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Novel fluoronucleoside analog NCC inhibits lamivudine-resistant hepatitis B virus in a hepatocyte model



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ABSTRACT

Antiviral drug resistance is the most important factor contributing to treatment failure using nucleos(t)ide analogs such as lamivudine for chronic infection with hepatitis B virus (HBV). Development of a system supporting efficient replication of clinically resistant HBV strains is imperative, and new antiviral drugs are needed urgently to prevent selection of drug-resistant HBV mutants. A novel fluorinated cytidine analog, NCC (N-cyclopropyl-4'-azido-2'-deoxy-2'-fluoro-β-D-cytidine), was recently shown to strongly inhibit human HBV *in vitro* and *in vivo*. This study was designed to evaluate the antiviral activity of NCC against lamivudine-resistant HBV. We generated a stable cell line encoding the major pattern of lamivudine-resistant mutations rtL180M/M204V and designated it "HepG2.RL1". Immunotransmission electron microscopic examination and enzyme-linked immunosorbent assay were used to detect secretion of HBV-specific particles and antigens. Quantification of extracellular DNA and intracellular DNA of HepG2.RL1 cells by quantitative real-time polymerase chain reaction revealed >625-fold and >5556-fold increases in the 50% inhibitory concentration of lamivudine, respectively, compared with that for the wild-type virus. The results showed that NCC inhibited DNA replication and HBeAg production in wild-type or lamivudine-resistant HBV in a dose-dependent manner. In conclusion, screening for antiviral compounds active against lamivudine-resistant HBV can be carried out with relative ease using hepG2.RL1 cells. NCC is a potential antiviral agent against wild-type HBV and clinical lamivudine-resistant HBV and deserves evaluation for the treatment of HBV infection.

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Introduction

Hepatitis B virus (HBV) infection is a global health problem, with more than 240 million individuals chronically infected worldwide and about 30 million in China.¹ Chronic infection with HBV is closely associated with liver cirrhosis and hepatocellular carcinoma, and carries high mortality.²⁻⁴ In recent years, on account of vaccines as well as treatment using interferon or nucleos(t)ide analogs,⁵ the prognosis of hepatitis B has improved significantly. However, HBV infection-related diseases remain a concern.

Covalently closed circular HBV DNA is a major determinant of persistence of the HBV genome in infected cells, and necessitates long-term antiviral treatment in those infected individuals.^{6,7} Unfortunately, prolonged administration of nucleos(t)ide analogs can result in the emergence of drug-resistant strains, especially if nucleos(t)ide analogs with low genetic barriers to resistance are used as monotherapies.⁸⁻¹⁰ For lamivudine, the prevalence of resistance rises rapidly, reaching 80% after five years of monotherapy.¹¹ The pattern of rtL180M/M204V is the most common lamivudine-resistant HBV variant, with a prevalence of 60%.¹² Emergence of lamivudine resistance leads to acute exacerbation or treatment failure of hepatitis and decompensation of liver function.^{13,14} Thus, novel drugs for the treatment of HBV, especially for rescue therapy in drug-resistant patients, are urgently needed.

NCC, N-cyclopropyl-4'-azido-2'-deoxy-2'-fluoro- β -D-cytidine, is a novel fluorinated cytidine analog. Recent studies have demonstrated that NCC is an efficient inhibitor of wild-type HBV in HepG2.2.15 and HepG.CW cells.¹⁵ However, the activity of NCC on a lamivudine-resistant HBV mutant remains unclear. The commonly used experimental approach is transient transfection of human hepatoma cells.¹⁶ However, in this way, the HBV genome is not integrated into host cells and only temporarily produces HBV particles, and the level of HBV expression is unstable. Here, a novel cell line was generated through stable transfection of an HBV genome (1.3-unit length) with lamivudine-resistant mutations of rtL180M and rtM204V into hepatoma HepG2 cells.

In this study, HepG2.2.15 cells and the newly generated cell line were used as experimental models to investigate the anti-HBV activity of NCC. Our results demonstrated that NCC possesses significant inhibitory activity against lamivudine-resistant HBV.

Materials and methods

Patient specimen

The patient was a 49-year-old Chinese female with hepatitis B e antigen-positive chronic HBV infection. Initially, she received lamivudine monotherapy (100 mg per day, p.o.) and had serum levels of HBV DNA of $8.23 \log_{10}$ copies/mL. After receiving lamivudine for five months, the viral load decreased to $4.39 \log_{10}$ copies/mL, and then became undetectable after an additional three months. Unfortunately, HBV DNA rebounded to $6.86 \log_{10}$ copies/mL and rose continuously in the next two months. At this point, signature mutations

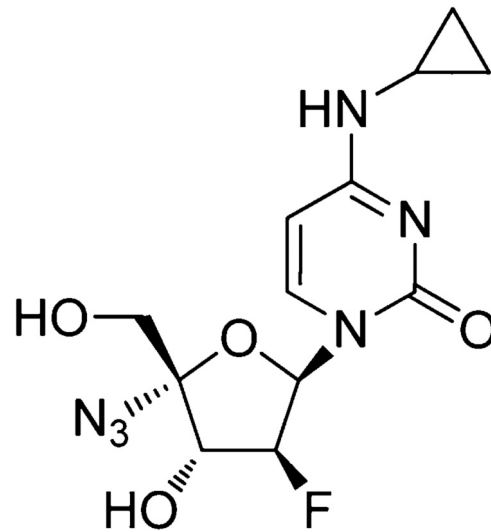


Fig. 1 – Chemical structure of NCC.

Molecular formula: C₁₂H₁₅FN₆O₄. **Molecular weight:** 326.28 g/mol.

of lamivudine resistance (rtL180M/M204V) were detected by DNA sequencing of the reverse transcriptase domain of circulating HBV, and the genotype was of clone B (Invitrogen, Shanghai, China). Blood samples were collected after viral breakthrough.

Compounds

Lamivudine was obtained from GlaxoSmithKline (Suzhou, China). Adefovir (GlaxoSmithKline, Tianjin, China) was used as a positive control for inhibition of the lamivudine-resistant HBV strain. NCC (Fig. 1) with 98.5% purity (as determined by high-performance liquid chromatography) was designed and synthesized in our laboratory (The College of Chemistry and Molecular Engineering, Zhengzhou University, Zhengzhou). Stock solutions (10 mM) of the three compounds were dissolved in phosphate-buffered saline.

Generation of plasmids expressing lamivudine-resistant HBV

HBV DNA was extracted using a Viral DNA Extraction kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. Briefly, patient serum was digested by OB protease and Buffer BL with linear acrylamide and RNase A, and then incubated sample at 65 °C for 10 min, followed by lysate with absolute ethanol (room temperature, 96–100%). The lysate was transferred into a HIBind DNA mini column and cleared of liquid by centrifugation at 8000 × g for 1 min to bind DNA. The HIBind DNA mini column was washed by Buffer HB and DNA wash Buffer and cleared of liquid by centrifugation at 15,000 × g for 2 min to dry the column. After incubated the column by preheated Elution Buffer for 5 min at room temperature, DNA was eluted by centrifugation at 8000 × g for 1 min. Polymerase chain reaction (PCR) amplification of the full-length HBV genome was performed according to the methods of Günther et al.,¹⁷ with

Table 1 – Primer sequence for generation of pcDNA3.1-1.3 HBV recombinant plasmid.

Name		Sequence (5'–3') and restriction sites	Fragment size	Location in HBV genome
HBV genome	P1:	GGCACTAGTGAGCTCTTCTTTTCACCTCTGCCTAATCA	3215 bp	1–3215
	P2:	TAAGCGGCCGCGAGCTCTTCAAAAAGTTGCATGGTGTGG		
A	A1:	GCAAGCTTGGACGACCCGTCTCGGGGCCGTTT Hind III	370 bp	2846–3215
	A2:	GGCAGAGGTCAAAAAGTTGCATGGTGTGGTGAACA		
B	B1:	ACCAGCACCATGCAACTTTTTTCACCTCTGCCTAATCATC	1393 bp	1–1393
	B2:	GTTGTGGAATTCACCTGCATGGCCTGAGGA EcoR I		
C	C1:	GCAGTGGAATTCACAACATTCCACCAAGCTCT EcoR I	1822 bp	1394–3215
	C2:	GGCAGAGGTCAAAAAGTTGCATGGTGTGGTGAACA		
D	D1:	ACCAGCACCATGCAACTTTTTTCACCTCTGCCTAATCATC	495 bp	1–495
	D2:	TATGCGGCCGAGGGGCATTGGTGTCTGTAA Not I		
A + B	A1:	GCAAGCTTGGACGACCCGTCTCGGGGCCGTTT Hind III	1763 bp	2846–1393
	B2:	GTTGTGGAATTCACCTGCATGGCCTGAGGA EcoR I		
C + D	C1:	GCAGTGGAATTCACAACATTCCACCAAGCTCT EcoR I	2317 bp	1394–495
	D2:	TATGCGGCCGAGGGGCATTGGTGTCTGTAA Not I		

the primers p1 and p2 (shown in Table 1). Cycling parameters: pre denaturation 94 °C for 5 min; 94 °C for 40 s, 60 °C for 78 s, 68 °C for 3 min, the extension time plus 1 min after every 10 cycles, 30 cycles total; final additional extension at 72 °C for 10 min; hold at 4 °C. Amplicons were cloned into pMD18-T (TaKaRa Bio, Dalian, China) to generate the T-HBV recombinant for sequencing the reverse transcriptase domain. Results showed that the cloned HBV genome harbored rtL180M/M204V mutations, and that the genotype was clone B.

HBV DNA is circular and partially overlapped, and its genome comprises four open reading frames named S, C, P, and X. According to this feature of HBV genome structure, we designed and amplified four fragments designated A, B, C, and D, and constructed a recombinant plasmid harboring 1.3 units length of HBV genome (4080 bp) to ensure efficient HBV replication and expression. Fragment A is located from nt 2846 to nt 3215 of HBV genome, fragment B is located from nt 1 to nt 1393 of HBV genome, fragment C is located from nt 1394 to nt 3215 of HBV genome, and fragment D is located from nt 1 to nt 495 of HBV genome (A + B + C + D = 4080 bp) (Table 1). T-HBV recombinant was used as a template for amplification of A, B, C, and D gene fragments using the corresponding primers (shown in Table 1) with the following reaction procedure: 94 °C for 4 min, then 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, and 72 °C for 6 min. The A + B and C + D gene fragments were obtained by recombinant PCR with the primers (shown in Table 1) and A, B and C, D gene fragments, respectively, as templates, and the amplification condition was the same as above. Fragment A + B was then digested with HindIII and EcoRI, and fragment C + D was digested with EcoRI and NotI. A 1.3-fold-over-length HBV genome containing fragments A + B and C + D was then ligated into HindIII and NotI cleaved pcDNA 3.1 plasmids by T4 DNA Ligase at 16 °C for 16 h, resulting in pcDNA3.1-1.3HBV.

Generation of stable cell line and detection of HBV antigens and HBV DNA

The human HepG2 was obtained from the China Center for Typical Culture Collection (Wuhan, China). Cells were cultured in DMEM (Dulbecco's modified Eagle's medium, Gibco, New York, NY, USA). HepG2 cells were transfected with pcDNA3.1-1.3HBV encoding mutant HBV using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Two days after transfection, cells were selected in medium supplemented with 600 µg/mL G418 (Sigma-Aldrich, Saint Louis, MO, USA) for three weeks and renewed every three days. The G418-resistant clone was transferred to a T25 culture flask and maintained in the same Dulbecco's modified Eagle's medium with G418 at 300 µg/mL. The novel stable cell line was designated "HepG2.RL1 cell." The surface antigen of HBV (HBsAg) and e antigen of HBV (HBeAg) in cell culture supernatants were detected using an enzyme-linked immunosorbent assay (ELISA) kit (Kehua Bio-engineering, Shanghai, China), and intracellular HBV DNA was extracted from the cells by Tissue viral DNA Extraction Kit (Omega Bio-Tek, USA) according to the manufacturer's instructions, respectively. The amount of intracellular and extracellular HBV DNA were determined by quantitative real-time PCR (qPCR) based on the TaqMan technology and performed in a Light-Cycler (Roche, Mannheim, Germany) with the HBV Fluorescent Quantitative PCR Detection Kit (Qiagen CO., Ltd. Shenzhen, China) following the manufacturer's protocol. After an initial denaturation at 95 °C for 90 s, 40 cycles of denaturation at 94 °C for 5 s and annealing/extension at 56 °C for 45 s were conducted.

Examination of HBV-specific particles

Cell medium (30 mL) was collected and centrifuged at 3000 × g for 30 min at room temperature. The supernatant was

filtered through a 0.22 μm filter membrane. HBsAg (+) healthy blood serum (4 mL) was diluted 1:5 with PBS and also filtered through a 0.22 μm filter membrane. Subsequently, filtrates were mixed at 1:1 and incubated overnight at 4 °C, then was centrifuged and resuspended in culture medium. Suspensions were placed on a copper network supported by carbon membranes. They were examined under immuno-transmission electron microscopy (ITEM) after negative staining with 2% phosphotungstic acid for observation of virus particles.

Cell culture and cytotoxicity assay

HepG2.2.15 cell line (wild-type HBV in the stable HBV-producing) was obtained from the Wuhan Institute of Virology (Wuhan, China) and maintained in the same manner as HepG2 cells except that 200 $\mu\text{g}/\text{mL}$ G418 was added to the culture medium in our laboratory. Compound cytotoxicity was determined by the MTT assay on HepG2.2.15 cells and HepG2.RL1 cells.¹⁸ Cells were treated with lamivudine, adefovir, and NCC at various concentrations (62.5, 125, 250, 500, and 1000 μM).

Drug susceptibility assays¹⁹

HepG2.2.15 cells and HepG2.RL1 cells were seeded in 24-well culture plates at 3×10^4 cells per well and cultured for 24 h. Further, lamivudine and adefovir at various concentrations and NCC (0.04, 0.2, 1, 5 μM) were added to cells for six days. On day 6, HBV DNA was quantified as described previously by qPCR to assess the inhibitory efficiency of drugs on viral replication. HBsAg and HBeAg production were assessed by ELISA. The 50% effective concentration (EC_{50}) was calculated by regression analyses. Fold resistance was calculated as the ratio of mutant EC_{50} to wild-type EC_{50} .

Statistical analyses

Statistical analyses were performed using SPSS v17.0 (IMB, Armonk, NY, USA). Results are the mean \pm SD. One-way ANOVA was used to determine the statistical significance of differences between groups. $p < 0.05$ was considered significant.

Results

Obtainment of HBV fragments and the pcDNA3.1-1.3HBV recombinant plasmid

As shown in Fig. 2A, the full-length HBV genome was extracted from the patient serum and amplified by PCR. The amplicon was cloned into pMD18-T, and used as a template for amplification of A, B, C, and D gene fragments. The PCR products were purified with Gel Extraction Kit (Omega Bio-Tek, USA) from an agarose gel, with a single strip (Fig. 2B). The A+B and C+D gene fragments were obtained by recombinant PCR, and digested with HindIII and EcoRI, EcoRI and NotI, respectively, then purified with Gel Extraction Kit (Fig. 2C). The gel purification of double-enzyme A+B and C+D gene fragments were ligated into the Hind III and Not I-digested pcDNA 3.1 plasmid (Fig. 2D), producing a recombinant clone termed

pcDNA3.1-1.3HBV (Fig. 3). For restriction double-enzyme digestion by Hind III and Not I, the recombinant plasmid pcDNA3.1-1.3HBV was separated into two bands with pcDNA 3.1 plasmid (5428 bp) and A+B+C+D (4080 bp) (Fig. 2E), as well as digestion by Hind III, the 9.5 kb linear plasmid pcDNA3.1-1.3HBV was shown (Fig. 2F). The full sequence of cloned pcDNA3.1-1.3HBV was uploaded as a supplementary file (Doc.1).

Time-course of secretion of HBV antigen and quantification of HBV DNA

To further characterize HepG2.RL1 cells, the supernatants and cells of each generation were collected for 100 continuous passages. HBeAg and HBsAg were secreted continuously at a stable level (Fig. 4A). The level of HBeAg produced by HepG2.RL1 cells was higher than the level of HBsAg under identical conditions. Amounts of viral DNA increased markedly at the 20th generation during the culture period. At the 10th and 100th generations, HepG2.RL1 cells produced 5.67 and 8.08 \log_{10} copies/mL of intracellular DNA, as well as 4.51 and 5.61 \log_{10} copies/mL of extracellular DNA, respectively (Fig. 4B).

Identification of HBV particles

Samples containing medium of the HepG2.RL1 cells were visualized by ITEM analyses. Immune complexes were obtained from samples that had been immunoprecipitated with anti-serum directed against HBsAg. Some of these immune complexes had several Dane-like particles of size ~ 42 nm and a few spherical HBsAg particles (~ 22 nm) (Fig. 4C). However, when samples containing medium of HepG2 cells were examined, virus-associated particles were not observed.

Effects of compounds on cell viability

Upon exposure to various concentrations of NCC for six days, the results from the MTT assay showed that NCC inhibited the growth of HepG2.2.15 cells and HepG2.RL1 cells with CC_{50} of 725.81 ± 23.34 μM and 782.38 ± 12.17 μM , respectively, indicating almost the same extent of toxicity on the two cell lines, whereas CC_{50} for adefovir was 259.81 ± 13.49 μM and 290.57 ± 21.31 μM in HepG2.2.15 cells and HepG2.RL1 cells, respectively.

Drug susceptibility assays

The HBsAg level from HepG2.RL1 was very low even in the drug-free group (data not shown) and hence the evaluation was based only on HBeAg. The dose-response relationship showed that lamivudine did not inhibit HBeAg secretion at the highest concentration (100 μM) in HepG2.RL1 cells, whereas wild-type HBV showed considerable inhibition (Fig. 5A). However, adefovir treatment dose-dependently reduced the secretion of HBeAg in wild-type and lamivudine-resistant HBV (Fig. 5B).

Viral DNA was extracted and subjected to qPCR analyses at the end of treatment. In terms of inhibitory potency on extracellular DNA replication, EC_{50} of lamivudine from wild-type

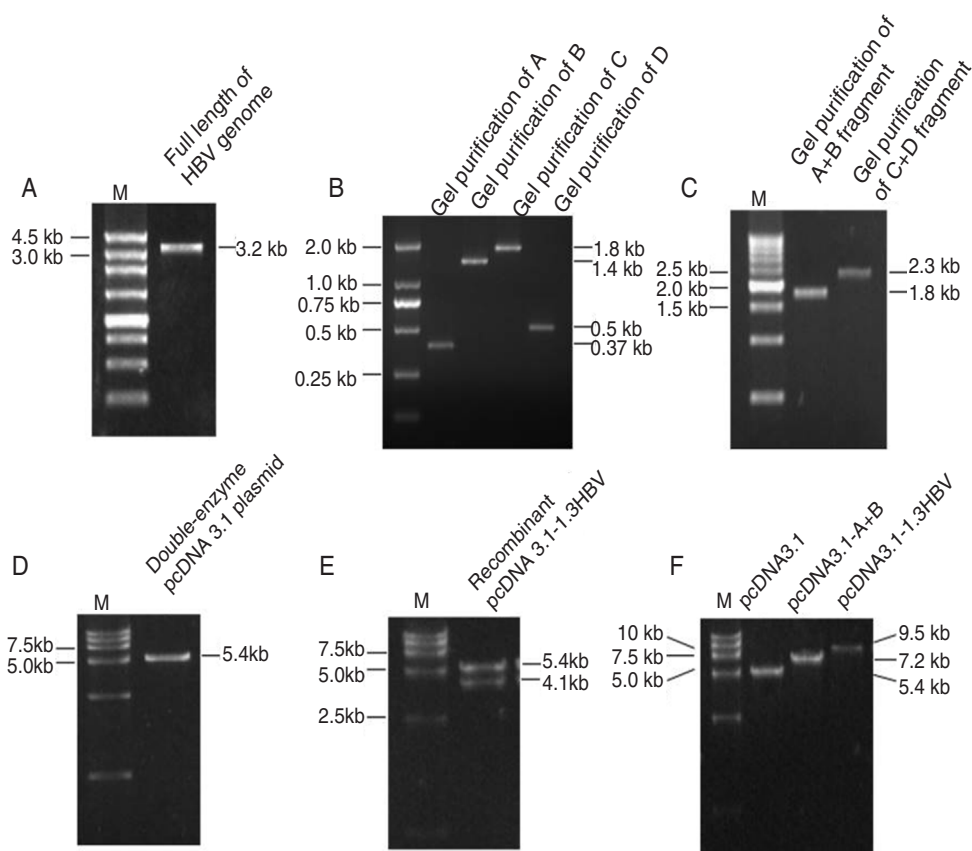


Fig. 2 – The PCR products of agarose gel electrophoresis analysis.

(A) Full-length HBV genome amplified by PCR. Lane M: 250 bp DNA Ladder Marker; Lane 1: the full length HBV genome (3215 bp). (B) Gel purification of A, B, C and D PCR fragments. Lane M: DL2000 DNA Marker; Lane 1, 2, 3, 4: gel purification of A (370 bp), B (1393) bp, C (1822 bp), and D (495 bp) PCR fragment, respectively. (C) Gel purification of A + B and C + D restriction enzyme digestion fragments. Lane M: 500 bp DNA Ladder Marker; Lane 1: gel purification of A + B fragment digested by *Hind* III and *Eco*R I; Lane 2: gel purification of C + D fragment digested by *Eco*R I and *Not* I. (D) Gel purification of double-enzyme digested pcDNA 3.1 plasmid. Lane M: DL15000 DNA Marker; Lane 1: gel purification of double-enzyme pcDNA 3.1 plasmid (5428 bp) digested by *Hind* III and *Not* I. (E) Restriction double-enzyme digestion identification of recombinant plasmid pcDNA3.1-1.3HBV by *Hind* III and *Not* I. Lane M: DL15000 DNA Marker; Lane 1: digestion of recombinant plasmid pcDNA3.1-1.3HBV by *Hind* III and *Not* I as pcDNA 3.1 plasmid (5428 bp) and A + B + C + D (4080 bp). (F) Enzyme digestion identification of recombinant plasmid pcDNA3.1-1.3HBV by *Hind* III. Lane M: DL15000 DNA Marker; Lane 1: digestion of plasmid pcDNA3.1 by *Hind* III; Lane 2: digestion of recombinant plasmid pcDNA3.1-A + B by *Hind* III; Lane 3: digestion of recombinant plasmid pcDNA3.1-1.3HBV by *Hind* III.

HBV and HepG2.RL1 cells were $0.16 \pm 0.06 \mu\text{M}$ and $>100 \mu\text{M}$, respectively (Fig. 5C), which indicates a >625 -fold-resistance. Based on the dose-response histogram, adefovir inhibited the replication of mutant HBV almost as efficiently as it did with wild-type HBV, with EC_{50} of $4.18 \pm 0.18 \mu\text{M}$ and $3.69 \pm 0.50 \mu\text{M}$, respectively (Fig. 5D). With respect to intracellular DNA, lamivudine was not active on the rtL180M/M204V double mutation carrying HepG2.RL1 cells ($\text{EC}_{50} > 100 \mu\text{M}$) compared to the wild-type HBV strain ($\text{EC}_{50} = 0.018 \pm 0.01 \mu\text{M}$), which represents a decreased susceptibility of more than 5556-fold (Fig. 5E). Adefovir inhibited replication of intracellular DNA in HepG2.RL1 cells, consistent with that seen in wild-type HBV, with EC_{50} of $2.24 \pm 0.11 \mu\text{M}$ and $1.36 \pm 0.08 \mu\text{M}$, respectively, both in a dose-dependent manner (Fig. 5F).

Effect of NCC on antigen production and viral replication in the two cell lines

Inhibitory efficiency of antiviral agents was assessed only with HBeAg. NCC dose-dependently inhibited HBeAg production by HepG2.2.15 cells and HepG2.RL1 cells (Fig. 6A). Consistent with the inhibitory effect on HBeAg production, NCC inhibited the replication of wild-type HBV and resistant HBV intracellular DNA in a dose-dependent manner. Importantly, there had no significant difference of the inhibitory rate between HepG2.2.15 cells and HepG2.RL1 cells (Fig. 6B), and EC_{50} s for the inhibition of replication of intracellular HBV DNA by NCC were $2.23 \pm 0.79 \mu\text{M}$ and $0.84 \pm 0.12 \mu\text{M}$, respectively. Moreover, NCC exposure produced dose-dependent inhibition

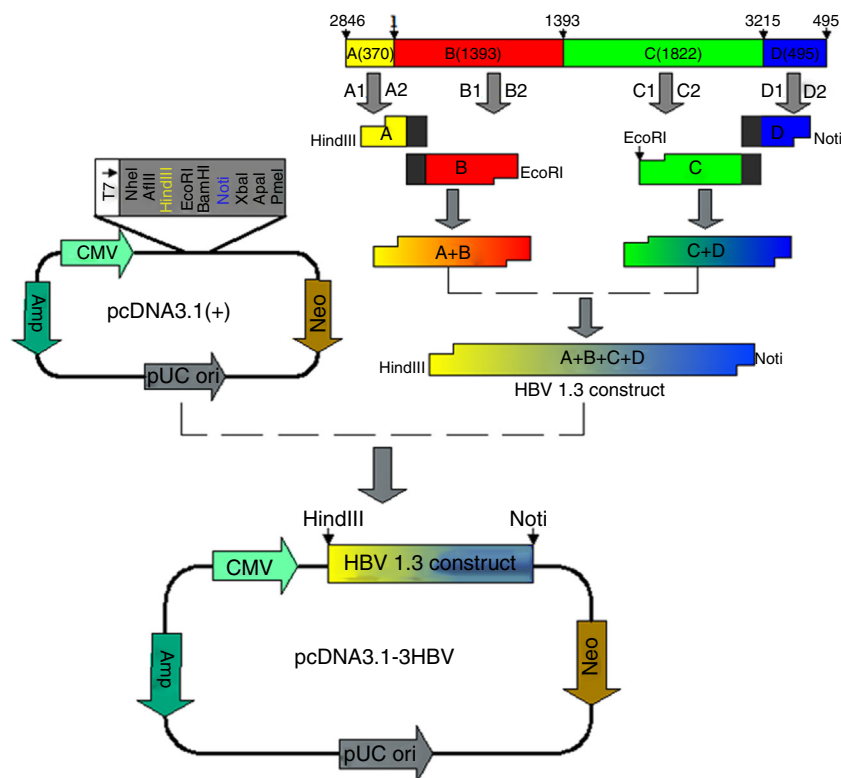


Fig. 3 – Construction of the pcDNA3.1-1.3HBV recombinant plasmid.

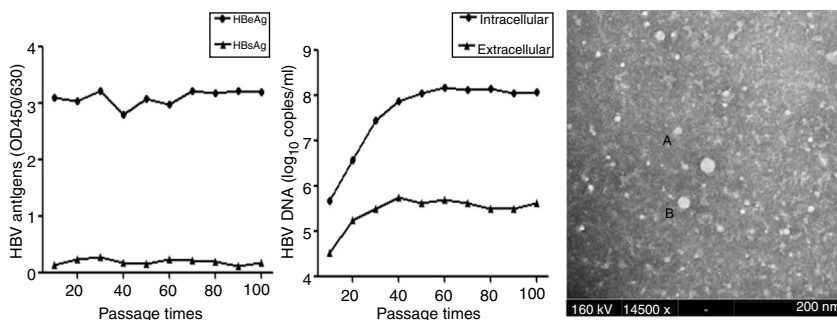


Fig. 4 – Time-course of secretion of viral antigens and HBV DNA, and electron micrograph of HBV particles in the HepG2.RL1 cell line

(A) Levels of HBeAg and HBsAg in the culture supernatants of the HepG2.RL1 cell line according to generation. OD_{450/630}, optical density at 450 nm and 630 nm. (B) Quantity of intracellular and extracellular DNA in HBV according to generation. (C) Shown here is a typical complex that includes some spherical HBsAg particles (diameter, 22 nm) (A) and a few Dane-like particles (42 nm) (B) (magnification 14,500×; bar: 200 nm; accelerating voltage: 160 kV).

of the replication of extracellular HBV DNA (Fig. 6C), with EC_{50s} of $0.14 \pm 0.07 \mu\text{M}$ and $0.46 \pm 0.09 \mu\text{M}$, respectively, for the HepG2.2.15 cells and HepG2.RL1 cells.

Discussion

In recent years, lamivudine has still remained widely used in the treatment of chronic hepatitis B either singly or in combination with other agents, mostly owing to its low cost.^{20,21} The prolonged use of lamivudine is associated with

emergence of principal mutations in the tyrosine-methionine-aspartate-aspartate (YMDD) motif within the C domain of HBV polymerase, which are the substitutions of valine, isoleucine, or (rarely) serine for methionine (rtM204I/V/S).^{22,23} The rtM204I substitution has been detected in isolation, but rtM204V and rtM204S are found only in association with other changes, which are at domain A (L80V/I, L82M, L91I)²⁴ and B (rtV173L, rtL180M).^{25,26} These additional compensatory mutations may stem from the natural evolution of the dynamics of quasispecies of HBV.²⁷ HBV genotypes have distinct geographical distributions and correlate with different clinical

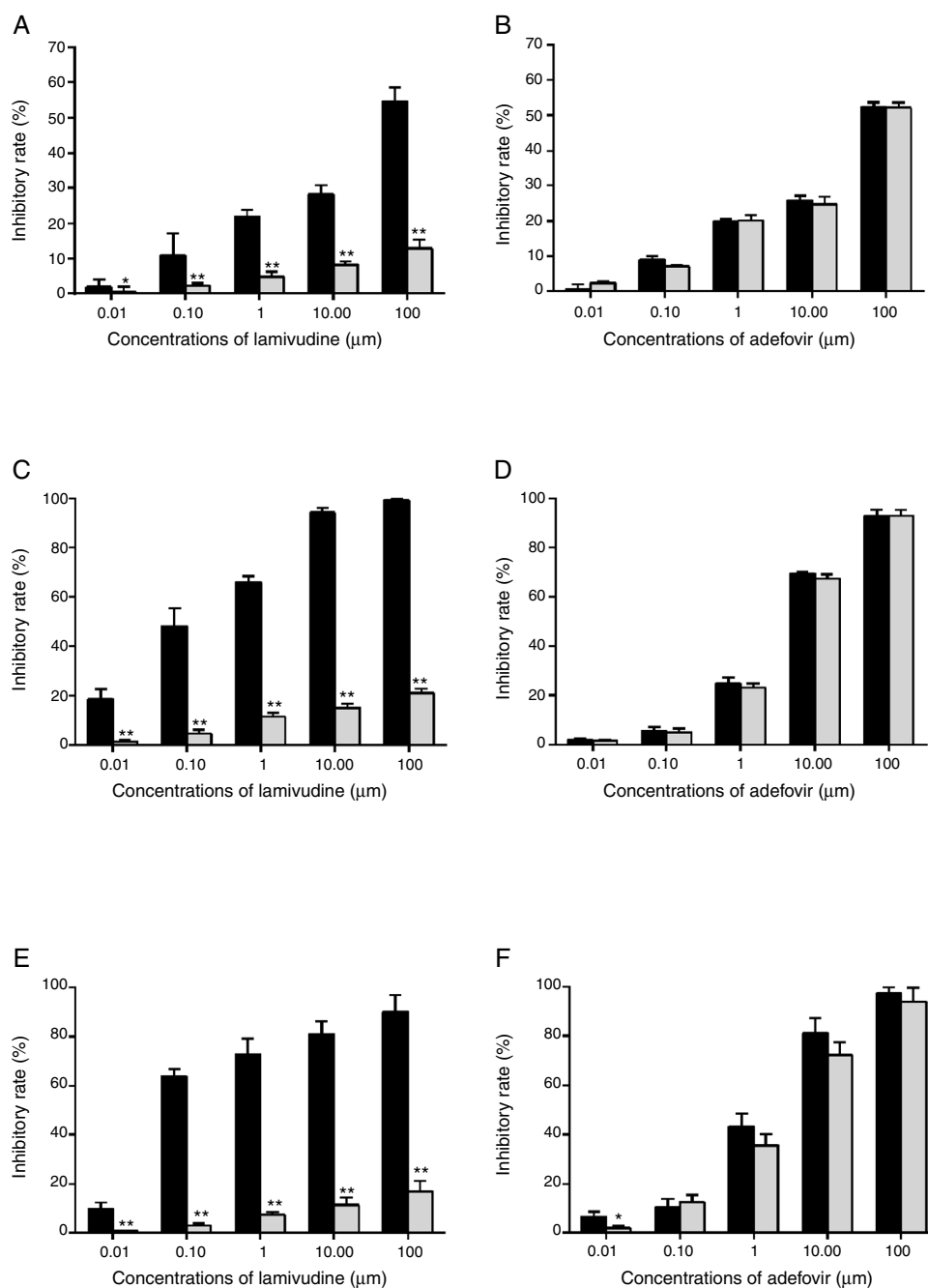


Fig. 5 – Drugs susceptibility to antigens secretion and viral replication.

HepG2.2.15 cells and HepG2.RL1 cells were treated with lamivudine and adefovir for six days. Cell-culture supernatants were assayed using a specific ELISA kit. Viral DNA was extracted from the culture medium and cells, and then quantified by qPCR using a commercial kit. Experiments were done from wild-type and lamivudine-resistant HBV isolates, on the inhibitory effects of lamivudine (A) and adefovir (B) on HBeAg production; inhibitory efficiency of lamivudine (C) and adefovir (D) on extracellular production of HBV DNA; inhibitory efficiency of lamivudine (E) and adefovir (F) on intracellular production of HBV DNA. Mean values of inhibition from three independent measurements are shown as bars with standard-error values indicated at the top. ***p* < 0.01, **p* < 0.05, compared with the no-drug control group. Black bar: wild-type HBV; gray bar: lamivudine-resistant HBV with rL180M/M204V mutations.

outcomes. Previous reports indicated that genotypes B and C are predominant in China.²⁸ Therefore, serial plasmids containing a specific HBV genotype (e.g., genotype B) and lamivudine-resistant have more practical clinical value.

In the HBV genome, the surface gene is overlapped completely by the polymerase gene.²⁹ Hence, mutations in the reverse transcriptase domain associated with lamivudine-resistance might produce sequence/structural changes in the

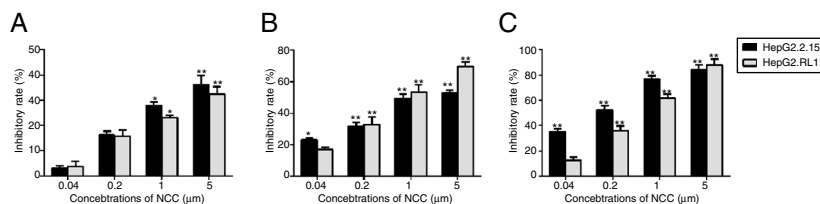


Fig. 6 – Effect of NCC on antigen secretion and viral replication.

HepG2.2.15 cells and HepG2.RL1 cells were treated with the indicated concentrations of NCC for 6 days. HBeAg was tested using a specific ELISA kit (A). The intracellular DNA (B) and extracellular DNA (C) was quantified by qPCR using a commercial kit. Experiments on the inhibitory effects of NCC on HBeAg production and replication of HBV DNA from HepG2.2.15 cells (black bar) and HepG2.RL1 cells (gray bar) were performed in triplicate. Mean values of inhibition from three independent measurements are shown as bars with standard-error values indicated at the top. ** $p < 0.01$, * $p < 0.05$, compared with the no-drug control group.

surface gene and its envelope protein that result in reduced antigenicity of the protein in the surface antigen or provoke stop codons in the surface gene, and may even affect the replication and infectivity of the virus.³⁰⁻³² It has been suggested that ELISA cannot detect the I195 M HBsAg protein efficiently.³³ Thus, HepG2.RL1 cells secreted HBsAg at a lower level than HBeAg during the previous 100 generations, and the inhibitory efficiency of antiviral agents in this study was assessed based only on HBeAg production.

The impact of the lamivudine-resistant mutants on the observed efficacy of the drugs can best be described by the corresponding fold-resistance values in EC_{50} *in vitro*. For lamivudine, treatment failure is associated with >100-fold increase in EC_{50} . In our drug susceptibility testing, the fold changes in lamivudine activity against extracellular DNA and intracellular DNA in HepG2.RL1 cells were >625 and >5556, respectively, by qPCR. It is noteworthy that the EC_{50} values of lamivudine and adefovir tested under our *in vitro* conditions were different from those obtained in other observations with stable transfected cell lines.³⁴⁻³⁷ The EC_{50} variations among different studies may be because of differences in the HBV genome and plasmids transfected (by site-directed mutagenesis or from clinical isolates), the time of action and the viral DNA quantification methods used for phenotypic assays. Therefore, the EC_{50} values of each drug determined against wild-type and mutant HBV viruses should only be compared in parallel experiments. However, the fold-resistance values are comparable with those found in previous studies,^{34,36} and confirmed that viruses containing these mutations show a remarkably decreased sensitivity to lamivudine but not to adefovir. Moreover, unlike transient transfection assays, stable cell line-based assays are less variable among experiments and thus suitable for high throughput drug screening.

NCC was selected from a series of 4-N-alkyl-substituted fluoronucleosides, which have been synthesized in order to address the toxicity issue of the parent compound FNC (2'-deoxy-2'-β-fluoro-4'-azido-β-D-arabinofuranosyl cytidine),³⁸ and after *in vivo* evaluation, NCC exhibited a significantly improved toxicity profile in mice. The results from the MTT test showed that NCC inhibited the growth of HepG2.2.15 cells and HepG2.RL1 cells with CC_{50} s of $725.81 \pm 23.34 \mu\text{M}$ and

$782.38 \pm 12.17 \mu\text{M}$, indicating that NCC causes less toxicity on cells and lower cytotoxicity than adefovir. The *in vitro* anti-HBV activity of NCC had been assessed in the HepG2.2.15 cells and HepG.CW cells in the previous study.¹⁵ In this study, we showed that NCC is a potent inhibitor of wild-type HBV *in vitro*. Moreover, the present study showed that NCC inhibited the replication of lamivudine-resistant HBV clinical isolates in a dose-dependent manner. In HepG2.RL1 cells, NCC inhibited HBeAg expression almost as efficiently as adefovir, while inhibited intracellular DNA replication more efficiently than adefovir ($0.84 \pm 0.12 \mu\text{M}$ versus $2.24 \pm 0.11 \mu\text{M}$). It is interesting that the EC_{50} value of NCC on extracellular DNA replication is still much lower than that of adefovir (0.46 ± 0.09 versus $4.18 \pm 0.18 \mu\text{M}$), even though the resistance factor of NCC is a little higher than that of adefovir (3.29 versus 1.13). The promising activity of NCC against the lamivudine-resistant HBV may be associated with the novel chemical structure of NCC such as 3'-OH, 4'-azido and N-cyclopropyl, which do not share the same resistance profile as lamivudine. Furthermore, the *in vitro* therapeutic potency of a drug should take into account its antiviral capacity and cytotoxicity because a high therapeutic index (EC_{50}/CC_{50}) is preferable for a drug to have a favorable safety profile. In combination with the above results, the *in vitro* cross-resistance profile of NCC for the clinical lamivudine-resistant strain studied here was more favorable than those of adefovir.

Conclusion

In conclusion, the HepG2.RL1 cell line, established by introducing a clinical lamivudine-resistant HBV isolate with rTL180M/M204V mutations into HepG2 cells, was capable of stably producing HBV virions. The cell line should be helpful for screening antiviral agents active against lamivudine-resistant HBV and can streamline the screening protocol and directly evaluate the effects of new antiviral agents on clinical HBV strains. In addition, our studies indicate that NCC possesses antiviral activity against both wild-type and lamivudine-resistant HBV with low toxicity, and suggest that NCC deserves further development as a new and potential inhibitor of resistant HBV.

Conflicts of interest

The authors declare no conflicts of interest.

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