



Original Article

Histone Deacetylase Inhibitors Romidepsin and Vorinostat Promote Hepatitis B Virus Replication by Inducing Cell Cycle Arrest

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Abstract

Background and Aims: Chronic hepatitis B virus (HBV) infection is a global public health challenge. HBV reactivation usually occurs in cancer patients after receiving cytotoxic chemotherapy or immunosuppressive therapies. Romidepsin (FK228) and vorinostat (SAHA) are histone deacetylase inhibitors (HDACi) approved by the Food and Drug Administration as novel antitumor agents. The aim of this study was to explore the effects and mechanisms of HDACi treatment on HBV replication. **Methods:** To assess these effects, human hepatoma cell lines were cultured and cell viability after FK228 or SAHA treatment was measured by the CCK-8 cell counting kit-8 assay. Then, HBV DNA and RNA were quantified by real-time PCR and Southern blotting. Furthermore, analysis by western blotting, enzyme-linked immunosorbent assay (ELISA), immunohistochemistry, and flow cytometry was performed. **Results:** FK228/SAHA treatment significantly promoted HBV replication and biosynthesis in both HBV-replicating cells and HBV-transgenic mouse model. Flow cytometry assay indicated that FK228/SAHA enhanced HBV replication by inducing cell cycle arrest through modulating the expression of cell cycle regulatory proteins. In addition, simultaneous inhibition of HDAC1/2 by FK228 promoted HBV replication more effectively than the broad spectrum HDAC inhibitor SAHA. **Conclusions:** Overall, our results demonstrate that

cell cycle blockage plays an important role in FK228/SAHA-enhanced HBV replication, thus providing a potential avenue for rational use of HDACi in patients with chronic hepatitis B.

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Introduction

Hepatitis B virus (HBV) infection continues to be a serious public health problem worldwide. Chronic HBV infection is a major risk factor for developing cirrhosis and hepatocellular carcinoma (HCC).¹ The World Health Organization estimated that 257 million people were infected with HBV and approximately 887,000 people die from HBV/HCC complications every year.² HBV, the prototype virus of the *Hepadnaviridae* family that productively infects hepatocytes, contains a partially double-stranded DNA genome surrounded by an icosahedral capsid.³ The HBV genome is only 3.2 kb long and contains four partially overlapping open reading frames, which transcribe four different lengths of mRNA, including pregenomic RNA, precore mRNA, preS/S mRNA, and X (i.e. HBx) mRNA.⁴ HBV covalently closed circular DNA (cccDNA) serves as the transcriptional template for all viral RNAs, and accounts for HBV persistence.⁵ Despite the availability of effective anti-HBV drugs, reactivation of HBV infection is a challenging issue for patients with a chronic HBV infection who undergo cytotoxic chemotherapy or immunosuppressive therapies.⁶

HBV reactivation was firstly reported in patients with hematological malignancies by Wands et al.⁷ It is usually defined as a sudden increase in HBV DNA levels (≥ 10 -fold relative to baseline), or an absolute increase that is more than 10^5 copies/mL in patients undergoing chemotherapy or immunosuppressive therapy.^{8,9} Reactivation of HBV could lead to severe complications, such as acute liver failure or even death.¹⁰ However, eradication therapy for HBV is still unavailable, leading to great concerns about the potential consequences of HBV reactivation.

Histone deacetylase inhibitors (HDACi) are a family of

Keywords: HBV reactivation; Histone deacetylase inhibitor; Romidepsin; Vorinostat; Cell cycle.

Abbreviations: ALT/AST, alanine and aspartic aminotransferase; CCK-8, cell counting kit-8; CDK, cyclin-dependent kinase; C/EBP α , CCAAT/enhancer-binding protein α ; EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; FK228, romidepsin; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBV cccDNA, HBV covalently closed circular DNA; HBV-Tg mice, HBV-transgenic mice; HCC, hepatocellular carcinoma; HDACi, histone deacetylase inhibitors; HIV-1, reactivates human immunodeficiency virus type 1; NTCP, solute carrier family 10 member 1; PBS, phosphate-buffered saline; qRT-PCR, real-time polymerase chain reaction; SAHA, suberoylanilide hydroxamic acid; siRNA, small interfering RNA.

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natural or synthetic small-molecule inhibitors of histone deacetylases (HDACs) which are widely applied in treating disorders such as hematopoietic malignancies and psychiatric disorders in clinical trials.¹¹ However, some clinical studies have indicated that virus reactivation is one of the severe complications that occur after HDACi treatment. Romidepsin (FK228), a cyclic peptide that specifically inhibits Class I HDACs, can efficiently induce the lytic cycle reactivation of Epstein-Barr virus (EBV).^{12–14} Vorinostat (SAHA), a broad-spectrum HDACi,¹⁵ reactivates human immunodeficiency virus type 1 (commonly known as HIV-1) via activation of the PI3K/Akt pathway in infected patients receiving highly active antiretroviral therapy.^{16–19} However, the effects of FK228/SAHA on HBV replication are still unknown.

Herein, we investigated the role of two FDA-approved HDACi, FK228 and SAHA, in HBV replication *in vitro* and *in vivo*. Our results will provide useful information for further studies on chemotherapy-induced HBV reactivation, especially for patients undergoing HDACi treatment.

Methods

Antibodies and reagents

The antibodies used in this study were as follows: anti-hepatitis B core antigen (HBcAg) (B0586) and anti-HBsAg (NB100-62652) from Dako (Glostrup, Denmark) and Novus Biological (Littleton, CO, USA) respectively, anti- β -actin (BL005B) from Biosharp (Hefei, China). Antibodies to p21 (Cat. no. 2947T), p27 (Cat. no. 3686T) and p-cyclin-dependent kinase (CDK)2 (Cat. no. 2561S) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies to Rb (Cat. no. BS1310), p-Rb (Cat. no. BS4165P), cyclin A (Cat. no. BS1083), cyclin B1 (Cat. no. BS6874), cyclin E (Cat. no. BS1085), cyclin D1 (Cat. no. BS1741), CDK2 (Cat. no. BS1050), HDAC1 (Cat. no. BS5576), and HDAC2 (Cat. no. BS1162) were all from Bioworld (St. Louis Park, MN, USA).

Cell culture, transfection and viral infection

The human HCC cell lines HepG2 and HepAD38 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μ g/mL streptomycin (HyClone, Logan, UT, USA). In addition, HepAD38 cells were cultured in the presence of 500 ng/mL tetracycline to suppress HBV pregenomic RNA transcription and 500 μ g/mL G418 to maintain the stably transfected HBV genome. HepG2 cells were infected with adenovirus Ad-HBV1.3 (kindly provided by Prof. Michael Nassal, University Hospital Freiburg, Freiburg, Germany) for 12 h to sustain all processes of HBV replication. All transfections were performed by using Lipofectamine™ 3000 transfection reagent (Invitrogen, Carlsbad, CA, USA).

HepG2 cells stably expressing sodium taurocholate co-transporting polypeptide, termed as HepG2-NTCP cells, were inoculated with HBV virus, as previously described.²⁰ Briefly, the supernatants of HepAD38 cells were collected and precipitated with 8% polyethylene glycol 8000. Then, the HepG2-NTCP cells were infected with concentrated HBV virus for 16 h in the presence of 4% polyethylene glycol 8000 and 1% DMSO.

Animal models

HBV-transgenic, termed as HBV-Tg mice, raised by the Laboratory Animal Center of Chongqing Medical University

(SCXK (YU) 2017-0001), were kindly provided by Prof. Ning-shao Xia from the School of Public Health (Xiamen University, Xiamen, China). Mice (6–8 weeks-old, $n=6$ for each group) were intraperitoneally injected with FK228, SAHA (2.5 mg/kg, 40 mg/kg body weight, respectively) or phosphate-buffered saline (PBS; control) every other day for seven times. At the 14th-day after injection, all mice were euthanized. Then, mice serum and liver tissue specimens were collected for RT-qPCR, Southern blotting, and immunohistochemical staining. All the animal procedures were conducted in compliance with the protocols approved by the Laboratory Animal Center of Chongqing Medical University, following the national guidelines and regulations for experimental animal use and welfare of China.

Chemical inhibitors and small interfering RNAs

The HDAC1 and HDAC2 inhibitors romidepsin (FK228) and the broad-spectrum HDAC activity inhibitor vorinostat (SAHA) were purchased from Selleckchem (Houston, TX, USA). Both of the chemicals were dissolved in DMSO and stored at -20°C . Small interfering RNAs (siRNAs) were obtained from TranSheep Bio (Shanghai, China). The siRNA sequences targeting human HDAC1, HDAC2 are listed in Supplementary Table 1. Scrambled siRNA was used as a control. Cells were transfected with specific or non-specific control siRNAs at a concentration of 20 μM by Lipofectamine™ 3000 (Invitrogen) according to the manufacturer's protocol.

Cell growth curve and cell viability assay

The proliferation capacity of HepAD38 and HepG2 cells was measured by using a cell growth curve. Cells were seeded into 96-well plates (2,000–3,000 cells/well), with three replicate wells per group. Then, the cells were incubated with various concentrations of FK228 (0, 0.5, 1, 2.5, 5, 10, 20 and 40 nM) and of SAHA (0, 0.25, 0.5, 1, 2.5, 5, 10 and 20 μM) for 120 h. Cell number was enumerated automatically every 24 h, and the growth curve was plotted. The cell viability was measured by the CCK-8 cell counting kit-8 assay (Dojindo Molecular Technologies Inc., Kumamoto, Japan). Cells were seeded into 96-well culture plates for 12 h with three replicate wells per group, then various concentrations of FK228 and of SAHA were added to the cells for 120 h. The absorbance at 450 nm was measured after the treatment with 10 μL of CCK-8 for 1 h.

Quantification of HBV DNA via RT-qPCR

RT-qPCR was performed to detect the HBV DNA copies. Cells or liver tissues were first lysed at 37 $^{\circ}\text{C}$ for 30 m with cell lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 2% sucrose, and 1% NP-40). Then, the mixture was centrifuged at 13,000 $\times g$ for 5 m and the supernatant was treated with micrococcal nuclease (Cat. no. 70196Y; Affymetrix, Santa Clara, CA, USA) and CaCl_2 for 60 m at 37 $^{\circ}\text{C}$ to eliminate residual DNA. Then, EDTA was used to terminate the reaction. A 35% polyethylene glycol 8000 solution was used for precipitation and a 0.5 mg/mL proteinase K solution (Cat. no. 3115879001; Roche Diagnostics GmbH, Mannheim, Germany) was used for digestion of viral DNAs at 45 $^{\circ}\text{C}$ for 12 h. Nucleic acids were purified via phenol:chloroform:isoamyl alcohol (25:24:1) extraction three times and precipitated with ethanol. Then, the SYBR Green qPCR Master Mix (Bio-Rad, Hercules, CA, USA) was used to perform qPCR with the indicated primers (Supplementary Table 1). The pCH9/3091 plasmid (containing 1.1 copies of HBV genome) served as a

template for the standard curve.

Quantification of HBV cccDNA via RT-qPCR

Cells or liver tissues were lysed at 37 °C for 20 m with cell lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 150 mM NaCl, 1% SDS), and then the lysate was incubated with 2.5 M KCl overnight. After centrifugation at 14,000 × *g* for 30 m, the supernatant was extracted by phenol:chloroform:isoamyl alcohol (25:24:1) three times and precipitated with isopropanol. The extraction was treated with Plasmid-Safe ATP-Dependent DNase (Epicenter, Madison, WI, USA) to remove double-stranded DNA, and then the RT-qPCR was performed with the indicated primers (Supplementary Table 1) to determine HBV cccDNA.

RNA isolation and RT-qPCR

Total RNA was extracted using TRIzol (Invitrogen) and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (A3500; Promega, Madison, WI, USA), in accordance with the manufacturer's instructions. RT-qPCR was performed to quantify mRNA levels, using the SYBR Green qPCR Master Mix (Bio-Rad) and a Bio-Rad CFX Connect Real-time PCR Detection System (Bio-Rad), according to the manufacturer's instructions. HBV 3.5-kb mRNA was standardized to genomic β -actin. The primer sequences are listed in Supplementary Table 1.

Southern blotting

Southern blotting was performed as previously described.²¹ Briefly, extracted DNA samples were separated via electrophoresis in 1% agarose gel. After denaturation in a solution of 0.5 M NaOH and 1.5 M NaCl, and neutralization in a solution of 1 M Tris-HCl (pH 7.4) and 1.5 M NaCl, the DNA fragments were transferred onto a nylon membrane (Cat. no. 11417240001; Roche Diagnostics GmbH). Then, the membrane was fixed via ultraviolet-crosslinking. A digoxigenin-labeled full-length HBV genome probe (Digoxigenin High Prime DNA Labeling and Detection Starter Kit; Roche Diagnostics GmbH) was used to detect HBV DNA via hybridization.

Western blotting

For SDS-PAGE and immunoblotting, cells or liver tissues were lysed in whole cell lysis buffer (Cat. no. P0013; Beyotime, Nantong, China). The protein concentration of the homogenates was measured by BCA protein assay (Cat. no. BCA02; Dingguo, Beijing, China). Then, the protein samples were boiled at 100 °C for 10 m. The boiled protein samples were subjected to gel electrophoresis and then were electrotransferred to polyvinylidene difluoride membranes (Cat. no. IPVH00010; Millipore, Billerica, MA, USA). The immunoblots were incubated at 4 °C overnight with primary antibodies. Horseradish peroxidase-coupled secondary antibodies (Abcam, Cambridge, UK) were applied on the 2nd day. At last, the blots were visualized by using Clarity Western ECL Substrate (Bio-Rad).

Detection of HBV antigen, alanine and aspartic aminotransferase (ALT/AST)

Quantification of hepatitis B surface antigen (HBsAg) and

hepatitis B e-antigen (HBeAg) in culture supernatants and in mouse serum were assayed by ELISA kits (Kehua Bio-Engineering, Shanghai, China). Serum ALT/AST was measured with ELISA kits (Kanglang, Shanghai, China), according to the manufacturer's protocols.

Immunohistochemistry

After fixation in 4% paraformaldehyde for 24 h, liver tissue samples were embedded in paraffin according to standard procedures. The resultant sections were incubated with anti-HBcAg (Cat. no. B0586; Dako, Glostrup, Denmark) separately. Subsequently, the slides were incubated with secondary anti-rabbit IgG (Cat. no. ZB-2301; ZSGB-BIO, Beijing, China) and visualized using 3, 3'-diaminobenzidine (ZSGB-BIO). After rinsing, the samples were dehydrated, treated with xylene for transparency, and scanned with an Olympus BX61 microscope.

Flow cytometry analysis

Cells were synchronized by starvation with 1% fetal bovine serum for 72 h, after treatment with HDACi FK228/SAHA, or transfection with an siRNA; then, the cells were re-stimulated with 10% fetal bovine serum. The cells were then fixed with 70% alcohol at 4 °C overnight, and resuspended in PBS with propidium iodide and RNaseA for 30 m before application to a flow cytometry assay (FACS Calibur; BD Biosciences, San Jose, CA, USA).

Statistical analysis

Data were expressed as mean \pm standard deviation. Data were analyzed using one-way analysis of variance for multiple comparisons and Student's *t*-test for between-group comparisons. A *p*-value less than 0.05 was considered statistically significant.

Results

HDACi directly promotes HBV replication in vitro

To investigate the effects of FK228 and SAHA on HBV replication, we first explored the optimal dose of FK228 and SAHA in the stable HBV-expressing HCC cell line HepAD38 and transient HBV-replicating cells (HepG2 cells infected with AdHBV-1.3, HepG2-HBV1.3), respectively. As shown in Supplementary Fig. 1A and 1C, cell proliferation of HepAD38 and HepG2-HBV1.3 was not affected by FK228 up to a concentration of 10 nM, or SAHA up to 5 μ M. In addition, the cytotoxic effects of the two HDACi were measured by CCK-8 assay. The EC₅₀ values of FK228 and SAHA were 27.10 nM and 17.61 μ M in HepAD38 cells, and 29.59 nM and 18.18 μ M in HepG2 cells, respectively (Supplementary Fig. 1B and 1D). Thus, HepAD38 and HepG2-HBV1.3 cells were incubated with FK228 at concentrations between 1 and 5 nM, or with SAHA between 0.5 and 2.5 μ M, for 72 h to investigate their effects on HBV replication in a safe range.

We observed that FK228 and SAHA significantly stimulated HBV replication in a concentration-dependent manner (Fig. 1). Treatment with FK228 and SAHA accounted for a significant increase of HBV 3.5-kb RNA levels (Fig. 1A and Supplementary Fig. 2C), HBV cccDNA levels (Fig. 1B), and HBV DNA levels (*p* < 0.01; Fig. 1C and Supplementary Fig. 2D), as determined by qPCR. Moreover, FK228/SAHA treat-

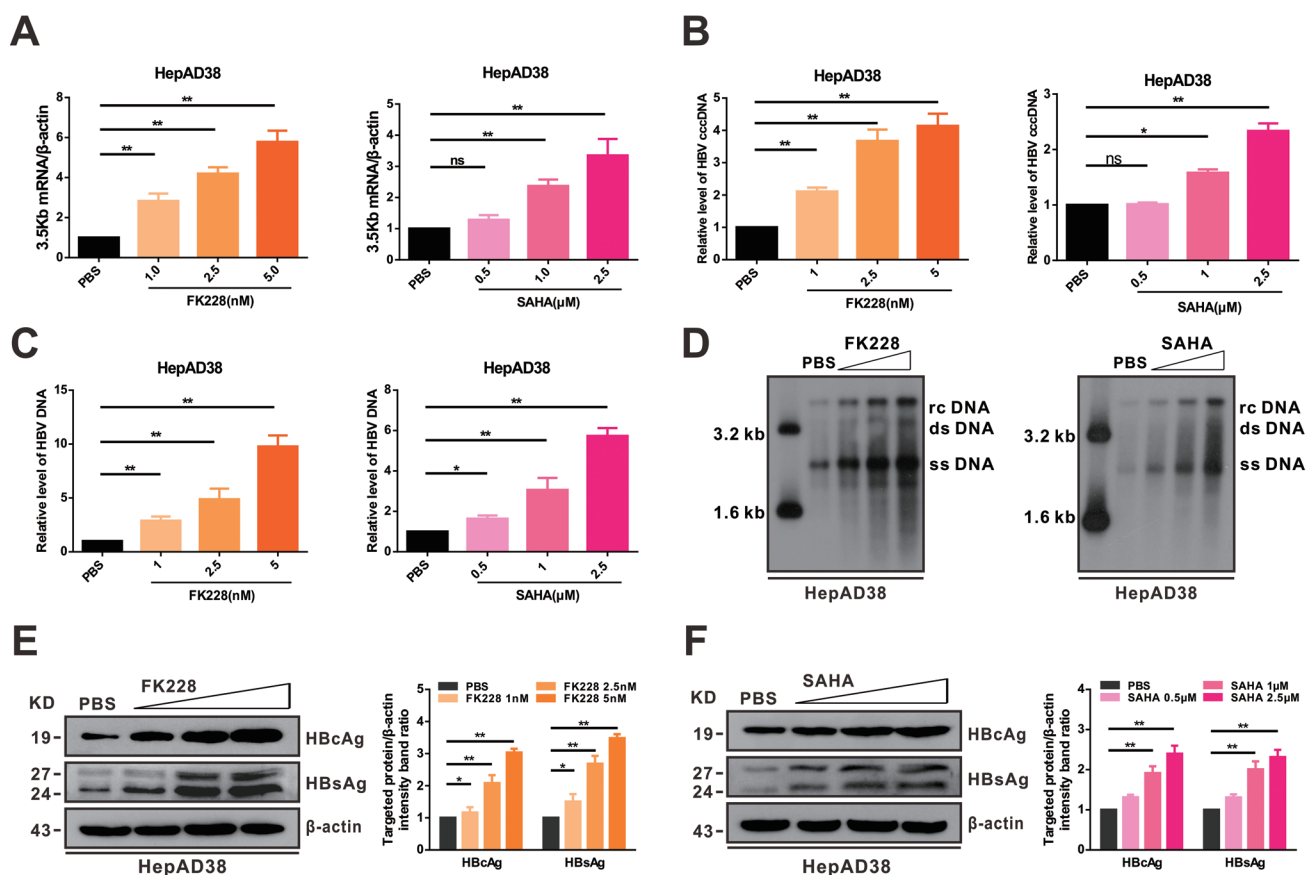


Fig. 1. HDACi enhance HBV DNA replication, viral protein production, and virion secretion. (A–C) Quantification of HBV 3.5-kb mRNA levels, intracellular HBV cccDNA levels, and HBV DNA levels by RT-qPCR assay in HepAD38 cells treated with FK228 (left) and SAHA (right). (D) Southern blotting to determine intracellular HBV replicative intermediates (RIs) treated as previously described in HepAD38 cells. rc DNA, relaxed circular DNA; ds DNA, double-stranded DNA; ss DNA, single-stranded DNA. (E–F) Western blotting to assess expression levels of HbcAg and HBsAg in HepAD38 cells treated with FK228 (left) and SAHA (right). Relative levels of HbcAg and HBsAg were measured by densitometry. Values represent the mean ± standard deviation ($n=3$, performed in triplicate), with statistical significance by comparison with PBS indicated by * $p<0.05$ and ** $p<0.01$.

ment enhanced HBV replicative intermediates (Fig. 1D and Supplementary Fig. 2F), intracellular expression of HBsAg and HbcAg (Fig. 1E, 1F and Supplementary Fig. 2E), and HBsAg and HBeAg levels in the supernatants of culture medium ($p<0.01$; Supplementary Fig. 2A–B). In addition, similar results were observed in HBV-infected HepG2-NTCP cells after HDACi treatment ($p<0.01$; Supplementary Fig. 5A–D). Interestingly, FK228 promoted HBV replication more significantly than SAHA, with approximately a 10-fold increase in HBV DNA levels compared to a 6-fold change obtained with SAHA treatment ($p<0.01$). These results demonstrate that FK228 and SAHA promote HBV replication and biosynthesis in HBV-expressing hepatoma cells.

FK228/SAHA block the cell cycle of HBV-replicating cells

HDACi contribute to apoptosis and growth arrest by inducing cell cycle arrest in cancer cells. To investigate the effects of FK228 and SAHA on cell cycle distribution during HBV replication, the HepAD38, HepG2-HBV1.3 and HBV-infected HepG2-NTCP cells were cultured with 5 nM FK228 or 2.5 μM SAHA for 72 h, respectively, and flow cytometry was performed. As shown in Fig. 2A and Supplementary Fig. 3A,

treatment with FK228/SAHA approximately induced a 2-fold decrease ($p<0.01$) in the S phase and a corresponding increase in the G1 phase in HBV-replicating cells, suggesting that FK228/SAHA treatment induced hepatocytes to stall at G1 phase and prevent cells from G1/S transition.

As mentioned earlier, FK228 is a HDAC1 and HDAC2 selective inhibitor, while SAHA is a broad-spectrum HDACi. To further elucidate the influence of HDAC1 and HDAC2 on the cell cycle in HBV-expressing hepatoma cells, siRNAs were used to lower HDAC1 and/or HDAC2 expression. As previously indicated, simultaneous silencing of HDAC1/2 significantly promoted HBV replication compared with inhibiting HDAC1 or HDAC2 alone ($p<0.01$; Fig. 3A–C, Supplementary Fig. 4A–B).

Additionally, we also examined the cell cycle-related protein levels after cells were treated with FK228/SAHA. The cyclin-dependent kinase inhibitors p21 and p27, which are key regulators of G1/S transition, were significantly upregulated (Fig. 2B, Supplementary Fig. 3B and Supplementary Fig. 5E) while the positive cell cycle regulators cyclin A, cyclin B1, cyclin D1, cyclin E, p-Rb, and p-CDK2 were apparently downregulated (Fig. 2C, Supplementary Fig. 3C, and Supplementary Fig. 5E), indicating that the cell cycle was arrested by FK228/SAHA treatment at G1 phase. Moreover, FK228 showed a more remarkable inhibitory effect on the cell cycle than SAHA; meanwhile, the siHDAC1+2 showed a

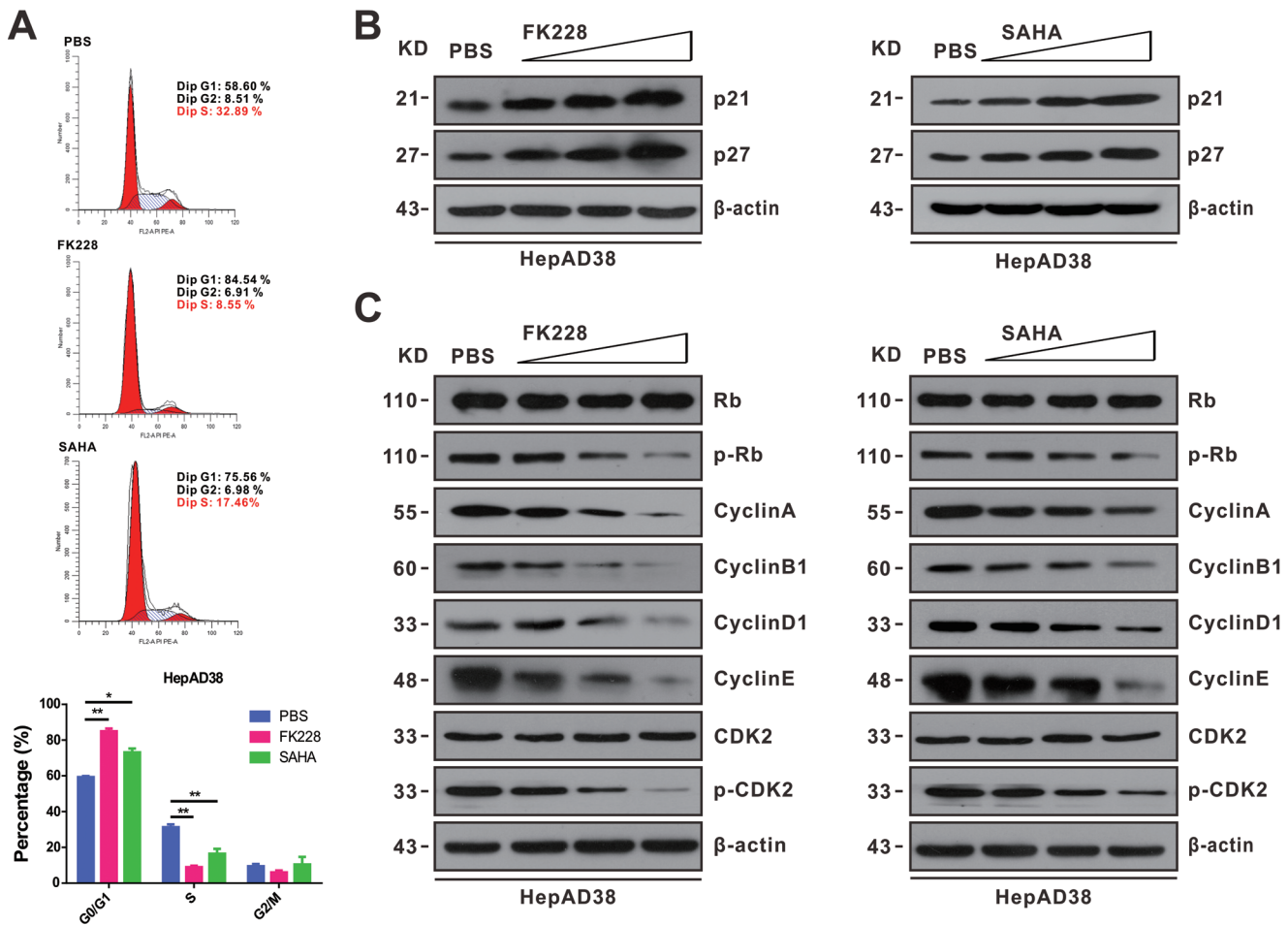


Fig. 2. Effect of HDACi on the cell cycle in HBV-expressing hepatoma cells. HepAD38 were incubated with different concentrations of FK228 (0, 1, 2.5, and 5 nM) and SAHA (0, 0.5, 1, and 2.5 μM) for 72 h. (A) Cell cycle distribution was detected by flow cytometry analysis. (B) Protein levels of CDK inhibitors p21 and p27 were detected by western blotting. (C) Protein levels of cell cycle-related proteins Rb, p-Rb, cyclin A, cyclin B1, cyclin D1, cyclin E, CDK2, and p-CDK2 were detected by western blotting. Values represent the mean ± standard deviation (n=3, performed in triplicate), with *p<0.05 and **p<0.01 vs. PBS control.

similar effect to FK228 (Fig. 3D–F and Supplementary Fig. 4C–D). Taken together, FK228/SAHA treatment, as well as transcriptional inhibition of HDAC1 and HDAC2, significantly promotes HBV replication by blocking the cell cycle at G1 phase.

FK228/SAHA promote HBV replication by inducing cell cycle arrest

Several studies have reported that HBV replication is cell cycle-dependent and highly associated with the growth status of hepatocytes.^{22,23} To further explore the relationship between enhanced HBV replication and cell cycle blockage induced by HDACi, we used siRNA to knockdown CDK inhibitors p21 and p27 to promote cell cycle conversion especially G0/G1 to S phase; then, we treated cells with FK228 or SAHA and examined HBV replication. We found that p21 and p27-knockdown significantly impaired the enhanced HBV replication induced by HDACi treatment, when compared with the negative control group which was followed with HDACi (Supplementary Fig. 6A–D). Conversely, when cells were first cultured with serum-free media to induce G0/G1 arrest and subsequently treated with FK228 or SAHA, HBV

replication showed a higher level than the serum-free group (Supplementary Fig. 6E).

FK228/SAHA enhance HBV replication in vivo

Finally, we examined the effects of FK228 and SAHA on HBV replication in the HBV-Tg mouse model. After the six HBV-Tg mice were treated with 2.5 mg/kg FK228, or 40 mg/kg SAHA or PBS (control) for 2 weeks, administration of FK228 and SAHA significantly increased serum levels of HBeAg and HBsAg secretion to varying degrees (p<0.05; Fig. 4A). Meanwhile, HBV 3.5-kb RNA, HBV cccDNA, and HBV DNA levels in liver tissues were also significantly upregulated by the FK228/SAHA treatment (p<0.01; Fig. 4C), consistent with results in the HBV-replication cell model. Furthermore, HBcAg levels in hepatocytes (examined by immunohistochemistry) also increased significantly after HDACi treatment (Fig. 4D).

A previous study had indicated that HBV reactivation after chemotherapy could generally induce liver injury.²⁴ In order to determine whether HDACi treatment could induce liver injury in HBV-Tg mice, we examined a serum inflammation marker (i.e. ALT/AST level) after FK228/SAHA treat-

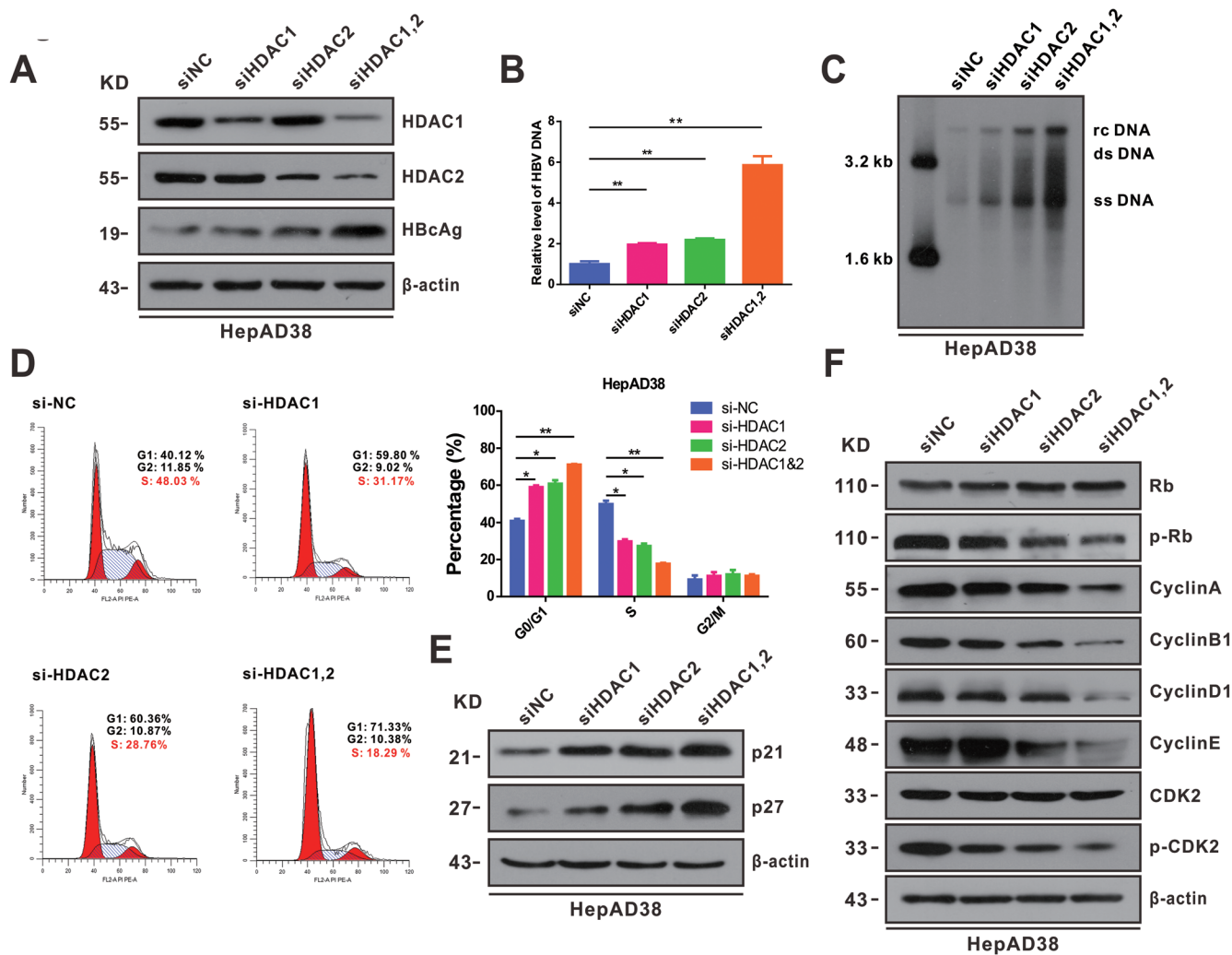


Fig. 3. Transcriptional HDAC1/2 inhibition leads to cell cycle blockage in HBV-expressing hepatoma cells. HepAD38 cells were transfected with 20 μ M siRNA duplexes against HDAC1, HDAC2, or HDAC1+2 for 72 h. (A) Protein levels of HDAC1, HDAC2, and HbcAg detected by western blotting. (B) Intracellular HBV DNA level detected by RT-qPCR assay. (C) Intracellular HBV replicative intermediates (RIs) by Southern blotting. (D) Cell cycle distribution detected by flow cytometry analysis. (E) Protein levels of p21 and p27. (F) Protein levels of Rb, p-Rb, cyclin A, cyclin B1, cyclin D1, cyclin E, CDK2, and p-CDK2. Values represent the mean \pm standard deviation ($n=3$, performed in triplicate), with $*p<0.05$ and $**p<0.01$ vs. siNC control.

ment. The ALT and AST levels were significantly elevated ($p<0.05$; Fig. 4B). Overall, administration of FK228/SAHA enhanced HBV replication and aggravated liver damage in the HBV-Tg mice. Additionally, FK228 showed a more significant damage effect than SAHA in the HBV mouse model.

Discussion

Previous studies have suggested that the immune system may be suppressed by systemic chemotherapy, leading to HBV reactivation. Moreover, it has been reported that chemotherapy can enhance the interactions between the promyelocytic leukemia protein and HBV core protein, which inhibit promyelocytic leukemia-associated HDAC activity, eventually leading to HBV exacerbation.²⁵ In fact, as early as 2009, it was reported that HDACi induce HBV reactivation and liver damage,²⁶ but the detailed molecular mechanisms remain to be explored to date. In this study, we investigated the effects of HDACi FK228 and SAHA on HBV reactivation.

Further analysis revealed that cell cycle arrest played an important role in FK228/SAHA-induced HBV replication.

The HDAC family is composed of 18 different enzymes, which are divided into four separate classes on the basis of their homology to yeast proteins.²⁷ Several HDACs are involved in HBV replication. For example, Pollicino *et al.*²⁸ found that recruitment of HDAC1 onto the HBV cccDNA can inhibit HBV replication. Moreover, inhibition of HDAC4 by miRNA-548ah can inhibit the deacetylation of histones combining with cccDNA, therefore, enhancing the replication of cccDNA.²⁹ Additionally, acetylated histone H3 also participates in HBV DNA replication.³⁰ However, the exact roles of HDACi in HBV replication are still unknown. In this study, we demonstrated that both the selective HDAC1 and HDAC2 inhibitors romidepsin and broad-spectrum HDACi vorinostat promoted HBV replication in a concentration-dependent manner *in vitro*; FK228 had a more significant effect than SAHA. We further investigated the effects of FK228 and SAHA *in vivo*, and found that these two HDACi could also induce HBV reactivation in HBV-Tg mice.

HDACi represent a new wave of anticancer drugs due to

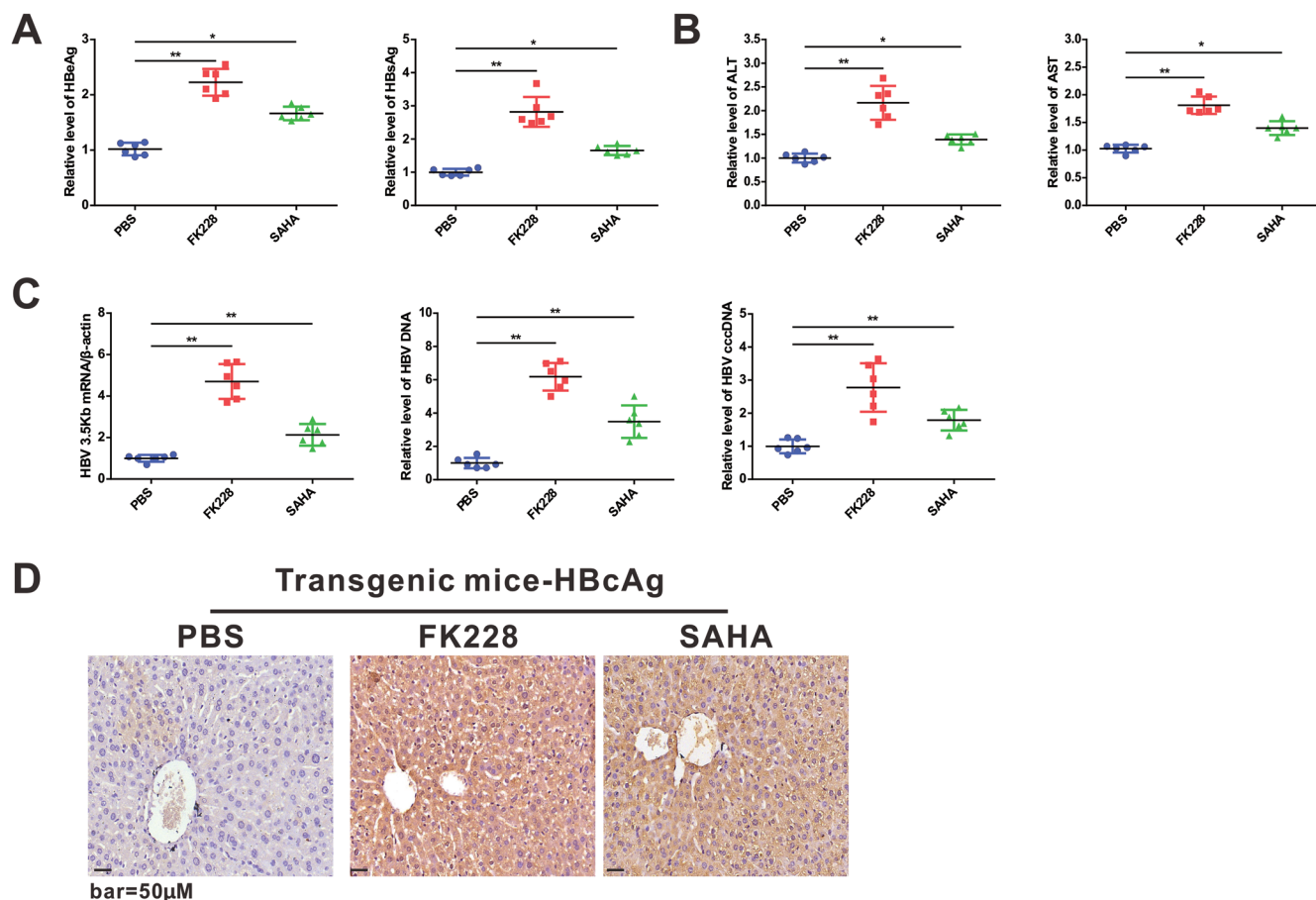


Fig. 4. Effect of HDACi in C57-HBV-Tg mice. HBV-Tg mice (6–8 weeks-old, $n=6$) were intraperitoneally injected with FK228, SAHA (2.5 mg/kg and 40 mg/kg body weight, respectively) or PBS (control) every other day for seven times. After the final injection, serum and liver tissue specimens were collected. (A–B) Quantification of HBeAg, HBsAg, and ALT/AST in mouse serum by ELISA. (C) Quantification of 3.5-kb mRNA, HBV DNA, and HBV cccDNA in liver tissues by RT-qPCR. (D) Immunohistochemistry analysis of HBcAg in liver tissues, scale bar: 50 µm. Values represent the mean ± standard deviation ($n=3$, performed in triplicate), with $*p<0.05$ and $**p<0.01$ vs. PBS control.

their biological effects, such as regulation of gene expression, cell cycle progression, and apoptosis.^{31,32} Increased expression of p21/p27 and, subsequently, cell cycle arrest are common responses to HDACi treatment.³³ Consistent with a previous study, our results showed that the expression of cyclin-dependent kinase inhibitors, such as p21 and p27, were significantly increased, while cyclin D1/E and CDK2, which are required for the G1/S checkpoint complex, were significantly decreased after FK228/SAHA treatment.

In fact, several studies have indicated that HBV replication is cell cycle-dependent and highly associated with the growth status of hepatocytes.^{22,23} For example, HBV replication was more active in quiescent cells but slowed down when cells started to divide,²³ the number of viral replicative intermediates was significantly increased after cells reached confluence,³⁴ and the chemotherapy drug vincristine could strongly stimulate HBV replication through S-phase arrest.³⁵ Therefore, our results strongly suggest that FK228/SAHA treatment enhances HBV replication mainly by stalling cell cycle progression at the G1 phase and preventing its transition to S phase. It is well known that viruses such as influenza A virus,³⁶ EBV,³⁷ and hepatitis C virus³⁸ usually utilize different strategies to deregulate cell cycle checkpoint controls, and regulate cell proliferation in order to replicate in cells and produce new progeny. HDACi-induced G1/S phase arrest might contribute to *de novo* HBV

replication before cells enter into mitosis, and regulation of some transcriptional factors which are involved in cell growth and differentiation, such as E2F transcription factor 5, CCAAT/enhancer-binding protein α (C/EBPα), hepatocyte nuclear factor 4 alpha, etc.³⁹ Increased p21 has been shown to recruit C/EBPα to the HBV promoter after doxorubicin treatment;⁴⁰ thus, FK228/SAHA-induced elevated p21/p27 expression might stimulate HBV replication by increasing the recruitment of C/EBPα or other transcriptional factors to HBV promoters. In addition, HDACi treatment has been reported to induce the depletion of uracil DNA glycosylase, which can counteract APOBEC3-induced hypermutations.⁴¹ As such, the enhanced host genome mutation may also, subsequently, help the virus to escape the immune system and evolve.⁴² The exact mechanism needs further investigation.

Recent reports have indicated that FK228 could potentially induce the lytic cycle of EBV through inhibition of HDAC1/2, which subsequently leads to G2/M phase arrest.¹⁴ Moreover, FK228 was able to induce the EBV lytic cycle at a lower concentration and showed a more significant effect than SAHA. Our results on cell cycle distribution also verified that FK228 treatment induced cell cycle arrest at a much higher degree through simultaneous inhibition of HDAC1/2, compared to the global inhibitor SAHA. This may be because, although inhibition of HDAC1/2 increases the promoter activities of

p21 and p27,⁴³ there is a limitation of specific HDAC-targeted inhibition when treated with SAHA. Therefore, FK228 treatment showed a stronger promotion of HBV replication than SAHA.

In conclusion, FK228 and SAHA significantly promoted HBV replication in a dose-dependent manner in HBV-expressing HCC cell lines and the HBV-Tg mice model. Further analysis showed that cell cycle blockage played an important role in HDACi-induced HBV reactivation. Higher HBV replication levels were found after FK228 treatment when compared with SAHA, suggesting simultaneous inhibition of HDAC1/2 had a stronger effect on the cell cycle arrest.

Conclusions

In summary, we have proposed herein a possible mechanism for FK228/SAHA-mediated HBV reactivation. According to our study's findings, FK228/SAHA induce cell cycle arrest to enhance HBV replication through the inhibition of HDAC1/2. Pharmacological or transcriptional inhibition of HDAC1/2 exhibits more significant effects than broad-spectrum inhibition of HDACs by increasing of p21/p27 and decreasing cyclins and CDKs, thereby stimulating HBV replication more robustly. Further studies in HBV-infected and reactivated animal models and clinical patients are required to verify and supplement the current information.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conception and design of the research (ZZ, XL), acquisition of the data (YY, YuY, ZC, JH), analysis and interpretation of the data (YY, YuY, ZC), drafting of the manuscript (YY, XL), materials and technical support (KW, NT), critical revision of the manuscript for important intellectual content (YY, ZZ), supervision (ZZ). All authors read and approved the final version for publication.

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