this study.

**Consent for publication**

Not applicable.

**Author Contributions**

WS, LX and XW designed the research; MZ and LX performed experiments, DT, ZL, TL, YZ, SZ, YM and DY helped to perform the experiments. DM performed histological analysis, XW and WS analyzed and interrupted the results; XW and MZ wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**Figures**

**Figure 1**
Chidamide arrests cell cycle progression in human BL cells.

Raji, Namalwa and CA46 cells were incubated with 0, 4 or 8 µM of chidamide for 48 hours, then the cells were harvested. a. cell cycle analysis was conducted using flow cytometry. b. Percentages of cells in different cell cycle phases were determined from three independent experiments. c. The harvested cells were subjected to western blotting using Cyclin D1, Cyclin E1, p21 and p27 antibodies. CHI: chidamide.

Figure 2

Chidamide induces apoptosis and activates the apoptotic pathway in human BL cells.

Raji, Namalwa and CA46 cells were incubated with 0, 4 or 8 µM of chidamide for 48 hours, then the cells were harvested. a. The cells were subsequently stained with Annexin-V-FITC/PI and analyzed for apoptosis by flow cytometry. b. Percentages of apoptotic cells were determined from three independent experiments. Error bars, mean ±SD. *P < 0.05; **P < 0.01, ***P < 0.001. c and d. The harvested cells were subjected to western blotting using DR5, Caspase8, Caspase3, Caspase9, PARP, Mcl-1, XIAP and Bax antibodies. CHI: chidamide.

Figure 3

Chidamide inhibits c-Myc expression and regulates MAPK pathway in human BL cells.

a-b. Raji, Namalwa and CA46 cells were incubated with 0, 4 or 8 µM of chidamide for 48 hours. Then whole cells were harvested and subjected to western blotting analysis using c-Myc, p-JNK, JNK, p-P38, P38, p-ERK and ERK antibodies. c. Raji Namalwa and CA46 cells were pretreated with SP600125 (20 µM) for 1 hour and incubated with chidamide (8 µM) for 48 hours. Afterward, the whole cells were harvested and subjected to western blotting analysis using the p-JNK, JNK, Caspase3 and PARP antibodies. CHI: chidamide.

Figure 4

Chidamide promotes EBV lytic genes expression in EBV positive BL cells.

a-b. Raji and Namalwa cells were incubated with 0, 4 or 8 µM of chidamide for 48 hours. The EBV DNA copies and mRNA level of EBV related genes (EBNA1, RPMS1, TK, BZLF1, BMRF1, BMRF2 and BMLF1) were detected by qPCR. Error bars, mean ±SD. CHI: chidamide. *P < 0.05; **P < 0.01; ***P < 0.001.
Tenofovir synergistically with chidamide induce growth arrest of EBV positive BL cells.

Raji, Namalwa and CA46 cells were treated with the incremental concentrations of chidamide (2,4,8,16,32 μM) combined with tenofovir (5,10,20,40,80,160 μM) for 48 hours, and the proliferation of cells were determined using CCK-8 assay. Composite index (CI) of chidamide and tenofovir was calculated in the above BL cells. Boxes represent median values in the first and third quartiles. The whiskers represent maximum and minimum values. CHI: chidamide.

Tenofovir synergistically with chidamide induce apoptosis of EBV positive BL cells through regulating MAPK pathway.

a. Raji and Namalwa cells were treated with chidamide (4 μM), tenofovir (40 μM) or the combination of both for 48 hours, then the whole cells were harvested and detected with Annexin V-FITC/PI by flow cytometry for apoptosis. b. The percentage of apoptotic cells was determined from three independent experiments. Error bars, mean±SD. **p < 0.01; ***p < 0.001. c and d. Raji and Namalwa cells were harvested and subjected to western blotting analysis using PARP, Caspase3, Caspase9, Bax, Bcl-2, p-JNK, JNK, p-ERK, ERK, p-P38 and P38 antibodies. Ctrl: control; CHI: chidamide; TFV: tenofovir.
Figure 7

**Tenofovir blocks EBV DNA replication and lytic genes transcription induced by chidamide in EBV-positive BL cells.**

Raji or Namalwa cells were treated with chidamide (4 µM), tenofovir (40 µM) or the combination of both for 48 hours, then the whole cells were harvested. **a.** The EBV DNA copies were assessed by qPCR. **b.** The mRNA expression levels of **TK** and EBV lytic genes (**BZLF1, BMRF1, BMRF2** and **BMLF1**) were assessed by real-time PCR. Error bars, mean ±SD. Ctrl: control; CHI: chidamide; TFV: tenofovir. *P < 0.05; **P < 0.01; *** P < 0.001.

Figure 8

**Tenofovir synergistically with chidamide exert enhancive tumor-suppression effect in BL cell tumor-bearing mice model.**

Female NPG mice of 6-7 weeks were injected subcutaneously with **1×10^6** Raji-GFP-Luc cells per mouse in the right armpit, and the systemic tumor-bearing mice model was established on day 8. The mice were randomly divided into 4 groups (n=3 in each group): mice that received vehicle phosphate buffered saline (PBS) intraperitoneally were defined as the control group. Mice that received chidamide, tenofovir, chidamide combined with tenofovir were defined as the chidamide group, tenofovir group and chidamide + tenofovir group, respectively. From the 8th day, chidamide was given to the mice intraperitoneally (30 mg/kg per day) and tenofovir was given to the mice orally (75 mg/kg per day) for 2 weeks. **a.** D- fluorescein was injected into the mice intraperitoneally on the 21st day, and IVIS imaging was performed to measure the tumor load. Total counts of luminescence from Raji cells in each group were recorded. **b.** Then the mice were sacrificed, xenograft tumors were dissected and photographed. The tumor weights were measured in each group. **c.** From the 8th day, the long diameter (a) and short diameter (b) of xenografts were measured every other day till the 20th day. Tumor volume was calculated according to the following formula: tumor volume(V)=1/2ab^2. **d.** H&E staining of the removed tumor grafts in mice (magnification ×200). The part circled by the dotted line represents necrotic cells. **e.** Part of xenograft tumor tissues were sent for western blotting analysis using Caspase3, Caspase9, PAPR, Mcl-1, XIAP, Bax, p-JNK, JNK, p-ERK, ERK, p-P38 and P38 antibodies. Error bars, mean ±SD. Ctrl: control; CHI: chidamide; TFV: tenofovir. *P < 0.05; **P < 0.01; *** P < 0.001.

Supplementary Files

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