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Distinct dual antiviral mechanism that enhances hepatitis B virus mutagenesis and reduces viral DNA synthesis

Yumeng Z. McDaniel[a,b], Steven E. Patterson[b,f], Louis M. Mansky[a,b,c,d,e,f,*]

[a] Veterinary Medicine Graduate Program, University of Minnesota-Twin Cities, Minneapolis, MN, 55455, USA
[b] Institute for Molecular Virology, University of Minnesota-Twin Cities, Minneapolis, MN, 55455, USA
[c] Division of Basic Sciences, University of Minnesota School of Dentistry, Minneapolis, MN, 55455, USA
[d] Masonic Cancer Center, University of Minnesota-Twin Cities, Minneapolis, MN, 55455, USA
[e] Department of Microbiology & Immunology, University of Minnesota-Twin Cities, Minneapolis, MN, 55455, USA
[f] Center for Drug Design, University of Minnesota-Twin Cities, Minneapolis, MN, 55455, USA

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ABSTRACT

Reverse transcriptase (RT) is an essential enzyme for the replication of retroviruses and hepadnaviruses. Current therapies do not eliminate the intracellular viral replication intermediate termed covalently closed circular (ccc) DNA, which has enhanced interest in hepatitis B virus (HBV) reverse transcription and cccDNA formation. The HBV cccDNA is generated as a plasmid-like episome in the host cell nucleus from the protein-linked relaxed circular (rc) DNA genome in incoming virions during HBV replication. The creation of the cccDNA via conversion from rcDNA remains not fully understood. Here, we sought to investigate whether viral mutagens can effect HBV replication. In particular, we investigated whether nucleoside analogs that act as viral mutagens with retroviruses could impact hepadnaviral DNA synthesis. We observed that a viral mutagen (e.g., 5-aza-2′-deoxycytidine, 5-aza-dC or 5-azacytidine, 5-aza-C) severely diminished the ability of a HBV vector to express a reporter gene following virus transfer and infection of target cells. As predicted, the treatment of 5-aza-dC or 5-aza-C elevated the HBV rcDNA mutation frequency, primarily by increasing the frequency of G-to-C transversion mutations. A reduction in rcDNA synthesis was also observed. Intriguingly, the cccDNA nick/gap region transcription was diminished by 5-aza-dC, but did not enhance viral mutagenesis. Taken together, our results demonstrate that viral mutagens can impact HBV reverse transcription, and propose a model in which viral mutagens can induce mutagenesis during rcDNA formation and diminish viral DNA synthesis during both rcDNA formation and the conversion of rcDNA to cccDNA.

1. Introduction

Nearly 257 million people worldwide are chronically infected with hepatitis B virus (HBV), a number 8 times greater than the number of people infected with human immunodeficiency virus type 1 (HIV-1) (Choi et al., 2018; Hutin et al., 2018). Chronic HBV infection can develop into serious liver disease, including cirrhosis or liver cancer. Although infection is preventable by vaccination and some antiviral treatments are available, there are up to 1 million deaths per year from HBV-associated hepatocellular carcinoma and complications of cirrhosis. The currently approved therapeutics for chronic HBV infection cannot cure HBV infection and efficiently eliminate HBV covalently closed circular (ccc) DNA (Lucifora and Protzer, 2016; Tang et al., 2017). Therefore, there is a need for basic research to better understand HBV biology, in order to help develop effective drug treatments. Since antiviral drug development is a time-consuming and costly process, drug repositioning has been applied for treatment of many conditions, which has been utilized to identify potential anti-HIV-1 drug treatments (Ashburn and Thor, 2004; Clouser et al., 2010). Decitabine (5-aza-dC), 5-azacytidine (5-aza-C) and gemcitabine, commercially used as anticancer drugs, are reported to have anti-HIV-1 activity, mainly through targeting reverse transcription (Clouser et al., 2010, 2011; Dapp et al., 2009). Reverse transcription is a characteristic step for the replication life cycle of retroviruses. Importantly, reverse transcription is also a crucial step in the life cycle of hepadnaviruses (Tang et al., 2017). HBV is a hepadnavirus and upon infection of a permissive target cell, the rcDNA enters into the nucleus, and is converted into covalently closed circular (ccc) DNA, which exists as a minichromosome that can transcribe HBV mRNAs. These HBV mRNAs are translated into viral proteins, including reverse transcriptase (RT). The pre-genomic RNA

* Corresponding author. Institute for Molecular Virology, University of Minnesota-Twin Cities, Minneapolis, MN, 55455, USA.
E-mail address: mansky@umn.edu (L.M. Mansky).

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List of abbreviations

5-aza-C	5-azacytidine
5-aza-dC	decitabine
AML	acute myeloid leukemia
cccDNA	covalently closed circular DNA
HBV	hepatitis B virus
HIV-1	human immunodeficiency virus type 1
pgRNA	pre-genomic RNA
rcDNA	relaxed circular DNA
RT	reverse transcriptase
RNRI	ribonucleotide reductase inhibitors

(pgRNA) and RT are packaged into HBV capsids. Reverse transcriptase (RT) activity generates a minus DNA strand synthesized from the pgRNA, which allows the synthesis of plus strand DNA to produce the rcDNA. Two nucleoside reverse transcriptase inhibitors (NRTIs), lamivudine (3TC) and tenofovir, have been used for the treatment of both HIV-1 and HBV infection. Given the similarities between HIV-1 and HBV reverse transcription, we hypothesized that 5-aza-dC, 5-aza-C and gemcitabine may target HBV reverse transcription.

5-aza-dC and 5-aza-C are cytidine analogs that are clinically approved for the treatment of myelodysplastic syndromes and acute myeloid leukemia (AML). Initial reports indicated that 5-aza-C and 5-aza-dC were found to inhibit HIV-1 infection in cell culture (Bouchard et al., 1990). More recent studies have shown that 5-aza-dC and 5-aza-C can induce G-to-C hypermutation in HIV-1, increasing the mutational load and extinguishing viral infectivity (Dapp et al., 2009; Rawson et al., 2015). Furthermore, 5-aza-C was reported to enhance HIV-1 mutagenesis by reduction of 5-aza-C to 5-aza-dC (Rawson et al., 2016). While the high rates of mutation can enable immune evasion and drive the emergence of drug resistance, quasispecies theory predicts that there is a maximum “error threshold” above which genetic information is not maintained, thus leading to virus extinction (i.e., production of non-infectious virus particles). This type of collapse in a viral population infectivity can be achieved through the use of viral mutagens, via lethal mutagenesis (Domingo et al., 2012). While hepatitis C virus, poliovirus, vesicular stomatitis virus, influenza virus, hepatitis E virus, herpes simplex virus and HIV-1 have been shown to be susceptible to lethal mutagenesis (de Avila et al., 2016; Graci et al., 2007; Greggs et al., 2012; Jiang et al., 2016; Moreno et al., 2012; Reina and Reina, 2017; Todt et al., 2016), no studies to date have been reported for HBV.

In this study, we have observed that the viral mutagens 5-aza-dC and 5-aza-C can cause an increase in viral mutagenesis concomitantly with reduced viral DNA synthesis. In particular, our observations support a model in which viral mutagens can induce mutagenesis during rcDNA formation and diminish viral DNA synthesis during both rcDNA formation and the conversion of rcDNA to cccDNA.

2. Materials and methods

2.1. Cell lines, plasmids and drugs

The HepG2 and HepAD38 cell lines were kind gifts from Dr. Daniel D. Loeb (University of Wisconsin-Madison, Madison, WI). The HepG2-NTCP cell line was graciously provided by Dr. Haitao Guo (Indiana University, Indianapolis, IN).

The pUC1.2xHBV/NL and pUCxHBV-D plasmids were provided by Dr. Kunitada Shimotohno (National Center for Global Health and Medicine, Ichikawa, Japan). 5-aza-C (Sigma-Aldrich, St. Louis, MO), gemcitabine (TCI, Tokyo, Japan), and 3TC (NIH-ARP, Germantown, MD) were obtained from the indicated sources. 5-aza-dC divalerate (abbreviated as 5-aza-dC), a more stable prodrug form of 5-aza-dC, was synthesized by the Center for Drug Design at the University of Minnesota as described previously (Clouser et al., 2014). All drugs were dissolved and were stored in aliquots at −20 °C.

2.2. Cell culture

HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal clone 3 (FC3) serum (HyClone, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin and 100 U/ml MEM-NEAA (Life Technologies). HepAD38 cells were maintained in RPMI-1640 with 10% FC3 serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 U/ml MEM-NEAA, 10 mM HEPES, 0.4 mg/ml G418 and 0.3 μg/ml tetracycline. HepG2-NTCP cells were maintained in Dulbecco's modified Eagle's medium (DMEM)-F12 supplemented with 10% FC3, 100 U/ml penicillin, 100 μg/ml streptomycin and 8 μg/ml blasticidin (Yan et al., 2015). All cell lines were maintained at 37 °C in 5% CO_2.

2.3. Production of virus stocks

HBV/NL virus was produced as described previously (Nishitsuji et al., 2015). Briefly, the pUC1.2xHBV/NL plasmids were co-transfected with pUCxHBV-D into HepG2 cells using Lipofectamine 3000 (Life Technologies). Media was replaced one day post-transfection and cells were treated with drugs or vehicle. Cell culture supernatants were collected five days post-transfection and concentrated to 24X by using a Vivaspin 20 ml 100,000 MWCO (Sartorius) centrifuged at 1000 x *g* at 20 °C. Virus was then aliquoted and stored at −80 °C until use.

2.4. Drug treatment and virus transfer to target cells

HepG2-NTCP cells were treated with drugs 2 h before adding virus to the cell cultures. HepG2-NTCP cells were infected with HBV/NL virus in the presence of 4% PEG8000 and 2% dimethyl sulfoxide (DMSO) overnight. After 24 h, media was replaced with fresh media containing 2% DMSO and drugs. Drugs were removed and media was changed at three days post-infection. At six or seven days post-infection, cells were washed with PBS once and lysed in 1X passive lysis buffer (Promega Corp., Madison, WI). The lysate was measured for Nano luciferase activity with the NL Luciferase Assay Kit (Promega) according to the manufacturer's protocol. The HepG2-NTCP cell viability was also measured with the lysate using CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega).

2.5. Cellular proliferation

HepG2 cells (50,000 cells per well) or HepAD38 cells (4500 cells per well) were plated in a 96-well plate 24 h prior to drug treatment. Cells were treated individually or in combination for five days, and proliferation was quantified using the CellTiter-Glo® kit from Promega according to the manufacturer's instructions. Cells treated with DMSO were used as an untreated control. The data were expressed as a percentage of the untreated control to normalize for differences in luciferase activity between experiments.

2.6. qPCR of rcDNA in released viral particles

The HepAD38 cell line produces HBV particles under control of a tetracycline operator/CMV promoter (Ladner et al., 1997). Five days following removal of tetracycline and drug treatment of cells, HBV rcDNA was purified from cell culture supernatants using the E.Z.N.A. Blood DNA Mini Kit (Omega Bio-tek). The purified DNA was quantified by qPCR as previously described (Pas et al., 2000). Primers were designed to amplify an 89 bp product in the pre-S gene (F: 5′-GGA CCC

CTG CTC GTG TTA CA-3' (nucleotides 184 to 203), R: 5′-GAG AGA AGT CCA CCA CGA GTC TAG A-3' (nucleotides 273 to 249)). HBV DNA was calculated by dividing the relative DNA copy number of the drug-treated group by that of the untreated group. A series of drug concentrations were tested, and each experiment was independently repeated three times.

2.7. qPCR of cccDNA

At seven days post-infection, HepG2-NTCP cells were lysed in 50 mM Tris/0.5% NP-40/150 mM NaCl (pH 7.4) as previously described (Ni et al., 2014). Cytosolic and nuclear fractions were separated by centrifugation (4000 rpm for 5 min). HBV cccDNA was extracted from the nuclear fractions by using the E.Z.N.A. Blood DNA mini kit (Omega Bio-tek). Extracted cccDNA was treated for 2 h at 37 °C with plasmid safe DNase I (Epicentre Inc, Madison, WI), which was subsequently inactivated by incubation at 70 °C for 30 min. The extracted DNA was quantified by qPCR as described above using primers in the nick/gap region of the HBV minus-strand cccDNA generating a 308 bp product (F: 5′-CCG TGT GCA CTT CGC TTC A-3' (nucleotides 1575 to 1593), R: 5′-GCA CAG CTT GGA GGC TTG A-3' (nucleotides 1864 to 1882).

2.8. RT-qPCR of RNA from HepG2-NTCP cells infected with HBV vector

RNA was extracted from infected HepG2-NTCP cells with the High Pure RNA isolation Kit (Roche). Next, cDNA synthesis was performed using the iScript Select cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. The synthesized cDNA was diluted 10-fold and quantified by qPCR using iTaq Universal SYBR Green Supermix (Bio-Rad). RNA was quantified by both *nick/gap* gene (nucleotides 1575–1882) and pre *S* gene (nucleotides 184–249) primer sets.

2.9. DNA sequencing

A portion of the *pol/s* gene was chosen for sequencing from the rcDNA and the cccDNA, respectively. The primers used, F: 5′-ACC CTG TTC TGA CTA CTG CC-3' (nucleotides 87 to 106), and R: 5' -ACA GCG GTA AAA AGG GAC TCA-3' (nucleotides 800 to 780), were designed to overlap a region of both the polymerase and surface antigen genes and generated a product of 714 bp. The primers designed in the *nick/gap* region in cccDNA were also used as described above. Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific) was used for PCR reactions, and the initial PCR reaction was purified with QIAquick Gel Extraction Kit (Qiagen). Amplicons were verified for correct size and purity by DNA gel imaging prior to ligation into the pGEM-T vector (Promega). Plasmids were transformed into *E. coli*, and insert-containing vectors were purified (DirectPrep 96 Miniprep Kit, Qiagen) and sequenced. Sequence alignments were performed using SeqMan assembler of Lasergene 7 software package (DNAStar; Madison, WI). DMSO-treated HepAD38 or HepG2-NTCP cells were used as a untreated, negative control.

2.10. Statistical analysis

Statistical analyses were, in part, implemented with GraphPad Prism, version 5.0 (GraphPad Software, Inc., La Jolla, CA). To determine the half maximal inhibitory concentration (IC_{50}), and the 50% cytotoxic concentration (CC_{50}) of 5-aza-dC, 5-aza-C, and gemcitabine, infectivity data were normalized to the data for the untreated control, plotted against log-transformed drug concentrations, and subjected to nonlinear regression in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). The MacSynergy II program was used for isobologram analysis in order to determine the nature of drug interactions between two compounds, e.g., additive, antagonistic, or synergistic (Clouser et al., 2012; Prichard and Shipman, 1990). Data were analyzed by calculating the mean ± standard deviation. Differences between groups were analyzed by either a one-way ANOVA test or by a "N-1" Chi-squared test, as indicated.

3. Results

3.1. Treatment of HBV-producing cells with 5-aza-dC, 5-aza-C, or gemcitabine, and virus infection of target cells

Viral mutagens and ribonucleotide reductase inhibitors (RNRIs) have been shown to be effective antivirals with several viruses, but have not been tested to date against HBV. HBV is difficult to study in cell culture. To determine the effect of viral mutagens on HBV transfer to permissive cells, we used a reporter system that was recently

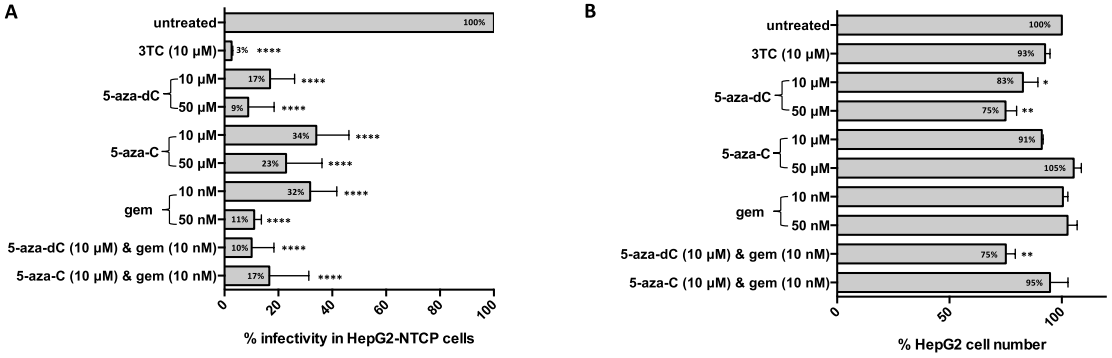

Fig. 1. Treatment of HBV-producing cells with 5-aza-dC, 5-aza-C and/or gemcitabine reduce viral infectivity. A. Analysis of virus infectivity. HepG2 cells (1.4×10^6 cells per well) were plated in a 6-well plate 24 h prior to transfection. HBV/NL vector virus is produced by the co-transfection of HBV/NL and HBV/D plasmids into HepG2 cells. One day post-transfection, the HepG2 cells were treated with 3 TC (10 μM), 5-aza-dC (10, 50 μM), 5-aza-C (10, 50 μM), gemcitabine (10, 50 nM), 5-aza-dC (10 μM) & gem (10 nM) or 5-aza-C (10 μM) & gem (10 nM). Virus was collected 5 days post-transfection. HepG2-NTCP cells (5.0×10^4 cells/well) were plated in a 96-well plate 24 h prior to infection. Next, HepG2-NTCP cells were infected with vector virus. At 6 days post infection, the infectivity was tested by using a luciferase assay and normalized to infected, untreated cells. **B. Cell proliferation analysis.** Cell proliferation was examined using the CellTiter-Glo kit from Promega according to the manufacturer's instructions. HepG2 (50,000 cells/well) cells were plated in a 96-well plate 24 h prior to drug treatment. Cells were treated with either an individual drug or a combination of two drugs for 5 days, and then cell proliferation was assessed. Cells were treated with the equivalent volume of dimethyl sulfoxide (DMSO) for the untreated group. Data is shown with the average ± standard deviation from 3 independent replicate experiments. The number in the graph bar indicates the mean value. All drug-treated groups were compared to that of the untreated group and statistical analysis is shown. A one-way ANOVA test was used; "****" = $p \leq 0.0001$; "**" = $p \leq 0.01$; "*" = $p \leq 0.05$. Abbreviation: gem = gemcitabine.

developed to look at the early stage of the HBV viral life cycle (Nishitsuji et al., 2015). The HBV/NL plasmid contains the small NanoLuc luciferase gene in place of part of the core and *pol* region of the HBV genome. The HBV/D plasmid expresses all HBV protein but is not packaged into virus particles. Therefore, virus producer cells (HepG2) are co-transfected with HBV/NL and the HBV/D plasmid, resulting in generating reporter virus that can only progress to pgRNA during virus transfer to HBV-susceptible cell line HepG2-NTCP. Virus transfer to permissive target cells can be measured by analyzing luciferase activity in these cells.

To determine if 5-aza-dC, 5-aza-C, or gemcitabine could inhibit HBV transfer, HepG2 cells were co-transfected with HBV/NL and HBV/D plasmids; one day later they were treated with 5-aza-dC, 5-aza-C, or gemcitabine at the concentrations shown in Fig. 1. Virus was collected 5 days post-transfection and used to infect the HBV-susceptible cell line HepG2-NTCP. Virus transfer was quantified at 6 days post infection using the NL Luciferase Assay Kit (Promega). As expected, we found that virus transfer of HBV/NL virus treated with 3 TC, a known RT-inhibitor, was almost completely ablated (Fig. 1A). Virus produced in the presence of 5-aza-dC, 5-aza-C, or gemcitabine also showed significant and concentration-dependent reductions in virus transfer. As RNRIs can potentiate the activity of 5-aza-dC and 5-aza-C (Clouser et al., 2010; Rawson et al., 2013), producer cells were also treated with these drugs in combination with gemcitabine. This resulted in virus that had reduced degree of virus transfer compared to either drug alone, indicating potentiation.

To exclude the possibility that the reduced expression of the NL reporter gene was caused by cytotoxicity, HepG2 cell proliferation was quantified in the presence of the drugs. Neither 5-aza-C nor gemcitabine treatment resulted in any significant cytotoxicity (Fig. 1B). Although 5-aza-dC did cause significantly decreased cell proliferation, the 25% decrease in proliferation caused by 50 μM 5-aza-dC does not account for the 91% reduction in reporter expression seen at that drug concentration. These findings indicate that 5-aza-dC, 5-aza-C, and gemcitabine are able to directly inhibit HBV virus transfer.

3.2. HBV rcDNA mutation frequency is significantly increased in the presence of 5-aza-dC, 5-aza-C, or the combination of 5-aza-dC and gemcitabine

Previous studies have shown that 5-aza-dC and 5-aza-C disrupt reverse transcription in HIV-1, inducing G-to-C and C-to-G transition mutations that lead to lethal mutagenesis, and that this can be potentiated by gemcitabine (Clouser et al., 2010; Dapp et al., 2009; Rawson et al., 2016). We thus sought to determine whether this mechanism of action also applied to HBV. As the majority of HBV reverse transcription takes place in the producer cells, we used the chronically infected HepAD38 cells, where expression of HBV particles is inhibited by tetracycline (Ladner et al., 1997). Production of virus was initiated by removing tetracycline at the same time that cells were treated with 5-aza-dC, 5-aza-C, gemcitabine, or the combination of azacytidines and gemcitabine. Supernatant containing viral particles was harvested 5 days later and HBV rcDNA was extracted from the supernatants and used as a template for Sanger Sequencing. We sequenced a portion of the *pol/s* gene (nucleotides 87–800) of rcDNA to determine if these drugs are able to increase the virus mutation frequency.

We found that 5-aza-C increased HBV rcDNA mutation frequency almost three-fold, while 5-aza-dC increased it by five-fold (Fig. 2 and Supplemental Table 1). Gemcitabine, as an RNRI, did not impact the mutation frequency of HBV rcDNA, as predicted. However, the addition of gemcitabine to 5-aza-dC increased the mutation frequency to eight times that of the untreated control, indicating that gemcitabine enhances 5-aza-dC-mediated mutagenesis. This gemcitabine-mediated enhancement in mutagenesis was not seen with 5-aza-C. As predicted, the treatment of 5-aza-dC and 5-aza-C significantly enhanced the level of G-to-C and C-to-G transition mutations (Fig. 2). Interestingly, G-to-T transition mutations were also observed at an increased frequency. These results indicate that 5-aza-dC and 5-aza-C inhibit HBV replication at least in part by increasing the mutation frequency in rcDNA.

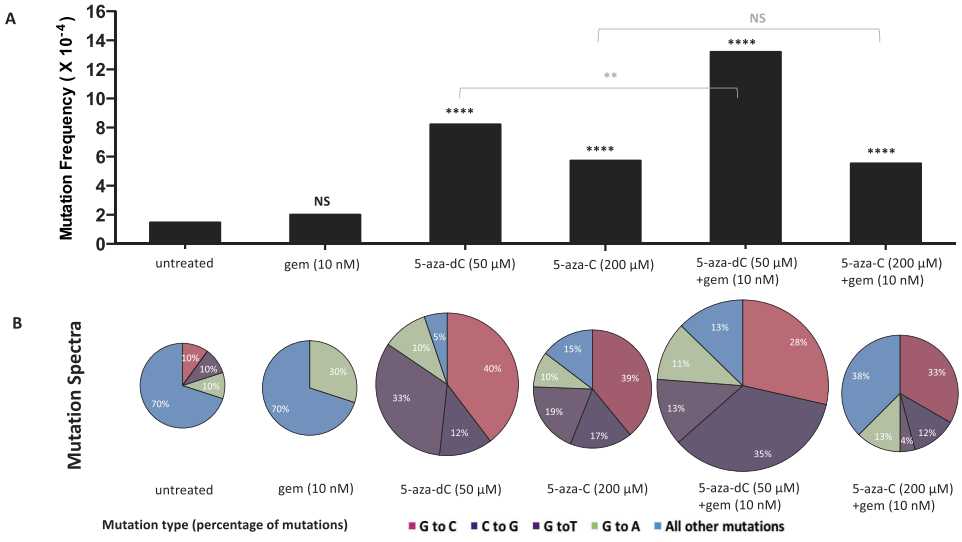

Fig. 2. Significant increase in HBV rcDNA mutation frequency in the presence of either 5-aza-dC or 5-aza-C. HepAD38 cells (1.0×10^5 cells per well) were plated in a 24-well plate 24 h prior to virus-producing induction. Five days following removal of tetracycline from cultured HepAD38 cells and cell treatment with 10 nM gemcitabine, 50 μM 5-aza-dC, 200 μM 5-aza-C or the combination of 5-aza-dC/5-aza-C with gemcitabine, HBV rcDNA was purified from cell culture supernatants. A DNA product of 714 bp (HBV sequence 87–800) was amplified by PCR. After gel purification, the 714 bp product was ligated to the pGEM-T vector. Ligated DNAs introduced into *E. coli* by transformation, and insert containing-vectors were subjected to DNA sequencing, with 70–100 colonies were sequenced per each treatment (see Supplemental Table 1). Sequence alignments were done by using SeqMan assembly tool in the Lasergene 7 software package (DNAStar; Madison, WI). **A. Mutation frequency analysis.** Mutation frequency was calculated by the number of mutations divided by the total number of bases sequenced. The mutation frequency of the untreated was 1.46×10^{-4} mutations per basepair (see Supplemental Table 1). **B. Mutation spectra analysis.** The spectra of mutations from each experimental group is depected by a pie chart, which shows the main mutation types and percentages observed. The size of the pie chart was normalized to the mutation frequency of untreated controls. Results are shown from 3 independent experiments. The gray asterisks and brackets associating any two bars indicate the significant difference between the experimental pairs. The statistical significance compared to the untreated control is shown above the bar with black asterisks. Significance was analyzed with the "N-1" Chi-squared test; "ns" = not significant; "****" = $p \leq 0.0001$; "**" = $p \leq 0.01$; "*" = $p \leq 0.05$. Abbreviation: gem = gemcitabine.

3.3. 5-aza-dC, 5-aza-C, and gemcitabine inhibit HBV rcDNA synthesis with minimal cytotoxicity and demonstrate anti-HBV synergy

To further assess the effects of 5-aza-dC, 5-aza-C, and gemcitabine on reverse transcription, rcDNA from viral particles produced from drug-treated HepAD38 cells was quantified by using qPCR. Similar to 3 TC, 5-aza-dC inhibited rcDNA synthesis with minimal cytotoxicity (Fig. 3). The half maximal inhibitory concentration (IC_{50}) of 5-aza-dC was found to be 15 μM. Inhibition of rcDNA synthesis was also observed for 5-aza-C, however, both the IC_{50} and 50% cytotoxic concentration (CC_{50}) were high (180 μM and 411 μM, respectively) (Fig. 3). Gemcitabine caused inhibition of rcDNA synthesis with a 47 nM IC_{50} (Fig. 3). Gemcitabine and 5-aza-dC or 5-aza-C have previously been observed to possess synergistic antiviral activity against HIV-1 (Clouser et al., 2010). To determine if drug synergy occurs with HBV, HepAD38 cells were treated with increasing concentrations of gemcitabine (1–100 nM) and 5-aza-dC (1–50 μM). The addition of 1 nM gemcitabine to 5-aza-dC treatment did not have a significant difference on antiviral activity compared to individual 5-aza-dC treatment, while an increased inhibition of rcDNA synthesis was observed in combinations with gemcitabine concentrations over 5 nM (Fig. 4A). MacSynergy II plot analysis confirmed the synergistic activity of gemcitabine and 5-aza-dC against HBV rcDNA synthesis (Fig. 4B). This synergy was also observed between 5-aza-C and gemcitabine (Fig. 5A and B). In particular, the combination of 5 nM gemcitabine with 100 μM 5-aza-dC led to greater inhibition of rcDNA synthesis than that caused by the same amount of each drug individually. Moreover, cytotoxicity analysis indicated that there is no enhancement in cytotoxicity between 5-aza-dC or 5-aza-C and gemcitabine (Fig. 4C, D, 5C and 5D). These results indicate that 5-aza-dC or 5-aza-C and gemcitabine synergistically inhibit HBV rcDNA synthesis with no enhancement in cytotoxicity.

3.4. 5-aza-dC, 5-aza-C, and gemcitabine have antiviral effects on HBV early-stage replication

In addition to their effects on reverse transcription, the effects of 5-aza-dC, 5-aza-C, and gemcitabine on the early stages of HBV replication was determined, particularly the generation and function of cccDNA. As indicated above, infection of HepG2-NTCP cells using the HBV/NL reporter virus is an *ex vivo* model of early-stage HBV replication, since the HBV/NL reporter virus has defective *pol* and *preC/C* genes needed for reverse transcription (Nishitsuji et al., 2015). With a defective polymerase and capsid, HBV infection can progress only to the HBV pgRNA stage. Therefore, HepG2-NTCP cells were treated during HBV/NL virus infection to study the effects on HBV early-stage replication. HepG2-NTCP cells were treated with 3 TC, 5-aza-dC, 5-aza-C, gemcitabine, or the drugs in combination for three days during HBV/NL replication. Six- or seven-days post-infection, NanoLuc luciferase activity in the lysate was measured using the NL Luciferase Assay Kit. 5-aza-dC, 5-aza-C, and gemcitabine were all found to be able to inhibit HBV early-stage replication, though to a lesser extent than late stages of replication (Fig. 6A). For example, treatment of 100 μM 5-aza-dC resulted in 50% loss of virus infection; while treatment of 10 nM gemcitabine led to virus infection that was about 40% that of the untreated group. Both 5-

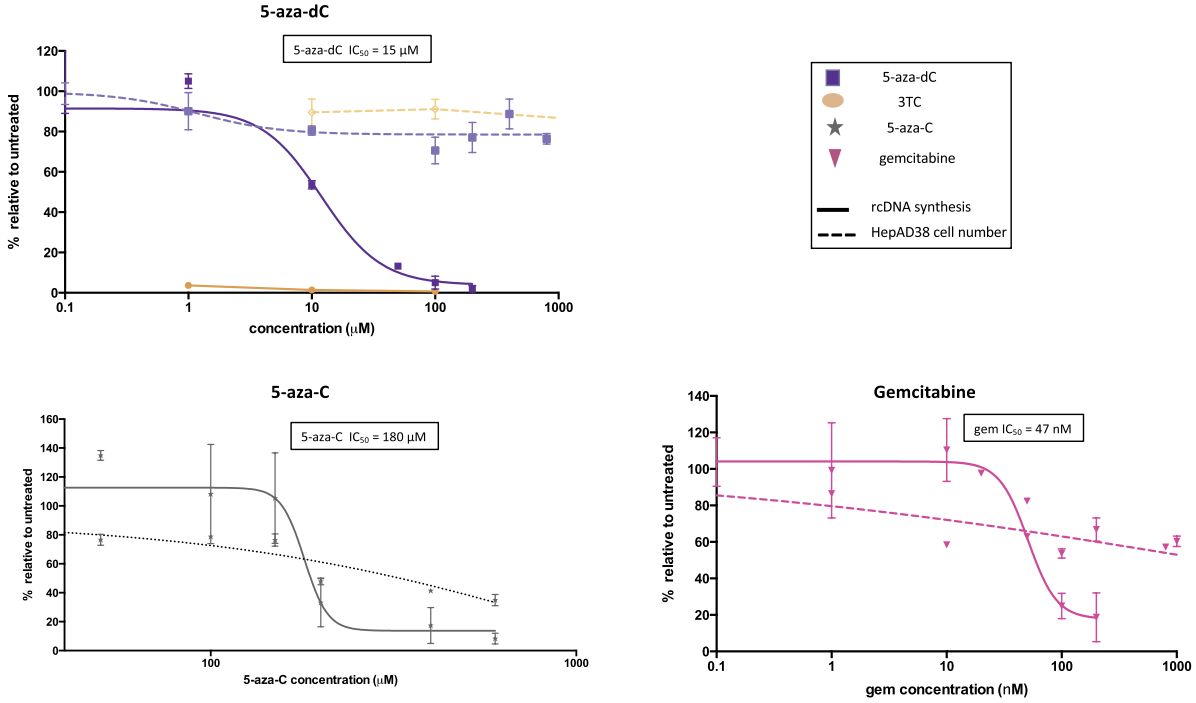

Fig. 3. 5-aza-dC, 5-aza-C and gemcitabine inhibit HBV rcDNA synthesis. HepAD38 cells (1.0×10^5 cells per well) were plated in a 24-well plate 24 h prior to virus-producing induction. Following removal of tetracycline, HepAD38 cells were treated with 3TC (1, 10, 100 μM), 5-aza-dC (0.001, 0.01, 0.1, 1, 10, 50, 100, 200 μM), 5-aza-C (10, 20, 50, 100, 150, 200, 400, 600 μM), or gemcitabine (gem) (0.1, 1, 10, 20, 50, 100, 200 nM). At 5 days post-induction of virus particle production, HBV rcDNA was purified from cell culture supernatants. The extracted rcDNA was quantified by qPCR. HBV DNA synthesis was normalized by dividing the DNA relative copy number from the drug-treated experiment by that of the untreated experiment. Cell proliferation was determined by using the CellTiter-Glo kit from Promega according to the manufacturer's instructions. HepAD38 cells (4500 cells per well) were plated in a 96-well plate 24 h prior to drug treatment. Cells were treated with 3TC (10, 100, 1000 μM), 5-aza-dC (0.1, 1, 10, 50, 100, 200, 400, 800 μM), 5-aza-C (0.1, 1, 10, 20, 50, 100, 150, 200, 400, 600 μM) or gem (0.1, 1, 10, 50, 100, 200, 800, 1000, 10000 nM) for 5 days, and cell proliferation was then assessed. Cells were treated with an equivalent volume of DMSO for the untreated (i.e., no drug) group. The data were converted to relative cell numbers by setting the value for untreated cells at 100 for each experiment and then multiplying the data for the other samples by the number used to convert the untreated cells to 100. The data represents the average ± standard deviation for 3 independent replicate experiments. To determine the half-maximal inhibitory concentration (IC50) and 50% cytotoxic concentration (CC50) of 5-aza-C, 5-aza-dC, and gemcitabine, the infectivity data were normalized to the data for the untreated control, plotted against log-transformed drug concentrations, and subjected to nonlinear regression. IC50s are indicated for each drug, with 3TC as a positive control.

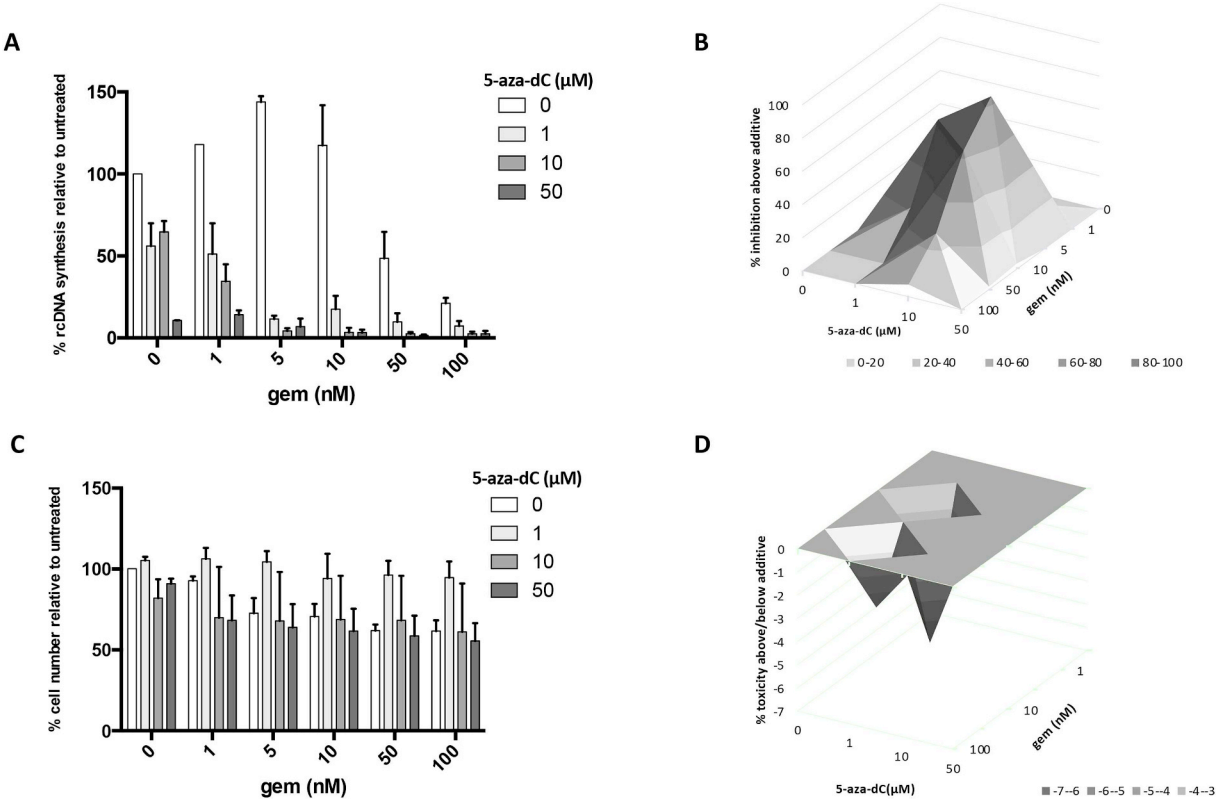

Fig. 4. Anti-HBV drug synergy of 5-aza-dC and gemcitabine. A. rcDNA synthesis. HepAD38 cells (1.0×10^5 cells per well) cells were plated in a 24-well plate 24 h prior to induction of virus production. Following removal of tetracycline, HepAD38 cells were treated with various concentrations of combinations of gem (1, 5, 10, 50, 100 nM) and 5-aza-dC (1, 10, 50 μM). At 5 days post-virus induction, HBV rcDNA was purified from cell culture supernatants, and the rcDNA was analyzed by qPCR. HBV DNA synthesis was normalized by dividing the relative DNA copy number of the drug-treated group to that of the untreated group. **B. Isobologram analysis of drug synergy.** Analysis of drug synergy was determined by using the MacSynergy II program. **C. Cell proliferation analysis.** HepAD38 cells (4500 cells per well) were plated in a 96-well plate 24 h prior to drug treatment. HepAD38 cells were treated with various concentrations of gem (1, 5, 10, 50, 100 nM) and 5-aza-dC (1, 10, 50 μM) for 5 days, and cell proliferation was then assessed. DMSO-treated cells were used as an untreated control. The data was converted to relative cell numbers by setting the value for untreated cells at 100. **D. Isobologram analysis of drug synergy in cell toxicity.** Analysis of drug synergy was determined by using the MacSynergy II program. The results are presented as the average ± standard deviation from 3 independent experiments. Abbreviation: gem = gemcitabine.

aza-dC and gemcitabine showed no significant toxicity to HepG2-NTCP cells under functional concentrations (Fig. 6B). Although high concentrations of 5-aza-C showed strong toxicity to HepG2-NTCP cells, lower concentrations, e.g., 50 μM, can potently inhibit early-stage HBV infection (63% reduction of infectivity) with limited cytotoxic effect (26% reduction of cellular proliferation) (Fig. 6B). It was also observed that synergy occurred between gemcitabine and low concentrations of 5-aza-dC (10 μM). In contrast, drug synergy was not observed between 5-aza-C (10 μM) and gemcitabine, likely due to the low 5-aza-C concentrations.

3.5. Inhibition of HBV cccDNA transcription in the presence of 5-aza-dC

The mechanism by which 5-aza-dC and 5-aza-C inhibit early stage HBV virus transfer was investigated by testing whether these drugs could impact cccDNA synthesis and transcription. To address this question, cccDNA in the infected HepG2-NTCP cells was quantified by using qPCR. It was observed that 50 μM 5-aza-dC led to a trend towards reduced cccDNA synthesis, which was not statistically significant (Fig. 7). While 10 μM 3 TC inhibited early-stage virus transfer at nearly the same level as 50 μM 5-aza-dC, it was observed to have no impact on cccDNA synthesis (Figs. 6 and 7). Intriguingly, gemcitabine increased the cccDNA synthesis. Viral RNA from infected cells was quantified by using RT-qPCR with primer sets in both the nick/gap region and pre S gene. 50 μM 5-aza-dC decreased nick/gap region transcription by 43%, but did not affect pre S gene RNA synthesis (Fig. 7). As expected, 3 TC and gemcitabine did not affect the viral RNA synthesis in cells where HBV was transferred to via replication. It was observed that 5-aza-C did not inhibit cccDNA or RNA synthesis. These results demonstrate that 5-aza-dC can impact cccDNA synthesis and transcription, while 5-aza-C and gemcitabine were not observed to have effects on cccDNA synthesis or RNA transcription.

3.6. 5-aza-dC and 5-aza-C do not increase the mutation frequency of cccDNA

The formation of cccDNA from rcDNA is poorly understood. 5-aza-dC can be incorporated into DNA during production of either plus- or minus-strand DNA. Incorporation of 5-aza-dC into the plus strand DNA may lead to the destabilization of cccDNA, which would lead to decrease of RNA transcription of both the preS and nick/gap genes. However, only a reduction of nick/gap gene transcription was observed. Therefore, it was speculated that 5-aza-dC might interfere with the completion of minus stand DNA synthesis, which would result only in a decrease of nick/gap gene cccDNA synthesis and transcription. The completion of minus strand DNA synthesis includes RNA primer removal, polymerase removal, the terminal redundancy removal and DNA ligation (Guo et al., 2007). Except for DNA ligation, these

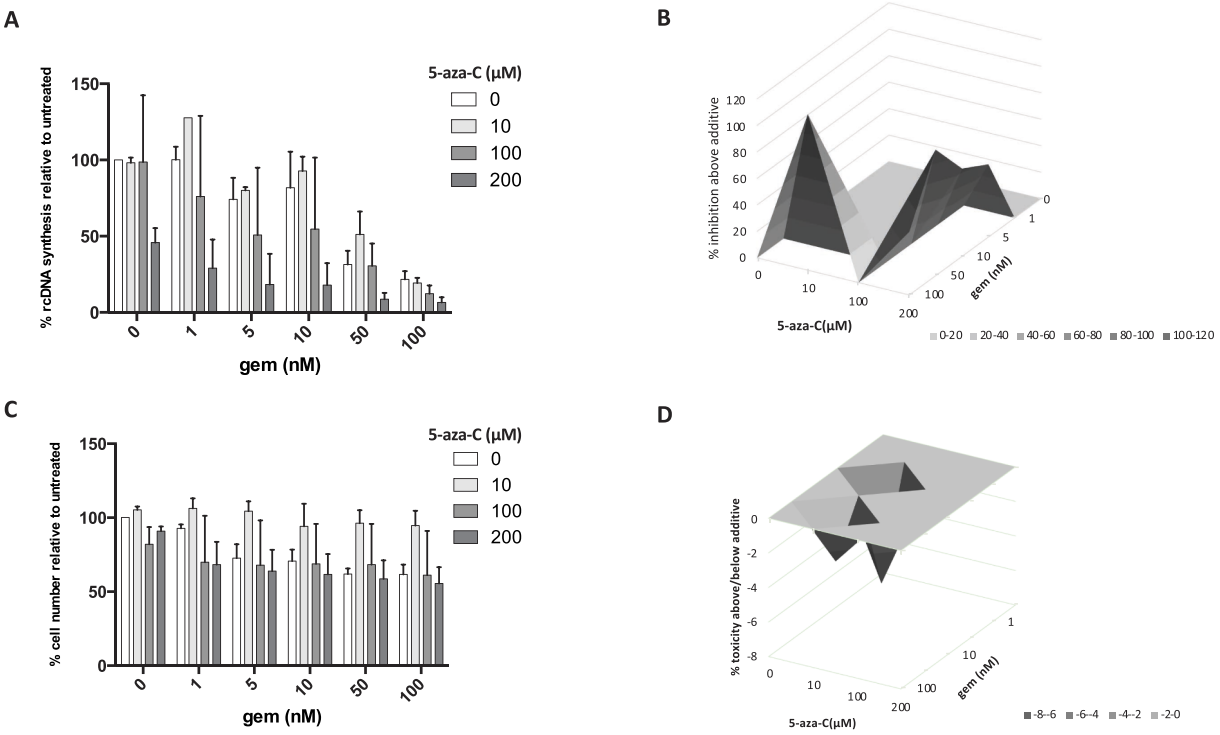

Fig. 5. Anti-HBV drug synergy of 5-aza-C and gemcitabine. A. rcDNA synthesis. HepAD38 cells (1.0×10^5 cells per well) were plated in a 24-well plate 24 h prior to induction of virus production. Following removal of tetracycline, HepAD38 cells were treated with various concentrations of gem (1, 5, 10, 50, 100 nM) and 5-aza-C (10, 100, 200 μM) in combination. At 5 days post-virus-induction, HBV rcDNA was purified from cell culture supernatants. The extracted rcDNA was quantified by qPCR, and the rcDNA was analyzed by qPCR. HBV DNA synthesis was normalized by dividing the relative DNA copy number of the drug-treated group to that of the untreated group. **B. Isobologram analysis of drug synergy.** Analysis of drug synergy was determined by using the MacSynergy II program. **C. Cell proliferation analysis.** HepAD38 cells (4500 cells per well) were plated in a 96-well plate 24 h prior to drug treatment. HepAD38 cells were treated with various concentrations of combination of gem (1, 5, 10, 50, 100 nM) and 5-aza-C (10, 100, 200 μM) for 5 days, and cell proliferation was then assessed. DMSO-treated cells were used as an untreated control. The data were converted to relative cell numbers by setting the value for untreated cells at 100. **D. Isobologram analysis of drug synergy in cell toxicity.** The results are presented as the average ± standard deviation from 3 independent experiments. Abbreviation: gem = gemcitabine.

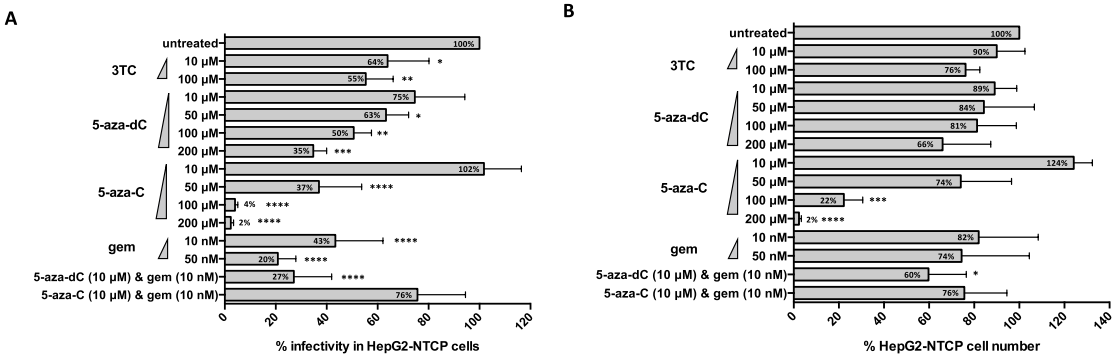

Fig. 6. Antiviral activity of 5-aza-dC, 5-aza-C and/or gemcitabine on the early phase of HBV infection. HepG2-NTCP cells (5.0×104 cells per well) were plated in a 96-well plate 24 h prior to infection. HepG2-NTCP cells were treated with 3 TC (10 or 100 μM), 5-aza-dC (10, 50, 100 or 200 μM), 5-aza-C (10, 50, 100 or 200 μM), gem (10 or 50 nM), 5-aza-dC (10 μM) & gem (10 nM) or 5-aza-C (10 μM) & gem (10 nM) for 2 h, then infected with HBV/NL vector virus in the presence of 4% PEG8000 and 2% DMSO and incubated overnight at 37 ˚C. At 24 h post-infection, fresh media with drugs were added. At 3 days post-infection, media was changed again, and at 6 or 7 days post-infection, cells were washed once with PBS and cells lysed. **A. Analysis of virus infectivity** To analyze virus infectivity, the cell lysates were tested for Nanoluciferase activity (NL Luciferase Assay), and normalized to that of untreated cells. **B. Cell proliferation analysis.** Cell lysates was also examined for cell proliferation (CellTiter-Glo assay). Shown are the average values ± standard deviation from 3 independent experiments. The number in each bar graph represents the mean value. Drug-treated experiments were compared to untreated controls. Statistical analysis was done using a one-way ANOVA; "ns" = not significant; "****" = $p \leq 0.0001$; "***" = $p \leq 0.001$; "**" = $p \leq 0.01$; "*" = $p \leq 0.05$. Abbreviation: gem = gemcitabine.

processes are poorly understood (Long et al., 2017). One hypothesis is that 5-aza-dC incorporates into the nick/gap region during synthesis, which would affect transcription of the nick/gap region but not the preS gene. To test this, the nick/gap region was sequenced, as well as a partially overlapping region of the polymerase and surface envelope of cccDNA from infected HepG2-NTCP cells. Interestingly, few mutations were observed in either the *nick/gap* region or partial *pol/s* gene in the 5-aza-dC treated, 5-aza-C-treated or untreated experiments, with no significant difference in mutation frequencies observed (Tables 1 and 2). These results suggest that while 5-aza-dC and 5-aza-C can induce mutations in rcDNA, they do not increase cccDNA mutagenesis.

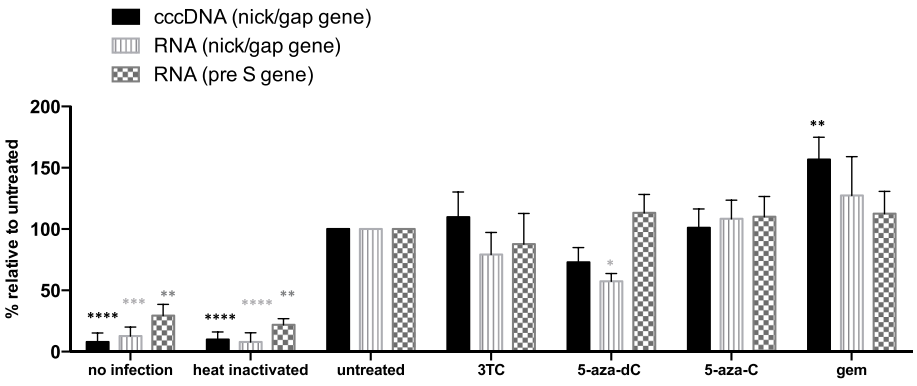

Fig. 7. Inhibition of HBV cccDNA transcription by 5-aza-dC. HepG2-NTCP cells (5.0×10^5 cells per well) were plated in a 24-well plate 24 h prior to infection. The HepG2-NTCP cells were treated with either 10 μM 3 TC, 50 μM 5-aza-dC, 50 μM 5-aza-C, 50 nM gem or untreated (DMSO only), respectively. At 2 h post-drug treatment, cells were infected by HBV/NL vector virus, and at 3 days post-infection, fresh media with drug was added. At 6–7 days post-infection, HepG2-NTCP cells were lysed, and nucleic acids were purified. The amount of cccDNA was quantified by qPCR using nick/gap gene primer sets (1575–1882). Viral RNA was quantified by qRT-PCR using both *nick/gap* gene and pre *S* gene (184–249) primer sets. Nucleic acids values were normalized to untreated, infected HepG2-NTCP cells. Nucleic acids from non-infected or heat-inactivated, virus-infected HepG2-NTCP cells were used as negative controls. Data is shown as the average ± standard deviation of 3 independent experiments. Statistical analysis was determined with a one-way ANOVA test; "****" = $p \leq 0.0001$; "***" = $p \leq 0.001$; "**" = $p \leq 0.01$; "*" = $p \leq 0.05$. Abbreviation: gem = gemcitabine.

4. Discussion

In 2016, the World Health Organization set a goal for global reduction of 90% in incidence and 65% in mortality for hepatitis B and C by 2030 (Hutin et al., 2018). Developing new therapeutics is therefore an important goal. Mutagens and RNRIs have been shown to have anti-HIV-1 activity through targeting of reverse transcription, which is a shared step in the lifecycle of HIV-1 and HBV. In this study, we found that the viral mutagens 5-aza-dC and 5-aza-C lead to a significant reduction in virus transfer to permissive target cells. In particular, 5-aza-dC or 5-aza-C elevated the HBV rcDNA mutation frequency, due primarily to an increase in the G-to-C transversion mutation frequency. Also, rcDNA synthesis was reduced. Intriguingly, cccDNA nick/gap region transcription was reduced by 5-aza-dC, but was found to not impact viral mutagenesis. Together, these observations demonstrate that viral mutagens are efficient for extinguishing HBV virus transfer to target cells, and supports a model in which viral mutagens can induce mutagenesis and synthesis reduction during rcDNA formation and diminish viral DNA synthesis during the conversion of rcDNA to cccDNA.

Lethal mutagenesis caused by mutagens, like 5-aza-dC and 5-aza-C, has been shown to be a promising antiviral strategy for HIV-1. After entering the cell, 5-aza-C is phosphorylated to 5-aza-CMP and then 5-aza-CDP. 5-aza-CDP can incorporate into the viral genome via one of two mechanisms (Rawson et al., 2016). The first is to incorporate into HIV-1 vRNA as 5-aza-CTP, causing C-to-G transversions. 5-aza-CDP can also be reduced to 5-aza-dCDP by ribonucleotide reductase and then phosphorylated to 5-aza-dCTP, which incorporate into viral DNA during reverse transcription, and leads to G-to-C or C-to-G transversions, depending on which strand it is incorporated into. The latter is believed to be the primary mechanism by which 5-aza-C incorporates into the HIV-1 genome. 5-aza-dC, on the other hand, exclusively incorporates into viral DNA directly after phosphorylation, also causing G-to-C and C-to-G transversions. Consistent with these findings on HIV-1, we observed significantly increased G-to-C and C-to-G mutations in HBV rcDNA treated with azacytidines, among which G-to-C is the most frequently observed mutation type. Surprisingly, a large portion of G-to-T mutations were also seen in HBV rcDNA with azacytidines treatment, which has not been reported for HIV-1. While we do not know the mechanisms for this type of mutagenesis at present, we suspect that this occurs during the incorporation of 5-aza-dCTP into viral DNA since there was an obvious reduction in the ratio of G-to-T mutations after the addition of gemcitabine (Fig. 2). For example, 200 μM 5-aza-C treatment resulted in 19% G-to-T mutations in rcDNA, while the addition of 10 nM gemcitabine to it led to only 4% G-to-T mutations. We also observed that mutations occur randomly throughout the *pol/s* (HBV sequence 87–800) of rcDNA, with no evidence of specific mutational "hot spots" (Supplemental fig. 1). This is consistent with what has been observed within the HIV-1 genome (Rawson et al., 2015).

Interestingly, 5-aza-dC was found to inhibit the transcription of cccDNA nick/gap region while 5-aza-C did not. It is possible that a higher concentration of 5-aza-C is needed to successfully inhibit cccDNA transcription than was used here, as our data suggested that 5-aza-C typically has a higher functional concentration than that of 5-aza-dC (Fig. 3). However, since 100 μM 5-aza-C treatment of HepG2-NTCP cells resulted in a nearly 80% reduction in cell proliferation, we have not tested higher concentrations of 5-aza-C to date. Nevertheless, our data demonstrate for the first time that a nucleotide analog is capable of interfering with cccDNA synthesis. The conversion from rcDNA to cccDNA involves completion of both plus and minus strand DNA, the latter of which requires RNA primer removal, polymerase removal, the terminal redundancy removal, and DNA ligation (Guo et al., 2007). The mechanism for this conversion is unclear. One recent study reported that host DNA polymerase κ contributes to cccDNA formation during de novo HBV replication (Qi et al., 2016). Given the high fidelity of the host polymerase, the possibility that 5-aza-dC incorporates into the plus strand of cccDNA is therefore low. Furthermore, even if 5-aza-dC could be incorporated into the plus strand DNA, the effect would be trivial as pgRNA is transcribed from minus strand DNA. It is also possible, however, that incorporation of 5-aza-dC into plus strand cccDNA destabilizes the cccDNA (due to the aza-C base inducing conformational changes in the DNA), thus resulting in defective or degraded cccDNA. This possibility could explain the decrease of cccDNA with 5-aza-dC

Table 1
Lack of increased mutation frequency in cccDNA *pol/s* gene in the presence of 5-aza-dC or 5-aza-C.

	cccDNA *pol/s* gene			
	No. of mutants/cloned sequence	No. of mutations/bases sequenced	Mutation frequency	Statistical significantce ($p \leq 0.05$)
untreated	2/55	2/41,939	4.77×10^{-5}	
5-aza-dC	3/65	4/47,007	8.51×10^{-5}	ns[a]
5-aza-C	4/63	4/45,726	8.75×10^{-5}	ns

[a] ns = not significant.

Table 2
Lack of increased mutation frequency in cccDNA *nick/gap* gene in the presence of 5-aza-dC or 5-aza-C.

	cccDNA *nick/gap* gene			
	No. of mutants/cloned sequence	No. of mutations/bases sequenced	Mutation frequency	Statistical significance ($p \leq 0.05$)
untreated	2/47	2/14,417	1.36×10^{-4}	–
5-aza-dC	0/45	0/12,440	0	ns[a]
5-aza-C	1/60	1/18,233	5.31×10^{-5}	ns

[a] ns = not significant.

treatment, but does not explain the unchanging amount of preS gene RNA. Therefore, we posit that 5-aza-dC incorporates during completion of minus strand cccDNA. To date, only host DNA ligases have been reported to be utilized by hepadnaviruses for cccDNA minus strand formation (Long et al., 2017). Other involved enzymes are not known yet. This incorporation may result in either a mutated or incomplete cccDNA. Both of these outcomes can explain the decrease in cccDNA nick/gap gene RNA observed. Nevertheless, we did not observe an increase in the mutation frequency of cccDNA after treatment with 5-aza-dC. However, given the limited size of our sequencing samples, we cannot conclude that 5-aza-dC does not cause mutagenesis of cccDNA. The nick/gap region of cccDNA is small, and a larger sample size as well as deep sequencing would help increase the sensitivity of mutation detection. 5-aza-dC incorporation may also lead to defective cccDNA formation, which could help explain the decreased nick/gap cccDNA synthesis and RNA with unchanged preS RNA levels.

While 5-aza-dC and 5-aza-C are both known to be viral mutagens, their mechanisms of action are not identical. As a deoxyribonucleoside, 5-aza-dC is only able to incorporate into DNA. However, 5-aza-C is a ribonucleoside, and is incorporated into RNA. However, 5-aza-C can be reduced to 5-aza-dC by ribonucleoside reductase, and can thus be incorporated into DNA as well (Rawson et al., 2016). These differences in template specificity likely explain why 5-aza-C inhibited the synthesis and increased the mutation frequency of rcDNA exclusively while 5-aza-dC also inhibited the synthesis and transcription of cccDNA. These results imply a difference in the anti-HBV mechanisms between 5-aza-dC and 5-aza-C (Fig. 8). In this model, 5-aza-dC incorporates into cccDNA minus strand nick/gap region during the conversion of rcDNA to cccDNA, resulting in formation of defective cccDNA. During rcDNA formation, 5-aza-dC incorporates into rcDNA, and leads to either chain termination or mutagenesis. In contrast, 5-aza-C interferes with gene translation of cccDNA and results in reduction of gene expression. During rcDNA formation, a majority of 5-aza-C is reduced to 5-aza-dC and then incorporated into rcDNA, with only a minority of 5-aza-C incorporating into rcDNA directly. This would explain why 5-aza-C has a much higher IC_{50} than that of 5-aza-dC in rcDNA synthesis. Gemcitabine was observed to inhibit rcDNA synthesis and inhibit early-stage HBV replication as well. It has been reported that the main mechanism of action of gemcitabine include masked chain termination and inducing dNTP pool imbalances in the cell (de Sousa Cavalcante and Monteiro, 2014). Given these activities, gemcitabine inhibit HBV cccDNA gene translation and cause rcDNA chain termination during reverse transcription.

Consistent with HIV-1, anti-HBV drug synergy between 5-aza-dC or 5-aza-C and gemcitabine was observed (Figs. 4 and 5) (Clouser et al., 2010). According to the synergistic analysis of mutagens and RNRIs on HIV-1 replication, it is thought that this synergy is due to induction of dNTP pool imbalances by RNRIs, which promotes the incorporation of mutagens. The results in our study are consistent with this model, as 5-aza-dC or 5-aza-C and gemcitabine acted together in synergy for inhibiting rcDNA synthesis. Gemcitabine was also observed to promote the mutagenesis caused by 5-aza-dC, but not 5-aza-C. This is likely due

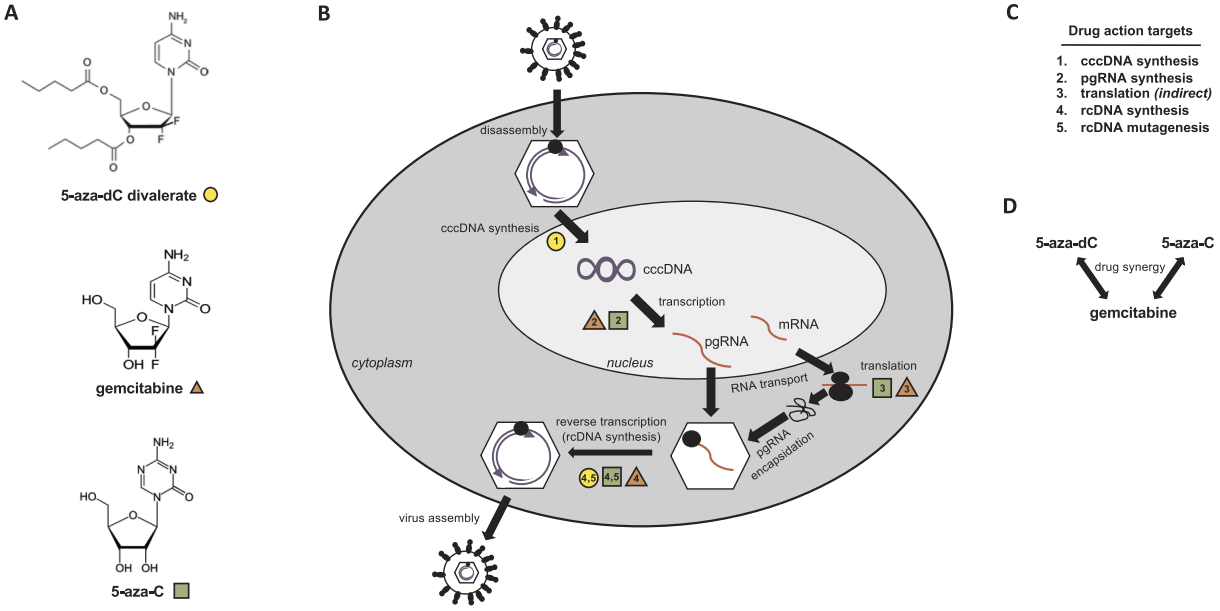

Fig. 8. Summary and model of observed antiviral activities of 5-aza-dC, 5-aza-C and gemcitabine against HBV. A. Drug structures. The chemical structures of 5-aza-dC divalerate, gemcitabine, and 5-aza-C are shown. The colored symbols, ○ = 5-aza-dC; □ = 5-aza-C and △ = gemcitabine, are indicated and refer to antiviral effects at particular steps in the HBV life cycle (see panel B.). **B. HBV life cycle and antiviral activities.** The HBV life cycle is shown, with the key steps where antiviral activity of 5-aza-dC, gemcitabine and 5-aza-C are indicated (colored symbols). The numbers in the symbols refer to particular drug action targets (see panel C). **C. Drug action targets.** The drug action targets of 5-aza-dC, gemcitabine, and 5-aza-C in the HBV life cycle are indicated. **D. Antiviral drug synergy.** The observed anti-HBV drug synergy between 5-aza-dC and gemcitabine and between 5-aza-C and gemcitabine are indicated.

to 5-aza-C being primarily incorporated as the reduced form; thus, inhibiting reduction with gemcitabine prevents incorporation and mutagenesis. This would also help explain why a lower mutation frequency of rcDNA was observed with the combined treatment of 5-aza-C (200 μM) and gemcitabine (10 nM) compared to individual treatment (Fig. 2 and Supplemental Table 1). Similarly, 5-aza-dC and gemcitabine exhibited synergistic activity in the inhibition of early-stage HBV replication, while 5-aza-C and gemcitabine did not. This is likely due to the lower functional concentration of 5-aza-C used.

Overall, our findings provide the first demonstration of the anti-HBV activity of viral mutagens and RNRIs. We found that the elevation of the HBV mutation rate can extinguish the virus transfer to target cells. Additionally, the anti-HBV synergy between mutagens and RNRIs should help to expand the application of both types of drugs in antiviral therapy. Notably, we propose a model in which viral mutagens like 5-aza-dC can induce mutagenesis and reduce rcDNA formation, as well as diminish viral DNA synthesis during the conversion of rcDNA to cccDNA. Taken together, these findings lay the foundation for future studies directed at studies of HBV DNA synthesis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104540.

References

Ashburn, T.T., Thor, K.B., 2004. Drug repositioning: identifying and developing new uses for existing drugs. Nat. Rev. Drug Discov. 3, 673–683.
Bouchard, J., Walker, M.C., Leclerc, J.M., Lapointe, N., Beaulieu, R., Thibodeau, L., 1990. 5-azacytidine and 5-azadeoxycytidine inhibit human immunodeficiency virus type 1 replication in vitro. Antimicrob. Agents Chemother. 34, 206–209.
Choi, E., Mallareddy, J.R., Lu, D., Kolluru, S., 2018. Recent advances in the discovery of small-molecule inhibitors of HIV-1 integrase. Future Sci OA 4, FSO338.
Clouser, C.L., Bonnac, L., Mansky, L.M., Patterson, S.E., 2014. Characterization of permeability, stability and anti-HIV-1 activity of decitabine and gemcitabine divalerate prodrugs. Antivir. Chem. Chemother. 23, 223–230.
Clouser, C.L., Chauhan, J., Bess, M.A., van Oploo, J.L., Zhou, D., Dimick-Gray, S., Mansky, L.M., Patterson, S.E., 2012. Anti-HIV-1 activity of resveratrol derivatives and synergistic inhibition of HIV-1 by the combination of resveratrol and decitabine. Bioorg. Med. Chem. Lett 22, 6642–6646.
Clouser, C.L., Holtz, C.M., Mullett, M., Crankshaw, D.L., Briggs, J.E., Chauhan, J., VanHoutan, I.M., Patterson, S.E., Mansky, L.M., 2011. Analysis of the ex vivo and in vivo antiretroviral activity of gemcitabine. PLoS One 6, e15840.
Clouser, C.L., Patterson, S.E., Mansky, L.M., 2010. Exploiting drug repositioning for discovery of a novel HIV combination therapy. J. Virol. 84, 9301–9309.
Dapp, M.J., Clouser, C.L., Patterson, S., Mansky, L.M., 2009. 5-Azacytidine can induce lethal mutagenesis in human immunodeficiency virus type 1. J. Virol. 83, 11950–11958.
de Avila, A.I., Gallego, I., Soria, M.E., Gregori, J., Quer, J., Esteban, J.I., Rice, C.M., Domingo, E., Perales, C., 2016. Lethal mutagenesis of hepatitis C virus induced by favipiravir. PLoS One 11, e0164691.
de Sousa Cavalcante, L., Monteiro, G., 2014. Gemcitabine: metabolism and molecular mechanisms of action, sensitivity and chemoresistance in pancreatic cancer. Eur. J. Pharmacol. 741, 8–16.
Domingo, E., Sheldon, J., Perales, C., 2012. Viral quasispecies evolution. Microbiol. Mol. Biol. Rev. 76, 159–216.
Graci, J.D., Harki, D.A., Korneeva, V.S., Edathil, J.P., Too, K., Franco, D., Smidansky, E.D., Paul, A.V., Peterson, B.R., Brown, D.M., Loakes, D., Cameron, C.E., 2007. Lethal mutagenesis of poliovirus mediated by a mutagenic pyrimidine analogue. J. Virol. 81, 11256–11266.
Greggs 3rd, W.M., Clouser, C.L., Patterson, S.E., Mansky, L.M., 2012. Discovery of drugs that possess activity against feline leukemia virus. J. Gen. Virol. 93, 900–905.
Guo, H., Jiang, D., Zhou, T., Cuconati, A., Block, T.M., Guo, J.T., 2007. Characterization of the intracellular deproteinized relaxed circular DNA of hepatitis B virus: an intermediate of covalently closed circular DNA formation. J. Virol. 81, 12472–12484.
Hutin, Y., Nasrullah, M., Easterbrook, P., Dongmo Nguimfack, B., Burrone, E., Averhoff, F., Bulterys, M., 2018. Access to treatment for hepatitis B virus infection-Worldwide, 2016. Am. J. Transplant. 18, 2595–2598.
Jiang, Y.C., Feng, H., Lin, Y.C., Guo, X.R., 2016. New strategies against drug resistance to herpes simplex virus. Int. J. Oral Sci. 8, 1–6.
Ladner, S.K., Otto, M.J., Barker, C.S., Zaifert, K., Wang, G.H., Guo, J.T., Seeger, C., King, R.W., 1997. Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication. Antimicrob. Agents Chemother. 41, 1715–1720.
Long, Q., Yan, R., Hu, J., Cai, D., Mitra, B., Kim, E.S., Marchetti, A., Zhang, H., Wang, S., Liu, Y., Huang, A., Guo, H., 2017. The role of host DNA ligases in hepadnavirus covalently closed circular DNA formation. PLoS Pathog. 13, e1006784.
Lucifora, J., Protzer, U., 2016. Attacking hepatitis B virus cccDNA–The holy grail to hepatitis B cure. J. Hepatol. 64, S41–S48.
Moreno, H., Tejero, H., de la Torre, J.C., Domingo, E., Martin, V., 2012. Mutagenesis-mediated virus extinction: virus-dependent effect of viral load on sensitivity to lethal defection. PLoS One 7, e32550.
Ni, Y., Lempp, F.A., Mehrle, S., Nkongolo, S., Kaufman, C., Falth, M., Stindt, J., Koniger, C., Nassal, M., Kubitz, R., Sultmann, H., Urban, S., 2014. Hepatitis B and D viruses exploit sodium taurocholate co-transporting polypeptide for species-specific entry into hepatocytes. Gastroenterology 146, 1070–1083.
Nishitsuji, H., Ujino, S., Shimizu, Y., Harada, K., Zhang, J., Sugiyama, M., Mizokami, M., Shimotohno, K., 2015. Novel reporter system to monitor early stages of the hepatitis B virus life cycle. Cancer Sci. 106, 1616–1624.
Pas, S.D., Fries, E., De Man, R.A., Osterhaus, A.D., Niesters, H.G., 2000. Development of a quantitative real-time detection assay for hepatitis B virus DNA and comparison with two commercial assays. J. Clin. Microbiol. 38, 2897–2901.
Prichard, M.N., Shipman Jr., C., 1990. A three-dimensional model to analyze drug-drug interactions. Antivir. Res. 14, 181–205.
Qi, Y., Gao, Z., Xu, G., Peng, B., Liu, C., Yan, H., Yao, Q., Sun, G., Liu, Y., Tang, D., Song, Z., He, W., Sun, Y., Guo, J.T., Li, W., 2016. DNA polymerase kappa is a key cellular factor for the formation of covalently closed circular DNA of hepatitis B virus. PLoS Pathog. 12, e1005893.
Rawson, J.M., Daly, M.B., Xie, J., Clouser, C.L., Landman, S.R., Reilly, C.S., Bonnac, L., Kim, B., Patterson, S.E., Mansky, L.M., 2016. 5-Azacytidine enhances the mutagenesis of HIV-1 by reduction to 5-aza-2'-deoxycytidine. Antimicrob. Agents Chemother. 60, 2318–2325.
Rawson, J.M., Heineman, R.H., Beach, L.B., Martin, J.L., Schnettler, E.K., Dapp, M.J., Patterson, S.E., Mansky, L.M., 2013. 5,6-Dihydro-5-aza-2'-deoxycytidine potentiates the anti-HIV-1 activity of ribonucleotide reductase inhibitors. Bioorg. Med. Chem. 21, 7222–7228.
Rawson, J.M., Landman, S.R., Reilly, C.S., Bonnac, L., Patterson, S.E., Mansky, L.M., 2015. Lack of mutational hot spots during decitabine-mediated HIV-1 mutagenesis. Antimicrob. Agents Chemother. 59, 6834–6843.
Reina, J., Reina, N., 2017. [Favipiravir, a new concept of antiviral drug against influenza viruses]. Rev. Española Quimioter. 30, 79–83.
Tang, L., Zhao, Q., Wu, S., Cheng, J., Chang, J., Guo, J.T., 2017. The current status and future directions of hepatitis B antiviral drug discovery. Expert Opin. Drug Discov. 12, 5–15.
Todt, D., Walter, S., Brown, R.J., Steinmann, E., 2016. Mutagenic Effects of Ribavirin on Hepatitis E Virus-Viral Extinction versus Selection of Fitness-Enhancing Mutations. Viruses 8.
Yan, R., Zhang, Y., Cai, D., Liu, Y., Cuconati, A., Guo, H., 2015. Spinoculation enhances HBV infection in NTCP-reconstituted hepatocytes. PLoS One 10, e0129889.