Chidamide induces EBV lytic infection and acts synergistically with tenofovir
to eliminate EBV-positive Burkitt lymphoma

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Abbreviations
ACV, acyclovir; BCA, bicinchoninic acid; BL, Burkitt lymphoma; CHI, Chidamide; CI, combination index; DLBCL, diffuse large B-cell lymphoma; EBNA1, Epstein–Barr nuclear antigen 1; EBV, Epstein Barr virus; ERK, extracellular regulated protein kinases; GCV, ganciclovir; HDAC, histone deacetylase; JNK, c-Jun N-terminal kinase; LMP1, latent membrane protein 1; MAPK, mitogen-activated protein kinase; NHL, non-Hodgkin’s lymphoma; OD, optical density; PBS, phosphate buffered saline; PBST, phosphate buffered saline with Tween 20; PCR, polymerase chain reaction; PVDF, polyvinylidene fluoride; qPCR, Quantitative polymerase chain reaction; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFV, Tenofovir; TK, Thymidine kinase.

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Abstract:

Epstein-Barr virus (EBV) is a type of human gamma-herpesvirus, and its reactivation plays an important role in the development of EBV-driven Burkitt lymphoma (BL). Despite intensive chemotherapy, the prognosis of relapsed/refractory BL patients remains unfavorable, and a definitive method to completely eliminate latent EBV infection is lacking. Previous studies have demonstrated that histone deacetylase (HDAC) inhibitors can induce the transition of EBV from latency to the lytic phase. The lytic activation of EBV can be inhibited by tenofovir, a potent inhibitor of DNA replication. Herein, we explored the anti-tumor effect and EBV clearance potential of a novel HDAC inhibitor called chidamide combined with tenofovir in the treatment of EBV-positive BL. In the study, chidamide exhibited inhibitory activity against HDAC. Moreover, chidamide inhibited BL cell proliferation, arrested cell cycle progression, and induced BL cells apoptosis primarily by regulating the MAPK pathways. Additionally, chidamide promoted the transcription of lytic genes including $\text{BZLF1}$, $\text{BMRF1}$, and $\text{BMLF1}$. Compared with chidamide alone, the addition of tenofovir further induced growth arrest and apoptosis in EBV-positive BL cells, and inhibited the transcriptions of EBV lytic genes induced by chidamide alone. Furthermore, our in vivo data demonstrated that the combination of chidamide and tenofovir had superior tumor-suppressive effects in a mouse model of BL cell tumors. The aforementioned findings confirm the synergistic effect of chidamide combined with tenofovir in inducing growth inhibition and apoptosis in EBV-positive BL cells, and provide an effective strategy for eliminating EBV and EBV-associated malignancies.

Keywords: Chidamide; Tenofovir; EBV; Burkitt lymphoma; MAPK.

Significance Statement:

High levels of EBV-DNA have consistently been associated with unfavorable progression-free survival (PFS) and overall survival (OS) in EBV-associated lymphomas. Therefore, identifying novel strategies to effectively eradicate tumor cells and eliminate EBV is crucial for lymphoma patients. In this study, we confirmed for the first time the synergistic effect of chidamide combined with tenofovir in the treatment of BL and the eradication of EBV virus.
Introduction

Burkitt lymphoma (BL), a highly aggressive non-Hodgkin lymphoma, is closely associated with Epstein-Barr Virus (EBV) infection (Coghill et al. 2020). Despite the advancements in intensive multi-drug chemotherapy, the prognosis of patients with relapsed/refractory BL remains grim, with long-term survival rates ranging from 10% to 20% (Du et al. 2020; Woessmann et al. 2020). Moreover, lymphoma patients with high EBV DNA loads were shown to have poor prognosis (Okamoto et al. 2015; Shen et al. 2022). Unfortunately, the current chemotherapy regimens are unable to eliminate EBV, thereby limiting the therapeutic efficacy of EBV-associated lymphomas, including BL.

EBV, a gamma-herpesvirus, has been demonstrated to promote clonal formation, occurrence, and development of EBV-associated lymphoma through multiple latent genes, including EBNA1 and LMP-1 (Nkosi et al. 2020; Smith et al. 2021; Soldan et al. 2021; L Sun et al. 2015). In addition, EBV-encoded lytic genes such as BZLF1, BMRF1, and BMLF1 play central roles in the development of EBV-driven BL (Chen et al. 2005). The lytic infection of EBV can be categorized into three stages: immediate-early lytic state, early lytic state, and late lytic state (Dasari et al. 2019). Each lytic stage of EBV generates multiple gene products, including the immediate-early genes BZLF1 and BRLF1. These gene products mutually activate each other and trigger the expression of other early lytic proteins (BMRF1, BALF1, BHRF1, etc.) (Wille et al. 2013). The resulting viral DNA replicates are then packaged into the viral progeny, and released to infect neighboring cells, thereby facilitating EBV lytic infection (Giunco et al. 2013).

EBV usually persists in latent form (Tangye et al. 2020). Antiviral drugs such as acyclovir (ACV) and ganciclovir (GCV), targeting EBV DNA polymerase, are unable to eliminate latent EBV at present (Kerr 2019). Fortunately, anti-virus treatment is expected to be effective in the EBV lytic state. Viral lytic reactivation from latency could be induced by various drugs or external stimuli (McKenzie et al. 2015), including histone deacetylase (HDAC) inhibitors (Chen et al. 2020). Switching the latent form of EBV infection to the lytic cycle intentionally represents a potentially effective therapeutic intervention for patients (Ghosh et al. 2012; Hui et al. 2016). Chidamide, a novel benzamide-type HDAC inhibitor, has been approved
in China for the treatment of recurrent/refractory peripheral T-cell lymphoma (PTCL) (Gao et al. 2017). Evidence of inducing $BZLF1$ and $BRLF1$ expression suggested that chidamide could promote the lytic cycle of EBV in NK/T cell lymphoma (Zhou et al. 2018). In addition, chidamide has demonstrated significant anti-tumor effects in colon cancer and gastric cancer (L Liu et al. 2016; Zhang et al. 2020). However, its role in BL needs to be further exploration.

Currently, most antiviral drugs, such as ACV and GCV, are based on broad-spectrum antiviral characteristics and are problematic in treating EBV infection (Drosu et al. 2020; Kerr 2019). Tenofovir is an acyclic nucleoside/nucleotide analog, mainly used to treat HIV and hepatitis B (Ueaphongsukkit et al. 2021). Intriguingly, tenofovir bisphosphate, an active component of tenofovir, can inhibit viral DNA polymerase by directly and competitively binding to natural deoxyribose substrates (Andrei et al. 2011; Duong et al. 2020). It has been reported that tenofovir dipivoxil fumarate inhibited EBV lytic DNA replication efficiently in vitro, exhibiting better efficacy and safety against EBV compared to other agents (Drosu, et al. 2020). These findings raise the question of whether tenofovir could be utilized in the treatment of EBV-associated lymphomas such as BL.

This study aims to investigate the effect of HDAC inhibitor chidamide on EBV lytic infection. In addition, we will explore the anti-tumor effect of the combination of chidamide and tenofovir in the treatment of EBV-positive BL, and assess their impact on EBV related lytic genes.

Material and methods

Cell lines and culture media

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

Antibodies and reagents

Chidamide and SP600125 powders were obtained from MedChemExpress (Monmouth Junction, NJ, USA) and dissolved in DMSO (Sigma Aldrich; Merck KGaA, Darmstadt, Germany) prior to storage in the
dark at −80 °C. Tenofovir was provided by Zhengda Tianqing Co. Ltd. (Nanjing, Jiangsu, China). The antibodies against DR5 (8074), Caspase8 (9746), Caspase9 (9502), Caspase3 (9664), PARP (9532), Bax (2772), Bcl-2 (15071), Mcl-1 (39224), 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 Group (Wuhan, China).

**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 and tenofovir for 48 hours. Then 10 µL of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added to each hole and incubated at 37 °C for 2 hours as directed by the manufacturer. The optical density (OD) value of viable cells was measured using the Synergy™ HTX Multi-Mode Microplate Reader at 450 nm. The combination index (CI), a drug interactivity measurement, is calculated by CompuSyn software. In general, CI < 1 is considered to be synergism.

**RNA extraction and real-time quantitative polymerase chain reaction**

Raji and Namalwa cells were exposed to varying concentrations of chidamide (0, 4, or 8 µM) alone or in combination with tenofovir (80 µM) for 48 hours. Total RNA was extracted using Trizol (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer’s instructions. NanoDrop was used to measure RNA concentrations and then reverse-transcribed into cDNA using M-MLV reverse transcriptase (Life Technologies, Gaithersburg, USA). Quantitative polymerase chain reaction (qPCR) was carried out on a LightCycler480 instrument using SYBR® Green Supermix (Roche, Indianapolis, IN, USA) according to the manufacturer’s instructions. Table 1 shows all the primers for PCR assay.

**Western blot analysis**

Raji, Namalwa, and CA46 cells were treated with different concentrations of chidamide (0, 4, or 8 µM) alone or in combination with tenofovir and SP600125. After 48 hours, cells were collected. The bicinchoninic acid (BCA) 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 (PVDF) 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 incubation with the Horseradish peroxidase antibodies. The images were captured using the GE Image Quant LAS4000 Mini system.

**Apoptosis analysis**

BL cell lines were seeded with 1 × 10⁶ cells per well in a 12-well plate and treated with chidamide (0, 4, or 8 µM) alone or in combination with tenofovir and SP600125 for 48 hours. After treatment, cells were collected and incubated with 5 µL Annexin V-FITC and 10 µL propidium iodide (PI) at room temperature in the dark for 10 minutes. Flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) is used to detect the samples, and FlowJo software is used to analyze data.

**Cell cycle analysis**

In 6-well plates (2 × 10⁶ cells per well), BL cell lines were inoculated and treated with chidamide (0, 4 or 8 µM) for 48 hours. Cells were then collected and immobilized in 70% precooled ethanol at –20 °C. On the following day, the cells were resuspended in 0.25 mL staining solution (50 µg/mL RNase A and 50 µg/mL propidium iodide) protected from light for 30 minutes. Furthermore, samples were detected using the FACS Calibur system and data were analyzed using ModFit LT 3.3 software (Verity Software House Inc., Topsham, ME, USA).

**Tumorigenicity assay in NSG mice**

NOD-SCID L2Rγnull (NSG) mice (female, 4–6 weeks old, approximately 20 g) were purchased from Beijing Vitalstar Biotechnology Co. Ltd. (Beijing, China) and maintained in a specific pathogen-free (SPF) facility. The study was approved by Xuzhou Medical University Institutional Ethics Committee. Each NSG mice underwent subcutaneous injection of 1 × 10⁶ Raji-GFP-Luc cells in the right armpit. The mice were randomly divided into four groups (n=3 per group): the control group received intraperitoneal administration of phosphate-buffered saline (PBS) vehicle, the chidamide group received intraperitoneal administration of chidamide, the tenofovir group received oral administration of tenofovir, and the chidamide + tenofovir group received both chidamide and tenofovir. The bodyweight of mice and the long (a) and short diameter (b) of
tumors were measured every other day. Starting from the 8th day after tumor cell injection, mice were intraperitoneally given chidamide at a dose of 30 mg/kg per day for 2 weeks, while tenofovir was orally administered at a dose of 75 mg/kg per day for the same duration. On the 21st day, D-fluorescein was injected intraperitoneally, and IVIS imaging was conducted to quantify tumor burden. Subsequently, the mice were euthanized and xenograft tumors were excised for weight, length, and diameter measurements. The tumors were fixed in a formaldehyde solution for pathological examination.

**Statistical analysis**

The data are presented as mean ± standard deviation (SD). One-way ANOVA and two-way ANOVA were used to analyze the statistical significance among 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 version 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

**Results**

**Chidamide inhibits BL cell proliferation and arrests cell cycle progression**

In Raji, Namalwa, and CA46 cell lines, chidamide downregulated the mRNA levels of HDAC1, 2, 3 and upregulated the acetylation of histone H3 expression in a concentration-dependent manner (Figures S1A and S1B). Initially, we investigated the effect of chidamide on the proliferation of BL cells. As shown in Figure S1C, chidamide exerted an anti-proliferative effect and significantly inhibited the viability of BL cells in a concentration- and time-dependent manner. To further explore the mechanism of chidamide in BL cells, cell cycle distribution was analyzed by flow cytometry. A higher percentage of G1 phase cells and a lower percentage of S phase cells were found in chidamide treated BL cells, while there was no significant change in the percentage of G2/M phase (Figures 1A and 1B). Moreover, western blotting revealed a concentration-dependent upregulation of p21 and p27 expression, as well as a downregulation of Cyclin D1 and Cyclin E1 expression in BL cells (Figure 1C).

**Chidamide induces apoptosis and activates the apoptotic pathways in BL cells**
Subsequently, we investigated the potential of chidamide to induce apoptosis in BL cells. As shown in Figure 2A and 2B, chidamide treatment induced apoptosis of Raji, Namalwa and CA46 cells in a concentration-dependent manner. Western blotting analysis showed upregulation of the cleavage of Caspase 3 and PARP expression in chidamide-treated BL cells (Figure 2C). Furthermore, apoptotic pathway investigation showed that DR5 and cleaved form of Caspase 8 were upregulated in chidamide-treated BL cells (Figure 2C), indicating that chidamide activated exogenous apoptotic pathway. Meanwhile, chidamide upregulated pro-apoptotic protein Bax and downregulated anti-apoptotic proteins Mcl-1 and XIAP expression in a concentration-dependent manner, suggesting chidamide induced endogenous apoptotic pathway activation (Figure 2D).

**Chidamide inhibits c-Myc expression and regulates MAPK pathways in BL cells**

Previous studies have shown that c-Myc activation is involved in the progression of BL and is associated with poor prognosis (Kanungo et al. 2006; Lopez et al. 2022). In the present study, chidamide significantly inhibited c-Myc expression in BL cells (Figures 3A and 3B). Further, chidamide affected MAPK pathways, upregulated p-JNK and p-P38, and downregulated the expression of p-ERK in BL cells (Figure 3C). To further investigate the regulation of chidamide on the JNK pathway, we utilized the JNK inhibitor SP600125. In the presence of SP600125, the upregulation of p-JNK induced by chidamide was blocked (Figure 3D). Moreover, the upregulation of the cleaved Caspase 3 and PARP induced by chidamide was also blocked in the presence of SP600125 (Figure 3D). The percentages of apoptosis induced by chidamide were also significantly reduced when cells were pretreated with SP600125 (Figures 3E and 3F), suggesting a MAPK pathway dependent manner of chidamide induced apoptosis in BL cells.

**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 EBV genes, EBV-positive Raji and Namalwa cells were treated with different concentrations of chidamide for 48 hours. As shown in Figures 4A and 4B, after chidamide treatment, the mRNA levels of latent genes (\textit{EBNA1} and \textit{RPMS1}) decreased in a concentration-dependent manner. In contrast, the expression levels of lytic viral transcripts such as \textit{BZLF1}, \textit{BMRF1}, and
BMLF1 significantly increased after chidamide treatment (Figures 4D-E). The mRNA expression of thymidine kinase (TK) gene, which is closely related to EBV lytic infection, showed a significant increase in the presence of chidamide (Figure 4C). The above results suggest that chidamide promotes the transition of EBV from the latent stage to the lytic stage in EBV-positive BL cells. We also investigated the effect of chidamide combined with SP600125 on the transcription of lytic genes in Raji and Namalwa cells. We found that adding SP600125 can inhibit the transcriptions of the EBV lytic genes (BZLF1, BMRF1, and BMLF1) and TK induced by chidamide (Figure S2), indicating the transition of EBV induced by chidamide maybe related to the MAPK pathways.

**Tenovir enhances the anti-proliferative effect of chidamide in EBV-positive BL cells**

To determine whether tenofovir can enhance the growth inhibitory effect of chidamide in EBV-positive BL cells, the proliferation ratio was measured using the CCK-8 assay. BL cells were incubated with incremental concentrations of chidamide and tenofovir for 48 hours, then growth suppression ratios of each combination were calculated, as shown in Figures 5A and 5C. The cell inhibition ratio in the combination drug group was higher compared to the single drug group in Raji and Namalwa cells, which are EBV positive, but not in CA46 cell, which is EBV negative, indicating the combination of these drugs is more toxic to EBV positive BL cells than the use of either agent alone. The CI of chidamide and tenofovir was obtained for each tested cell line using CompuSyn software. In Raji and Namalwa cells, the CI value was consistently less than 1 at all test concentrations, but not in CA46 cells (Figures 5B and 5C). These results suggest that combination with tenofovir can enhance the anti-proliferative effect of chidamide in EBV-positive BL cells.

**Tenofovir synergistically with chidamide induce apoptosis of EBV-positive BL cells by regulating MAPK pathways**

Furthermore, we investigated the apoptotic response of the combination of chidamide and tenofovir in Raji, Namalwa, and CA46 cells. Interestingly, the addition of tenofovir significantly enhanced chidamide-induced cell apoptosis, resulting in a synergistic pro-apoptotic effect in Raji and Namalwa cells (Figures 6A and 6B). In contrast, no synergistic effect was observed in EBV-negative CA46 cells (Figures 6A and 6B). We further investigated the effect of tenofovir on the changes of the apoptotic pathways induced by
chidamide in EBV-positive Raji and Namalwa cells. Western blot analysis revealed that the cleaved levels of Caspase 9, Caspase 3, PARP, and the pro-apoptotic protein Bax were significantly upregulated in the presence of both chidamide and tenofovir. Meanwhile, the expression of the anti-apoptotic protein Bcl-2 was downregulated (Figure 6C). Furthermore, western blot analysis showed that in Raji and Namalwa cells, the combination of chidamide and tenofovir significantly increased the levels of p-JNK and p-P38, while decreasing the level of p-ERK (Figure 6D). However, tenofovir did not further affect MAPK pathways in CA46 cells (Figures S3).

**Tenofovir blocks lytic genes transcription induced by chidamide in EBV-positive BL cells**

Further experiments were carried out to test the effect of tenofovir on the transcription of lytic genes induced by chidamide. Figure 7 demonstrated that the addition of tenofovir significantly inhibited the transcription of EBV lytic genes (*BZLF1, BMRF1, and BMLF1*) and *TK* induced by chidamide. These data suggest that chidamide may be sufficient to induce EBV lytic cycles and sensitize EBV-positive cells to tenofovir therapy. Furthermore, when cells were treated with chidamide, tenofovir, and SP600125 simultaneously, the transcriptions of the EBV lytic genes were further reduced (Figures S4), which maybe due to SP600125 interfering with the up-regulation of EBV lytic genes induced by chidamide.

**Tenofovir synergistically with chidamide enhance the tumor-suppression effect in BL cell tumor-bearing mouse model**

To investigate the *in vivo* anti-tumor effect of chidamide combined with tenofovir, this study used a Raji cell tumor-bearing NSG mouse model. As a single agent, tenofovir was insufficient to induce tumor remission, but chidamide alone exhibited partial anti-tumor activity. Strikingly, tumor inhibitory effect induced by chidamide was significantly enhanced by the addition of tenofovir, reflected in a significant reduction in tumor luminescence, as well as the reductions in tumor size, weight, and volume (Figures 8A-F). In contrast, the volume and growth rate of transplanted tumors in the combination group were similar to those in the chidamide group in EBV-negative CA46 mice model (Figure S5), indicating that tenofovir can effectively assist chidamide in inhibiting the growth of subcutaneous transplanted tumors in EBV-positive BL mice. Further pathological examination showed that the tumor tissue structures were almost full field of tumor...
cells and a small amount of 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 (Figure 8G). In particular, an enhanced tumor-suppression effect was observed in the chidamide + tenofovir group, with severely damaged tumor tissue structure accompanied by a large number of necrotic cells (Figure 8G). We additionally detected the apoptotic pathways and MAPK pathways in the extracted tumor tissue by western blot. Consistent with in vitro study, our in vivo study showed that compared to the monotherapy group, the cleavage levels of Caspase 9, Caspase 3, and PARP and the expression of Bax were upregulated, while the anti-apoptotic protein Mcl-1 was significantly downregulated in the chidamide + tenofovir group (Figure 8H). Meanwhile, in the EBV-positive BL cell tumor-bearing mouse model, the combination of chidamide and tenofovir significantly increased the levels of p-JNK and p-P38, and decreased the level of p-ERK (Figure 8I).

Discussion

EBV infection is closely associated with BL, posing challenges for the treatment of refractory/relapsed BL patients. In addition, high EBV DNA load is associated with poor prognosis in many EBV-positive lymphoma subtypes (Coghill, et al. 2020; Okamoto, et al. 2015; Shen, et al. 2022). However, current treatment approaches often overlook the EBV-positive status of BL due to difficulty in eliminating the latent EBV (Tikhmyanova et al. 2019). In this study, we confirmed that the novel HDAC inhibitor chidamide effectively induced EBV lytic reactivation, and found that the combination of chidamide and tenofovir has a synergistic effect on the growth inhibition, apoptosis and modulation of related signaling pathways in EBV-positive BL cells. In addition, we demonstrated that tenofovir significantly inhibited chidamide-induced transcription of EBV lytic genes. Furthermore, the synergistic anti-tumor effect of tenofovir combined with chidamide in vivo was verified in the EBV-positive BL cell tumor-bearing mouse model, providing a potent new therapeutic strategy for EBV-positive BL.

HDAC inhibitors are a class of anti-lymphoma therapeutics approved by FDA in the treatment of T-cell lymphoma (Gao, et al. 2017; Ye et al. 2019). In recent years, HDAC inhibitors have demonstrated efficacy in
diffuse large B-cell lymphoma (DLBCL), follicular lymphoma, Hodgkin lymphoma, and other types of lymphoma (Chen, et al. 2020; Zhang et al. 2021). Vorinostat, the first approved HDAC inhibitor, combined with rituximab or R-CHOP has shown enhanced efficacy in DLBCL patients (Persky et al. 2018; Straus et al. 2015). Belinostat, another HDAC inhibitor, was well tolerated and resulted in 10.5% overall response rate in relapsed/refractory aggressive B-NHL patients (Puvvada et al. 2016). The novel HDAC inhibitor chidamide has been shown to induce growth inhibition, apoptosis, DNA damage and cell cycle arrest in NK/T lymphoma cells (Song et al. 2021; Zhang, et al. 2020).

HDAC inhibitors have been reported to block cell cycle progression of many tumor cells in the G1 phase (Takai et al. 2007). TSA induced G1 phase arrest accompanied by upregulation of p21 and p27, and downregulation of CDK2 and Cyclin A in lymphatic endothelial cells, bladder cancer cells, cervix carcinoma cells (Hrgovic et al. 2016; Li et al. 2006). Vorinostat led to an increase in G1 cell cycle phase along with a decrease in S phase through downregulation of CDK2 and Cyclin E and upregulation of p21 (Xue et al. 2016). Studies has also shown that chidamide induced cell arrest in G1 phase in acute myeloid leukemia cells, myelodysplastic syndromes, and multiple myeloma cells (Deng et al. 2023; Z Liu et al. 2016; Yuan et al. 2019; Zhao et al. 2016). These reports are consistent with our results that chidamide arrests cell cycle progression in the G1 phase through upregulation of p21 and p27 expression, as well as downregulation of Cyclin D1 and Cyclin E1 expression.

Mitogen-activated protein kinase (MAPK) pathways, including extracellular signal regulated protein kinases (ERK), stress-activated protein kinase [SAPK/c-Jun N-terminal protein kinase (JNK)], and p38 MAPK, are known to be associated with the regulation of cell proliferation, apoptosis, differentiation, and migration (Guo et al. 2020; Y Sun et al. 2015). When cells are exposed to various physical, chemical, and biological stress stimuli, the JNK and p38 MAPK pathways are usually activated, leading to cell apoptosis; while ERK cascades are often deactivated (Sui et al. 2014; Yue et al. 2020). ERK cascades are mostly activated upon cell growth factor-stimulated and implicated in cell growth and differentiation, as well as anti-apoptotic function (Cagnol et al. 2010). In our experiment, chidamide affected the MAPK pathways, leading to upregulation of p-JNK and p-P38 expression, as well as downregulation of p-ERK expression in BL cells.
Furthermore, the JNK pathway is contributed to chidamide-induced cell apoptosis. These results are consistent with the study of chidamide inhibiting ERK phosphorylation in human colon cancer cells (Liu et al. 2010).

Lytic infection is a unique state of EBV, which represents the invasion to the host. The EBV lytic genes BZLF1, BMRF1, and BMLF1 could be detected in a variety of tumors, and their positivity reflects the cleavage and replication of EBV (Murata 2018). BZLF1 is an EBV-encoded immune early lysis gene product initiating reactivation of the EBV lytic cycle (Kusano et al. 2019). BMRF1 encodes the EA-D protein, which is essential for viral DNA synthesis (Salamun et al. 2019). The BMLF1 protein is a multifunctional RNA-binding protein that may increase the viral or cellular gene expression (Zhang et al. 1992). The EBV DNA load is closely related to the prognosis of lymphoma (Okamoto, et al. 2015). The elimination of EBV DNA has become an essential part that we must consider when treating EBV-related lymphoma. Lytic state of EBV is expected to be inhibited by antiviral drug nucleoside analogue (Amon et al. 2005; Novalic et al. 2017).

Previous studies have shown that HDAC inhibitors such as romidepsin and sodium valproate can induce the switch of EBV from latent to lytic cycle (Hui et al. 2010; Hui et al. 2012; Jones et al. 2010; Shin et al. 2015). In the study, we found that chidamide promotes EBV lytic gene expression, and induces EBV to enter the lytic cycle, which might render EBV-positive BL cells more susceptible to antiviral therapy.

EBV lytic DNA replication depends on DNA polymerase, but the clinical application of antiviral nucleoside/nucleotide analogs ACV or GCV in EBV infection is currently limited due to their low potency or high toxicity (Kerr 2019). Tenofovir, a new nucleoside compound primarily used for the treatment of HIV and hepatitis B, has demonstrated high efficacy against EBV lytic infection in vitro (Drosu, et al. 2020). In the study, we found an enhanced co-lethal effect of tenofovir and chidamide on BL cells. The significant changes observed in the MAPK signaling pathways further support the synergistic effect of the two drugs. Further exploration is needed to explore other signaling pathways related to the synergistic effect of the combination of chidamide and tenofovir. Notably, tenofovir significantly inhibited the transcriptions of EBV lytic genes induced by chidamide in EBV-positive BL cells, which is consistent with that other antiviral drug such as GCV, inhibited the transcription of EBV lytic genes (Figure S6). EBV, as a complex double-stranded DNA...
virus, its DNA polymerase is mainly responsible for the replication of DNA during the lytic infection process, which is different from latent infection. Tenofovir mainly inhibits EBV lytic DNA replication through targeting the virus DNA polymerase system. Intentionally converting the latent form of EBV infection into the lytic cycle could activate viral DNA polymerase and provide drug targets for antiviral drugs (Ghosh, et al. 2012; Hui, et al. 2016). It is possible that tenofovir prevents the release of infectious EBV particles from cells by chidamide, thereby blocking the transcription of lytic genes and synergistically with chidamide eliminating EBV positive tumor cells. However, it is currently unclear whether the downregulation of EBV lytic genes and the prevention of replicated viral particles release directly affect cell apoptosis, which requires further exploration in the future.

To further validate the synergistic effect of chidamide combined with tenofovir, we conducted experiments using a BL cell tumor-bearing mouse model. Consistent with our in vitro findings, tumor inhibitory effect induced by chidamide was significantly enhanced by the addition of tenofovir in vivo. Western blot analysis of apoptotic and MAPK pathways provided additional evidence to support the synergistic anti-tumor effect of this combination therapy in vivo. However, we observed that tenofovir alone did not exert anti-tumor effects, which raises the need for further investigation. In addition, due to the limitation of observation days, we did not observe the effect of chidamide combined with tenofovir on the overall survival of mice. Therefore, more experiments are needed to further explore the anti-tumor effects and mechanisms of these two-drug combination in vivo.

In conclusion, this study demonstrates the synergistic anti-tumor effects of the novel HDAC inhibitor chidamide in combination with tenofovir on EBV-positive BL cells. Most strikingly, we showed that the addition of tenofovir significantly reduced chidamide-induced transcription of EBV lytic genes, presenting a promising strategy for elimination EBV and related tumors. Given the complex molecular pathogenesis of EBV-related tumors, more research is warranted to establish this two-drug combination regimen as a first-line therapy for relapsed/refractory EBV-positive lymphoma.

Data Availability Statement
The data that support the findings of this study are available on request from the corresponding author.

Authorship Contributions

Participated in research design: Xu, M. Zhang, Wang, and Sang.


Contributed new reagents or analytic tools: Xu, M. Zhang, and Tu.

Performed data analysis: Xu, M. Zhang, Wang, and Sang.

Wrote or contributed to the writing of the manuscript: Xu, M. Zhang, Wang, and Sang.

Ethics statement

The animal study protocol was approved by the Institutional Ethics Committee of Xuzhou Medical University.

References


hTERT Inhibition Triggers Epstein-Barr Virus Lytic Cycle and Apoptosis in Immortalized and Transformed B Cells: A Basis for New Therapies. *Clinical Cancer Research, 19*(8), 2036-2047. doi: 10.1158/1078-0432.CCR-12-2537


Kanungo A, Medeiros LJ, Abruzzo LV, and Lin P (2006). Lymphoid neoplasms associated with concurrent t(14;18) and 8q24/c-MYC translocation generally have a poor prognosis. *Mod Pathol, 19*(1), 25-33. doi:
10.1038/modpathol.3800500


Nkosi D, Sun L, Duke LC, Patel N, Surapaneni SK, Singh M, and Meckes DG (2020). Epstein-Barr Virus...


Footnotes

a) This work was supported by Jiangsu Province’s Graduate Scientific Research Innovation Program (KYCX21–2685, KYCX21–2680); National Natural Science Foundation of China (81900177), Natural Science Foundation of Jiangsu Province (BK20190985) and Jiangsu Key Research and Development Project of Social Development (BE2019638).

b) The authors declare no conflicts of interest.

c) L Xu, and M Zhang contributed equally to this work.

d) This manuscript is posted on the Researchsquare site and the Researchsquare URL is https://www.researchsquare.com/article/rs-1952984/v1.
Figure Legends

Figure 1. Chidamide arrests cell cycle progression in human BL cells.
Raji, Namalwa, and CA46 cells were incubated with 0, 4, or 8 µM 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. Error bars, mean ±SD. *** P < 0.001 (significant between chidamide group with the control group, n=3). **C**. The harvested cells were subjected to western blotting using Cyclin D1, Cyclin E1, p21, p27, and GAPDH antibodies.

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 chidamide for 48 hours, then the cells were harvested. **A**. The cells were 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 < 0.001 (significant between chidamide group with control group, n=3). **C** and **D**. The harvested cells were subjected to western blotting using DR5, Caspase 8, Caspase 3, PARP, Caspase 9, Mcl-1, XIAP, Bax, and GAPDH antibodies.

Figure 3. Chidamide inhibits c-Myc expression and regulates the MAPK pathways in human BL cells.
**A-C**. Raji, Namalwa, and CA46 cells were incubated with 0, 4 or 8 µM 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, ERK, and GAPDH antibodies (A and C) or detected the mRNA levels of c-Myc by RT-qPCR (B). **D-F**. 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, Caspase 3, PARP, and GAPDH antibodies (D) or subjected to analyze by flow cytometry for apoptosis (E). Percentages of apoptotic cells were determined from three independent experiments (F). Error bars, mean ± SD. * P < 0.05; ** P < 0.01; *** P < 0.001 (significant between chidamide group with control group, n=3).

Figure 4. Chidamide promotes EBV lytic genes expression in EBV positive BL cells.
A-F. Raji and Namalwa cells were incubated with 0, 4 or 8 µM chidamide for 48 hours. The mRNA levels of EBV-related genes (EBNA1, RPMS1, TK, BZLF1, BMRF1, and BMLF1) were detected by RT-qPCR. Error bars, mean ±SD. * P < 0.05; ** P < 0.01; *** P < 0.001 (significant between the chidamide group and control group, n=3).

**Figure 5. Tenofovir enhances the anti-proliferative role of chidamide in EBV-positive BL cells.**

A. Drug dose matrix data in Raji, Namalwa and CA46 cells. BL cells were incubated with the incremental concentrations of chidamide and tenofovir for 48 hours, then CCK-8 assay was performed to assess cell viability. The numbers indicate the percentages of growth suppression of the cells treated with the corresponding compound combination relative to control-treated cells. The data are visualized over the matrix using a color scale. B. CI were calculated by CompuSyn software to predict the potential synergism of chidamide and tenofovir at the indicated combination concentrations in the three BL cells. Each point represents the CI value under the corresponding compound combination. CI < 1 is considered to be synergism. C. Raji, Namalwa and CA46 cells were incubated with chidamide (4 µM), tenofovir (80 µM), or a combination of both for 48 hours, then CCK-8 was added to detect the cell viability. Error bars, mean ±SD. * P < 0.05; *** P < 0.001 (significant between the chidamide group with combination treatment group, n=3). ns, not significant.

**Figure 6. Tenofovir synergistically with chidamide induce apoptosis of EBV-positive BL cells by regulating the MAPK pathways.**

A. Raji and Namalwa cells were treated with chidamide (4 µM), tenofovir (80 µM), or a 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 percentages of apoptotic cells were determined from three independent experiments. Error bars, mean±SD. *** p < 0.001 (significant between chidamide group with combination treatment group, n=3). ns, not significant. C and D. Raji and Namalwa cells were harvested and subjected to western blotting analysis using PARP, Caspase3, Bcl-2, Bax, Caspase9, p-JNK, JNK, p-ERK, ERK, p-P38, P38, and GAPDH antibodies.

**Figure 7. Tenofovir blocks lytic gene transcription induced by chidamide in EBV-positive BL cells.**
A-D. Raji or Namalwa cells were treated with chidamide (4 μM), tenofovir (80 μM) or a combination of both for 48 hours, then the whole cells were harvested. The mRNA expression levels of TK and EBV lytic genes were assessed by RT-qPCR. Error bars, mean ±SD. * P < 0.05; ** P < 0.01; *** P < 0.001 (significant between chidamide group with combination treatment group, n=3).

Figure 8. Tenofovir synergistically with chidamide exert enhanced tumor suppression effect in BL cell tumor-bearing mouse model.

Female NPG mice aged 6–7 weeks were subcutaneously injected with $1 \times 10^6$ Raji-GFP-Luc cells per mouse, and the systemic tumor-bearing mice model was established on the 8th day. From the 8th day, chidamide and tenofovir were given to the mice for 2 weeks. A. On the 21st day, D-fluoresein was intraperitoneally injected into mice and IVIS imaging was performed to measure tumor burden. B. Total counts of luminescence from Raji cells in each group were recorded. Error bars, mean ±SD. * P < 0.05; ** P < 0.01 (significant between individual drug group with combination group, n=3). C and D. The mice were sacrificed, xenograft tumors were dissected and photographed. The tumor weights were measured in each group. * P < 0.05; *** P < 0.001 (significant between individual drug group with combination group, n=3). E. The weights of the mice were measured every two days. F. 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($V$)= $1/2ab^2$. G. H&E staining of the removed tumor grafts in mice (magnification $\times$ 200). The part circled by the dotted line represents necrotic cells. H and I. Part of xenograft tumor tissues were sent for western blotting analysis using Caspase 3, Caspase 9, PAPR, Mcl-1, XIAP, Bax, p-JNK, JNK, p-ERK, ERK, p-P38, P38, and GAPDH antibodies.
Table 1. Primers used for real-time PCR.

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<td>5’-GCTGTCACCTCACCAGTCC-3’ (reverse)</td>
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Figure 1
Figure 2
Figure 3
Figure 4
**Figure 5**

(A) Heatmaps showing the effect of CHI and TFV on Raji, Namalwa, and CA46 cell lines. The color scale represents the percentage of cell viability.

(B) Graphs illustrating the synergistic effect of CHI and TFV on Raji, Namalwa, and CA46 cell lines. The x-axis represents CHI concentration, while the y-axis shows the cell viability index (CI).

(C) Bar charts depicting the cell viability of Raji, Namalwa, and CA46 cell lines treated with CHI and TFV. The CI values indicate the synergistic effect of the combined treatment.
Figure 6
Figure 7
Figure 8