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# The sirtuin inhibitor sirtinol inhibits hepatitis A virus (HAV) replication by inhibiting HAV internal ribosomal entry site activity

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ABSTRACT

Epigenetics plays a role in the regulation of gene expression. Epigenetic changes control gene expression at the transcriptional level. Our previous study suggested that the La protein, which is mainly localized in the nucleus, was associated with hepatitis A virus (HAV) internal ribosomal entry site (IRES)-mediated translation and HAV replication. The aim of this study was to investigate whether epigenetic compounds have effects on HAV IRES-mediated translation and HAV replication. Sirtinol, a sirtuin inhibitor, inhibited HAV IRES-mediated translation in COS7-HAV-IRES cells. Treatment with 10 μM sirtinol resulted in a significant reduction in the intracellular RNA levels of HAV HA11-1299 genotype IIIA in Huh7 cells. Epigenetic treatment with a sirtuin inhibitor may represent a new treatment option for HAV infection. In conclusion, epigenetic control was involved in HAV IRES-dependent translation and HAV replication. Special attention should also be paid to underlying viral diseases in the clinical use of epigenetic treatments for malignancies.



## 1. Introduction

Hepatitis A virus (HAV) infection is a major cause of acute hepatitis and liver failure in both developing and developed countries [1–5]. In spite of the development of effective vaccines against HAV, in recent years, patients hospitalized for hepatitis A in the United States have been older and more likely to have liver diseases and other comorbid medical conditions, such as hypertension, ischemic heart disease, disorders of lipid metabolism, and chronic kidney disease [6]. The development of drugs against HAV may improve these conditions.

HAV is a single-stranded, positive-sense RNA virus that is 7.5 kb in length that belongs to the *Picornaviridae* family. The HAV genome is flanked by a 5′ non-translated region (NTR) and a 3′ NTR, and it encodes structural (VP4, VP2, VP3 and VP1) and non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D) [7]. It also possesses an internal ribosomal entry site (IRES) that is a responsible for cap-independent translation initiation. The nucleotide sequence of the HAV IRES is conserved among different HAV genotypes and is consequently a candidate antiviral target [7–9].

Recently, we reported that the Janus kinase (JAK) inhibitors SD1029, AG490 and AZD1480 reduced host factor La protein expression and inhibited HAV IRES activity and HAV replication [10,11]. Thus, human La protein, which is predominantly localized in the nucleus and is associated with RNA metabolism [12], is involved in HAV IRES-dependent translation and replication [10,11], although HAV seems to replicate in the cytoplasm of hepatocytes.

Epigenetic compounds such as histone deacetylase (HDAC) inhibitors have effects on the transcription of many genes and have also been shown to have anti-cancer [13] and/or anti-viral properties [14]. In the present study, we investigated the effects of epigenetic compounds on HAV IRES-dependent translation and HAV replication.

## 2. Materials and methods

### 2.1. Cell lines and reagents

The human hepatoma cell line Huh7 and the African green monkey kidney cell line COS7 were maintained in Dulbecco's

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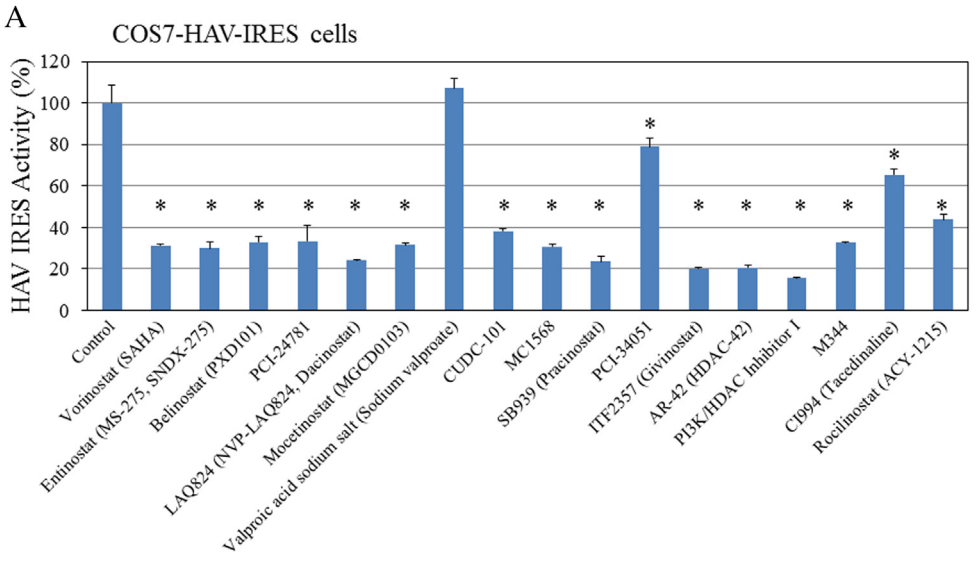
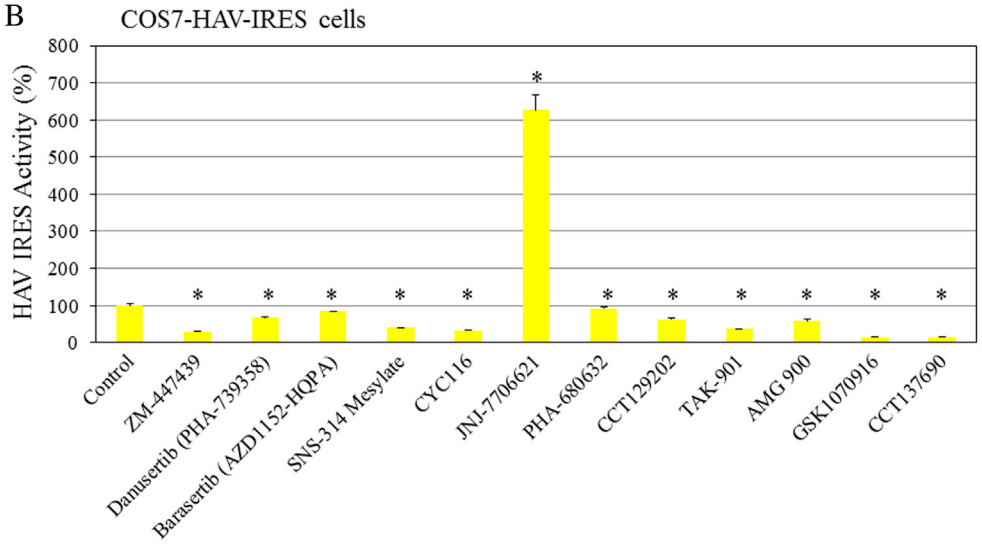

A
COS7-HAV-IRES cells
HAV IRES Activity (%)
120
100
80
60
40
20
0
Control
Vorinostat (SAHA)
Entinostat (MS-275, SNDX-275)
Belinostat (PXD101)
PCI-24781
LAQ824 (NVP-LAQ824, Dacinostat)
Mocetinostat (MGCD0103)
Valproic acid sodium salt (Sodium valproate)
CUDC-101
MC1568
SB939 (Pracinostat)
PCI-34051
ITF2357 (Givinostat)
AR-42 (HDAC-42)
PI3K/HDAC Inhibitor I
M344
CI994 (Tacedinaline)
Rocilinostat (ACY-1215)


B
COS7-HAV-IRES cells
HAV IRES Activity (%)
800
700
600
500
400
300
200
100
0
Control
ZM-447439
Danusertib (PHA-739358)
Barasertib (AZD1152-HQPA)
SNS-314 Mesylate
CYC116
JNJ-7706621
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AMG 900
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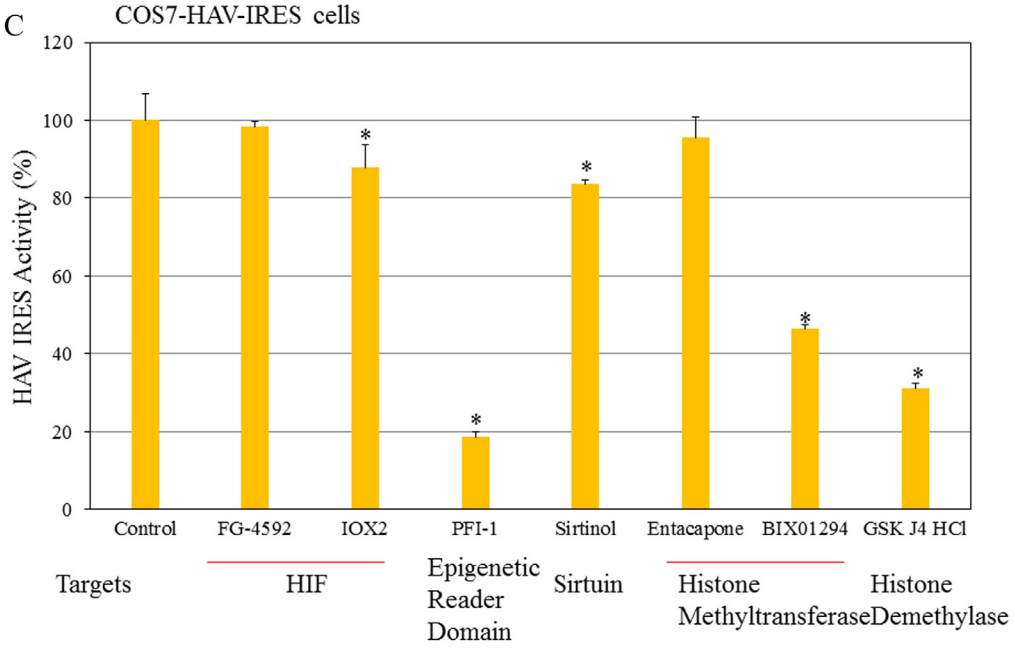
C
COS7-HAV-IRES cells
HAV IRES Activity (%)
120
100
80
60
40
20
0
Control
FG-4592
IOX2
PFI-1
Sirtinol
Entacapone
BIX01294
GSK J4 HCl
Targets
HIF
Epigenetic Reader Domain
Sirtuin
Histone Methyltransferase
Histone Demethylase

modified Eagle's medium (DMEM) (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Thermo Fisher Scientific, Yokohama, Japan), 100 units/mL penicillin and 100 μg/mL streptomycin (Thermo Fisher Scientific) at 37 °C in a 5% $CO_2$ atmosphere. COS7-HAV-IRES cells, which stably expressed pSV40-HAV-IRES, were used for the measurement of HAV IRES activity as previously described [11]. Epigenetic compounds were purchased from Selleckchem (Houston, TX, USA) (Supplementary Table 1). Interferon α-2a and amantadine were purchased from Sigma (St. Louis, MO, USA).

### 2.2. Infection with HAV into Huh7 cells

HAV infection was performed as previously described [10]. Briefly, Huh7 cells were incubated with or without the various aforementioned reagents for 24 h prior to infection at a density of $1 \times 10^5$ cells/well in 12-well plates (AGC Techno Glass, Shizuoka, Japan) or $1 \times 10^6$ cells/well in 6-well plates (Sigma). The cells were washed twice with PBS and infected with the HAV HA 11-1299 genotype IIIA strain at a multiplicity of infection (MOI) of 0.05–0.1 in DMEM supplemented with 2% FCS [10,11]. After 24 h of infection, the cells were washed three times with PBS, followed by the exchange of DMEM supplemented with 2% FCS. After 96 h of infection, total cellular RNA was extracted for the evaluation of HAV RNA.

### 2.3. RNA extraction and quantitation of HAV RNA

Total cellular RNA was extracted using an RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized using the Prime Script RT reagent (Perfect Real Time; Takara, Otsu, Japan). Reverse transcription was performed at 37 °C for 15 min, followed by 85 °C for 5 s. For HAV RNA quantification, the following primer set was used: sense primer 5′-AGGCTACGGGTGAAACCTCTTA-3′ and antisense primer 5′-GCCGCTGTTACCCTATCCAA-3'. For GAPDH RNA quantification, the following primer set was used: sense primer 5′-ACCCACTCCTCCACCTTTG-3′ and antisense primer 5′-CTCTTGTGCTCTTGCTGGG-3' [10,11]. Real-time PCR was performed with Power SYBR Green Master Mix (Applied Biosystems, Tokyo, Japan) on a StepOne Real-Time PCR system (Applied Biosystems). The PCR reaction was performed as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Specificity was confirmed by melting curve analysis. Data analysis was based on the ddCt method [11].

### 2.4. Reporter assay

For the determination of HAV IRES activity, $1 \times 10^5$ cells/well were seeded into 6-well plates (Sigma) with or without various reagents as outlined above (Supplementary Table 1). After 48 h of incubation, cells were lysed with reporter lysis buffer (Toyo Ink, Tokyo, Japan), and luciferase activity was determined with a Luminescencer JNR II AB-2300 (ATTO, Tokyo, Japan) [11]. All samples were run at least three times.

### 2.5. Evaluation of cell viability

Dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays were used to evaluate cell growth and cell viability with the CellTiter 96 Aqueous One-Solution cell proliferation assay (Promega, Madison, WI, USA) [11]. Enzyme activity was measured at a wavelength of 490 nm on a Bio-Rad iMark microplate reader (Bio-Rad, Hercules, CA, USA).

### 2.6. Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using Student's t-test with the Excel statistics program for Windows, version 7 (SSRI, Tokyo, Japan). P values of less than 0.05 were considered significant.

## 3. Results

### 3.1. Effects of epigenetic compounds on HAV IRES activity in COS7-HAV-IRES cells

To investigate the effects of epigenetic compounds on HAV IRES-dependent translation, we selected 36 epigenetic compounds, including reagents that had already been approved by the United States Food and Drug Administration, as follows: 17 HDAC inhibitors, 12 Aurora kinase inhibitors, 2 hypoxia inducible factor (HIF) inhibitors, 1 epigenetic reader domain inhibitor, 1 sirtuin inhibitor, 2 histone methyltransferase inhibitors and 1 histone demethylase inhibitor (Supplementary Table 1). First, COS7-HAV-IRES cells were treated with or without these reagents, and after 48 h of treatment HAV IRES activity was determined by a reporter assay. Among the tested compounds, at a concentration of 10 μM for each reagent, 32 drugs significantly down-regulated HAV IRES activity, and only one reagent, JNJ-7706621, significantly up-regulated HAV IRES activity (Fig. 1A–C).

### 3.2. Effects of 9 epigenetic compounds on HAV replication in Huh7 cells

Due to cell viability concerns, we chose to evaluate the effects of 9 epigenetic compounds (belinostat, PCI-24781, LAQ824, valproic acid sodium salt, PCI-34051, JNJ-7706621, FG-4592, sirtinol and entacapone) on HAV replication. Next, we determined whether these drugs had effects on HAV replication in Huh7 cells. Huh7 cells were incubated with or without these reagents for 24 h prior to infection at a density of $1 \times 10^5$ cells/well in 12-well plates. Then, the cells were infected with HAV at a multiplicity of infection (MOI) of 0.1. Real-time PCR suggested that 10 μM sirtinol could lead to a 67% reduction in HAV replication compared to that of untreated controls at 96 h of infection (Fig. 2). Fig. 1C also shows that 10 μM sirtinol inhibited HAV IRES activity at 83.6% of the level of the untreated controls. We observed that 10 μM JNJ-7706621 could lead to a 541% increase in HAV replication compared to that of untreated controls (Fig. 2). Of interest, 10 μM JNJ-7706621 enhanced HAV IRES activity at 627% of the level of the untreated controls (Fig. 1B).

### 3.3. Effects of sirtinol on Huh7 cell viability

The cytotoxicity of sirtinol treatment in Huh7 cells was determined using an MTS assay (Fig. 3). Cell viability was not affected by supplementation with 0.1–50 μM sirtinol. These results suggest that 10 μM sirtinol could safely inhibit HAV replication.

**Fig. 1.** Effects of epigenetic compounds on hepatitis A (HAV) internal ribosomal entry site (IRES) activity in COS7-HAV-IRES cells. (A) Histone deacetylase (HDAC) inhibitors, (B) aurora kinase inhibitors, (C) hypoxia inducible factor (HIF) inhibitors, epigenetic reader domain inhibitor, sirtuin inhibitor, histone methyltransferase inhibitors and 1-histone demethylase inhibitor. All drug concentrations are 10 μM. Data are expressed as the mean ± SD. *P < 0.05, compared with the untreated control by Student's t-test.

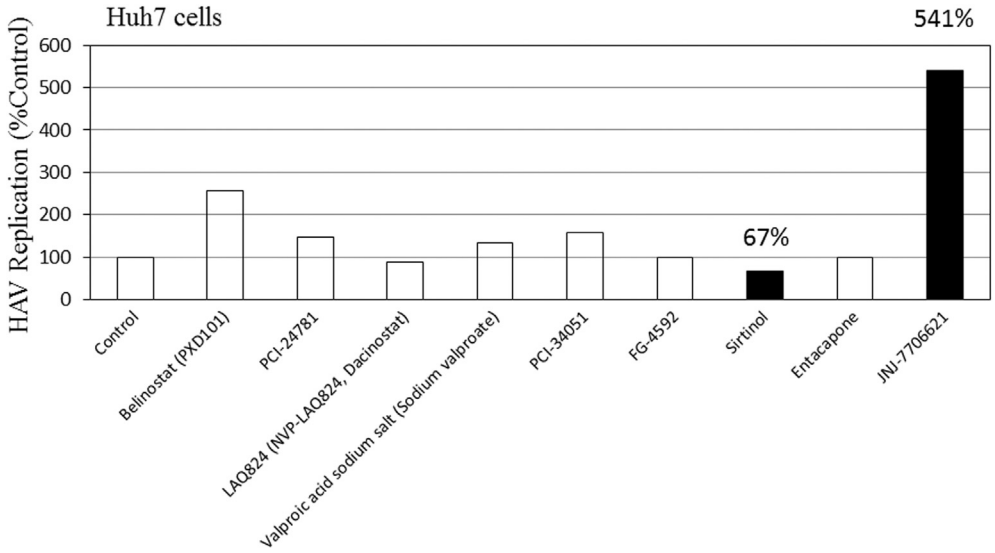


**Fig. 2.** Effects of 9 epigenetic compounds on HAV replication in Huh7 cells. Cells were treated with or without indicated reagents for 24 h before HAV infection. Cellular RNA was extracted and subjected to real-time RT-PCR 96 h after HAV HA 11-1299 genotype IIIA strain infection at a multiplicity of infection (MOI) of 0.1. All experiments were performed in triplicate.

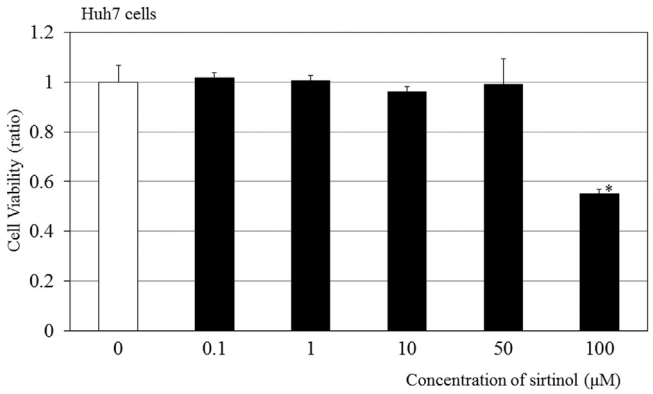


**Fig. 3.** Effects of sirtinol on cell growth and viability. MTS assays of cells 24 h after treatment with sirtinol in Huh7 cells. Data are expressed as the mean ± SD. *P < 0.05, compared with untreated control by Student's t-test. All experiments were performed in triplicate.

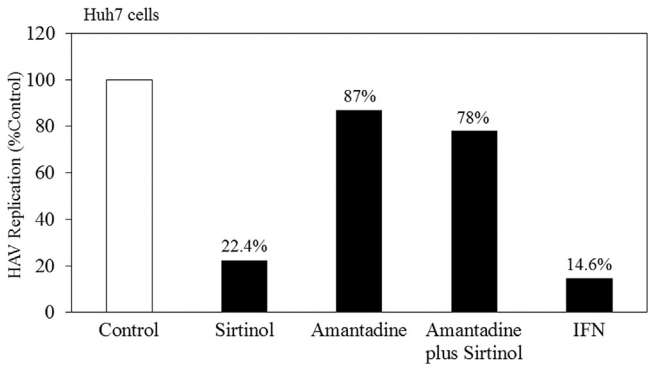


**Fig. 4.** Effects of sirtinol with or without amantadine on HAV replication in Huh7 cells. Cells were treated with or without indicated reagents for 24 h before HAV infection. Cellular RNA was extracted and subjected to real-time RT-PCR 96 h after HAV HA 11-1299 genotype IIIA strain infection at a multiplicity of infection (MOI) of 0.05. All experiments were performed in triplicate. The concentrations of sirtinol, amantadine and interferon (IFN) α-2a were 10 μM, 50 μg/mL and $1 \times 10^5$ IU/mL, respectively.

### *3.4. Effects of sirtinol with or without amantadine on HAV replication in Huh7 cells*

Next, we performed an infectivity assay using the virus to investigate the effects of the combination of amantadine and sirtinol on HAV propagation in Huh7 cells (Fig. 4). Huh7 cells were incubated with or without these reagents for 24 h prior to infection at a density of $1 \times 10^6$ cells/well in 6-well plates. Then, the cells were infected with HAV at a multiplicity of infection (MOI) of 0.05. Unfortunately, we did not observe enhanced suppression of HAV replication with the combination of amantadine and sirtinol.

## 4. Discussion

The present study suggested that certain epigenetic compounds have effects on HAV IRES-dependent translation and/or HAV replication, even though HAV is an RNA virus and replicates in the cytoplasm. These results also suggested that epigenetic control is involved in HAV IRES-dependent translation as well as in HAV replication.

Sirtinol, with a molecular weight of 394.47, could specifically inhibit sirtuin 1 (SIRT1) and SIRT2 at IC50 values of 131 μM and 38 μM, respectively. Both SIRT1 and SIRT2 are involved in telomere maintenance and hepatocyte growth [15]. Sirtinol suppressed hepatitis B virus (HBV) DNA replication intermediates as well as 3.5 kb mRNA at concentrations between 12.5 and 50 μM [16]. The HBV genome is primarily composed of double-stranded DNA. Because HBV utilizes covalently closed, circular DNAs as templates for HBV DNA replication in the nucleus, it is easy to understand the involvement of epigenetic mechanisms during HBV replication. Sirtinol also induced apoptosis at concentrations between 25 and 50 μM in human T-cell leukemia virus (HTLV-1)-related cell lines [17].

Sato et al. [14] reported that the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) inhibited hepatitis C virus (HCV) replication, which is a single-stranded RNA virus that replicates in the cytoplasm. They also reported that SAHA had a suppressive effect on HCV replication through alterations in the gene expression levels of osteopontin and apolipoprotein-A1 in host cells [14]. Our previous studies [10,11] suggested that La, which is mainly localized

in the nucleus, was involved in HAV IRES-dependent translation and HAV replication. In the present study, sirtinol had an inhibitory effect on HAV IRES-dependent translation and HAV replication at a concentration of 10 μM (Figs. 1C and 2). Sirtinol might interact with factors that are localized in the nucleus. Further studies will be needed to confirm this hypothesis.

Unexpectedly, we also found that JNJ-7706621, which has a molecular weight of 394.36, had an enhanced effect on HAV IRES-dependent translation and HAV replication at a concentration of 10 μM (Figs. 1C and 2). JNJ-7706621 could inhibit cyclin-dependent kinases (CDK) and aurora kinases [18]. It has also been reported that JNJ-7706621 delayed G1 phase in HeLa cells [18]. Huh7 cells in G1 phase produced greater amounts of infectious HAV than cells in G0 or G2/M phase. Our results may support previous reports [19]. It is well known that HBV reactivation is a common event in both HBsAg-positive and HBsAg-negative patients treated with anti-cancer therapies or corticosteroids [20,21]. As epigenetic treatments have already been used in the treatment of some malignancies [22], special attention should be paid to viral reactivation during the clinical use of reagents such as JNJ-7706621.

Epigenetics plays a role in the regulation of gene expression [23]. Epigenetic changes, such as DNA methylation, histone methylation or acetylation, control gene expression at the transcriptional level [24,25]. In conclusion, certain epigenetic compounds have effects on HAV IRES activity and/or HAV replication. The present study revealed that epigenetic control was involved in HAV IRES-dependent translation and HAV replication.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.09.083.

## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.09.083.

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