Original article

Novel nucleoside analogue FNC is effective against both wild-type and lamivudine-resistant HBV clinical isolates

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Background: HBV infection causes major public health problems worldwide. The clinical limitation of current antiviral drugs for HBV, such as lamivudine, is causing rapid emergence of drug-resistant viral strains during prolonged antiviral therapy. Therefore, new antiviral drugs are urgently needed to prevent or delay the selection of drug-resistant HBV mutants. A novel cytidine analogue, FNC (2'-deoxy-2'β-fluoro-4'azidocytidine), was recently shown to strongly inhibit human HBV and duck HBV (DHBV) replication in vitro and in vivo, respectively. The present study was designed to evaluate the in vitro antiviral activity of FNC against clinical wild-type and lamivudine-resistant HBV isolates in transiently transfected cells. Methods: HBV DNA was extracted from serum samples collected both before lamivudine therapy and at the time of viral breakthrough and was amplified by PCR. The amplicon was cloned into a novel expression vector, pHY106, which can initiate the intracellular HBV replication cycle after cell transfection. Following transfection of the cloned amplicon into HepG2 cells, a drug susceptibility assay was performed. Quantitative real-time PCR was used for determining the amount of intracellular HBV DNA, and the effective concentration required to reduce HBV replication by 50% (EC50) was calculated.

Results: FNC inhibited the replication of both wild-type and lamivudine-resistant HBV clinical isolates in a dose-dependent manner, with mean ±sd EC50 values of 0.12 ±0.01 μM and 0.27 ±0.01 μM, respectively.

Conclusions: FNC is a potential antiviral agent against both wild-type and lamivudine-resistant HBV clinical isolates, and therefore deserves further evaluation for the treatment of HBV infection.

Introduction

Despite the universal vaccination of neonates and infants during the past two decades and the subsequent decrease in the incidence of new infections with HBV, chronic hepatitis B remains a major health problem worldwide. During this period, the prognosis of chronic hepatitis B has been significantly improved due to the emergence of two formulations of interferon and five orally administered nucleoside/nucleotide analogues [1]. The orally active nucleoside/nucleotide analogues, such as lamivudine, adefovir, entecavir, telbivudine and tenofovir, are efficacious in suppressing viral replication, decreasing inflammation and preventing the progression of chronic hepatitis B to liver cirrhosis and hepatocellular carcinoma [2]. However, the prolonged use of nucleoside/nucleotide analogues frequently result in the emergence of drug-resistant HBV mutants, which are responsible for therapeutic failure and progression of liver disease [3]. It is reported that up to 76% of patients developed viral resistance after 5 years of lamivudine therapy [4]. The signature mutations rtM204V/I, which have been mapped in the conserved YMDD motif within the C domain of the reverse transcriptase (RT), are frequently associated with lamivudine resistance [5]. Furthermore, they are often associated with the compensatory mutations rtL180M or rtV173L in the B domain of the RT, which partially restore the replicative capacity of the YMDD mutation strains [6,7].
One in vitro study demonstrated that drug susceptibility decreased >10,000-fold once lamivudine-resistant HBV mutants were selected [8]. Moreover, the rapid progression or acute exacerbation of hepatitis usually occurs after the emergence of lamivudine resistance [9]. Thus, the discovery and development of novel anti-HBV drugs is urgently needed for the treatment of chronic hepatitis B, especially for rescue therapy of drug-resistant patients.

Fluoronucleosides have a history of being well-phosphorylated by cellular kinases and can be good substrates for RNA and DNA polymerases. FNC, 2′-deoxy-2′-β-fluoro-4′-azidocytidine, is a novel cytidine analogue that is an excellent substrate for deoxycytidine kinase and can be phosphorylated with higher efficiency than deoxycytidine [10–12]. In the early reports, FNC was proven to possess potent anti-HIV and anti-HCV activity [13,14]. Furthermore, our recent observations demonstrated that FNC was an efficient inhibitor of wild-type HBV in the stable HBV-producing cell line HepG2.2.15 (HepG2). Meanwhile, FNC showed a favourable safety profile and inhibited virus replication in duck HBV (DHBV)-infected ducks with significant reduction of serum DHBV DNA levels and restoration of liver histology [15]. However, the activity of FNC on lamivudine-resistant HBV mutant remains unclear.

Accordingly, the present study was designed to compare the antiviral activities of FNC with those of lamivudine and adefovir in parallel antiviral assays in hepatoma cells transiently transfected with either wild-type or lamivudine-resistant clinical isolates.

Methods

Patient
A 26-year-old chronically HBV-infected Chinese man initially received oral lamivudine monotherapy at 100 mg per day. The patient’s serum HBV DNA decreased from $1.4 \times 10^8$ copies/ml to an undetectable level after receiving 12 months of lamivudine monotherapy, but rebounded to $4.8 \times 10^6$ copies/ml after a total of 16 months of lamivudine monotherapy, and continued to rise to the initial level 3 months later. At this time point, a genotypic analysis by direct sequencing of the polymerase RT region of the circulating HBV was performed and revealed the presence of rtV173L/L180M/M204V triple mutations. The resistant HBV strain was genotype C. The patient was seropositive for hepatitis B surface antigen and hepatitis B e antigen. Serum samples were collected before lamivudine therapy and at the time when viral load increased to the initial level for drug susceptibility analysis. Written informed consent was obtained from the patient.

Compounds
FNC (Figure 1) with a purity of 98.5%, as determined by HPLC, was designed and synthesized in our laboratory (The College of Chemistry and Molecular Engineering, Zhengzhou, China). Lamivudine was provided by GlaxoSmithKline Co., Ltd (Suzhou, China). Adefovir, used as a positive control for the inhibition of the lamivudine-resistant HBV strain, was obtained from GlaxoSmithKline Co., Ltd (Tianjin, China). Stock solutions (10 mM) of FNC, lamivudine and adefovir were dissolved in phosphate-buffered saline (PBS) and diluted with culture medium to the desired working concentrations when used.

Cell line and plasmid
The human hepatoma cell line HepG2 was obtained from the China Center for Typical Culture Collection (Wuhan, China). The cells were cultured at 37°C in a humidified 5% CO₂ atmosphere in Dulbecco’s modified Eagle’s medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Birmingham, MI, USA), 100 units/ml penicillin G and 100 μg/
ml streptomycin. pHY106, a novel plasmid expression vector, was constructed and kindly provided by William Delaney and Huiling Yang (Gilead Sciences, Foster City, CA, USA). The plasmid contains 199 bp of HBV sequence and two SapI sites that allow insertion of the SapI-treated PCR-amplified HBV genome [16,17].

Isolation and sequence analysis of clinical HBV strains
HBV DNA was extracted from 200 µl of serum samples using a commercial viral DNA extraction kit (Omega Bio-Tek, Norcross, GA, USA). The full-length HBV genome was amplified by PCR using the primers and thermocycling conditions described by Günther et al. [18]. The PCR product was digested with SapI (MBI, Fort Collins, CO, USA) and then ligated into the SapI-digested and shrimp alkaline phosphatase-treated pHY106 plasmid, producing a recombinant clone termed pHY106-HBV. The recombinant clone served as a template for sequencing the conserved HBV polymerase RT region. The result indicated that the cloned HBV genome that was directly isolated from the serum of the lamivudine-resistant patient harboured the rtV173L/L180M/M204V triple mutation. As expected, the cloned HBV genome from the serum collected before lamivudine therapy contained no mutation and was used as a wild-type control.

Cytotoxicity assay
The cytotoxicity of the tested compounds to HepG2 cells was determined by the MTT assay as described by Wei et al. [19], except that the duration of drug administration was 6 days, with the media refreshed every 2 days. Each compound was assayed in triplicate with ≥5 concentrations. The cytotoxic concentrations of the compounds reducing cell viability by 50% (CC50) were determined.

Transfection and drug treatment
The selected recombinant clones containing wild-type or mutated HBV genome were transiently transfected into HepG2 cells. Briefly, 6-well tissue culture plates (Corning Inc., Corning, NY, USA) were seeded at 6×10^4 HepG2 cells/well 16 h earlier and transfected with 4 µg DNA using 10 µl lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. After determining the effects of the compounds on HepG2 cell viability, drug treatment was initiated the next day and lasted for 6 days, with replacement of fresh drug-free or drug-containing media every 2 days. The concentrations of FNC, lamivudine and adefovir used for the drug susceptibility assays were 0, 0.01, 0.1, 1 and 10 µM. On the last day of treatment, the cells were collected and stored at -20 °C for the subsequent experiments.

Quantification of intracellular HBV DNA
The quantity of intracellular HBV DNA was determined by quantitative real-time PCR based on the TaqMan technology. In brief, the cells were rinsed twice with cold PBS and lysed with 500 µl of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.5% (v/v) Nonidet P-40. After centrifugation at 13,000 rpm for 3 min, supernatants were transferred to fresh microcentrifuge tubes, and MgCl2 was added to give a final Mg2+ concentration of 10 mM. Any input plasmid contamination was then digested for 1 h at 37°C with 20 U of DNase I before the reaction was terminated with 10 mM EDTA. HBV DNA was purified using a commercially available viral DNA extraction kit (Omega Bio-Tek, Norcross, GA, USA). Quantitative real-time PCR was performed in a LightCycler (Roche, Mannheim, Germany) with the HBV Fluorescent Quantitative PCR Detection Kit (PG Biotech, Shenzhen, China) according to the manufacturer's instructions. 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. For each compound tested in the present assay, the effective concentration required to reduce HBV replication by 50% (EC50) was calculated by regression analyses.

Statistical analysis
Statistical analysis was performed using the SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). The data are expressed as the means ±SD. Student’s t-test and one-way ANOVA were used to determine the statistical significance of differences between groups. A value of P<0.05 was considered statistically significant.

Results
Effect of compounds on cell viability
Cell viability of HepG2 cells was evaluated by the MTT assay after exposure to varying concentrations of compounds for 6 days. Results showed that neither FNC nor lamivudine could cause 50% reduction in cell viability at the highest drug concentrations of 1,000 µM, whereas the CC50 value for adefovir was 215.06 ±18.75 µM (Figure 2).

Wild-type HBV is susceptible to FNC
To assess the inhibitory potency of antivirals on wild-type HBV replication, intracellular HBV DNA was extracted and submitted to real-time PCR analysis after exposure to varying concentrations of drugs for 6 days. As demonstrated in Figure 3, FNC, lamivudine and adefovir inhibited the replication of wild-type HBV in a dose-dependent manner. Based on the results of three independent experiments, the most potent inhibition of replication of wild-type HBV was observed with FNC (EC50=0.12 ±0.01 µM), followed by lamivudine (EC50=0.81 ±0.05 µM) and adefovir (EC50=4.19 ±0.38 µM; Table 1).
Lamivudine-resistant HBV is susceptible to FNC

The inhibitory activity of the tested compounds on the replication of lamivudine-resistant triple mutant was evaluated after transient transfection of HepG2 cells. The lamivudine sensitivity of mutant HBV and the wild-type strain was compared in order to confirm that the rtV173L/L180M/M204V triple mutations conferred lamivudine resistance to the HBV clinical isolates used in this study. As expected, lamivudine was not as active on the rtV173L/L180M/M204V triple mutant as it was on wild-type HBV. It was impossible to determine the accurate EC$_{50}$ value or resistance factor for the triple mutant, which could not be inhibited by 50%, even at the highest lamivudine concentration of 10 $\mu$M in this assay. As previously observed, adefovir inhibited the replication of mutant HBV almost as efficiently as it did that of wild-type HBV with an EC$_{50}$ value of 5.18 ±0.42 $\mu$M. Similarly, exposure to FNC produced dose-dependent inhibition of replication of the lamivudine-resistant HBV variant, although higher concentration was required to produce 50% inhibition (EC$_{50}$=0.27 ±0.01 $\mu$M; Figure 4, Table 1). It is noteworthy that the differences were not statistically significant, suggesting that lamivudine-resistant HBV mutant remains susceptible to FNC.

**Discussion**

The emergence of HBV strains resistant to antiviral treatments is a major clinical concern. With the expanding use and prolonged administration of lamivudine, rtV173L/L180M/M204V triple mutant, in which rtV173L and rtL180M act as compensatory mutations partially restoring the replicative capacity of rtM204V
mutation strains, was selected more and more frequently and became the next most common mutation profile associated with lamivudine resistance [20]. One alternative, delaying or overcoming the occurrence of drug-resistant mutants, is to switch to or add-on novel drugs that do not share the same resistance profiles. Our previous study has demonstrated that FNC was a potent inhibitor against human HBV and DHBV replication in vitro and in vivo, respectively [15]. However, the activity of FNC on lamivudine-resistant HBV mutant remains unclear. In our unpublished observation, we isolated four major patterns of lamivudine-resistant HBV variants, termed rtL180M/M204V, rtV173L/L180M/M204V, rtM204I and rtL80I/M204I, from four lamivudine-experienced patients, and determined the replication levels of these mutants. The rtV173L/L180M/M204V triple mutant was shown to have the most potent replication capacity (approximately 90% of wild-type isolates), which was consistent with the results reported by Delaney et al. [6]. Hence, in the present study, we investigated the in vitro antiviral activity of FNC against this lamivudine-resistant HBV clinical isolate in an rtV173L/L180M/M204V triple mutant transiently transfected cell line.

Due to the lack of a proofreading function of HBV polymerase, combined with the high level viral replication in infected liver cells of chronic hepatitis B patients, it is estimated that $10^{10}$ base-pairing errors occur daily [21]. Consequently, HBV in chronically infected patients harbours many virus quasispecies, which contain a diversity of genetic contexts. Presently, the in vitro drug susceptibility assessments of HBV mutants commonly depend on the methods that artificially reproduce one or a few resistant mutations, usually by site-directed mutagenesis of a laboratory strain, on a wild-type virus backbone [22]. However, viruses created by the site-directed mutagenesis of a laboratory strain would not contain the natural genetic context of a mutation identified in clinical strains, and discrepancies between the susceptibilities to antivirals of laboratory- and patient-derived HBV mutants will emerge [23]. Therefore, phenotypic analysis of HBV clinical mutants isolated directly from the sera of patients would offer more relevant and accurate information than that obtained from testing viruses with mutations introduced into laboratory strains.

Table 1. Antiviral activity of the tested compounds against wild-type and lamivudine-resistant HBV

<table>
<thead>
<tr>
<th>Compound</th>
<th>Wild-type EC₅₀ μM±sd</th>
<th>Lamivudine-resistant EC₅₀ μM±sd</th>
<th>Resistance factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>FNC</td>
<td>0.12 ±0.01</td>
<td>0.27 ±0.01</td>
<td>2.25</td>
</tr>
<tr>
<td>Lamivudine</td>
<td>0.81 ±0.05</td>
<td>&gt;10</td>
<td>&gt;12</td>
</tr>
<tr>
<td>Adefovir</td>
<td>4.19 ±0.38</td>
<td>5.18 ±0.42</td>
<td>1.24</td>
</tr>
</tbody>
</table>

Data are means ±sd of three independent experiments performed in triplicate. *The effective concentration required to reduce HBV replication by 50% (EC₅₀). †The ratio of mutant EC₅₀ and wild-type EC₅₀. FNC, 2′-deoxy-2′-β-fluoro-4′-azidocytidine.

Figure 4. Effects of antivirals on replication of lamivudine-resistant HBV

HepG2.2.15 cells were transiently transfected with lamivudine-resistant HBV genome. At 24 h after transfection, the cultures were treated with the indicated concentrations of lamivudine (3TC), adefovir (ADV) or FNC (2′-deoxy-2′-β-fluoro-4′-azidocytidine). After 6 days of treatment, viral DNA was extracted from the cultured cells and quantified by real-time quantitative PCR using a commercially available kit. The experiments were performed in triplicate, and the data are presented as the means ±sd of three independent experiments. *P<0.05 and †P<0.01 compared with the no-drug control group.
In the present study, a previously reported expression vector for cloning the entire HBV genome was used to facilitate the replication of clinical HBV isolates. The full-length HBV genome, amplified with high-fidelity PCR polymerase from the serum of a chronic hepatitis B patient who had well-documented clinical lamivudine resistance after receiving lamivudine monotherapy, was cloned into pHY106 and used for drug susceptibility assays. The sequences of our individual clone matched that of its respective patient serum HBV (data not shown), indicating that the constructed individual clone represented the dominant serum HBV quasispecies population. Thus, the drug susceptibility results obtained in this report would precisely reflect those of the serum HBV itself.

We recently assessed the in vitro potency of FNC for the inhibition of wild-type HBV in a stably expressing HBV cell line HepG2 [15]. In the present study, we confirmed the findings of our previous studies, which showed that FNC is a potent inhibitor of wild-type HBV in vitro. In HepG2 cells transiently transfected with the wild-type HBV genome, FNC inhibited HBV replication more efficiently than lamivudine, whereas adefovir displayed a relatively weaker antiviral activity. Moreover, we provided new information that showed that FNC inhibited the replication of lamivudine-resistant HBV clinical isolates in a dose-dependent manner. Although a higher concentration was required to produce 50% inhibition of the viral replication, this mutant was still sensitive to FNC according to the criteria defined by Chin et al. [24] (resistance factor <5). It is interesting that the absolute EC50 value of FNC is still much lower than that of adefovir for lamivudine-resistant HBV mutant (0.27 ±0.01 μM versus 5.18 ±0.42 μM), even though the resistance factor of FNC is a little higher than that of adefovir to this variant (2.25 versus 1.24). This promising activity of FNC against lamivudine-resistant HBV may be associated with the novel chemical structure of FNC (for example, 3′-OH and 4′-azido; Figure 1) [13], which does not share the same resistance profile as lamivudine.

It is noteworthy that the EC50 values of compounds tested under our in vitro conditions were different from those obtained in our previous study with a stable cell line derived from HepG2 cells and those obtained in other observations with transiently transfected cell lines [8,15,23,24]. The EC50 variations among different studies may be explained by differences in the cell lines used, the HBV genome and plasmids transfected, the duration of drugs administration and the intracellular viral DNA measuring methods employed for phenotypic assays. However, the ranking of antiviral potency of the tested compounds was not affected. In addition, in our drug susceptibility testing, the fold shift in lamivudine activity against the rtV173L/L180M/M204V triple mutant was only >12, which was much lower than that reported by other researchers [6]. Actually, we evaluated the antiviral activity of lamivudine against this triple mutant using the maximum concentration of 100 μM in the present study. As expected, the replication capability of this mutant could not be reduced by 50% after exposure to 100 μM of lamivudine for 6 days. Accordingly, the resistance factor of this triple mutant to lamivudine is >120. Furthermore, compared with the 10 μM group, the inhibitory efficacy of wild-type HBV replication was not enhanced significantly in the 100 μM group. In other words, lamivudine reaches the peak of its antiviral activity at the concentration of 10 μM, as well as adefovir and FNC. Thus, the 100 μM data for lamivudine, adefovir and FNC were not shown in our manuscript. Another reason for the lower fold shift in the present study is the relative higher wild-type EC50 of lamivudine in our drug susceptibility assay.

Of course, the in vitro therapeutic potency of a compound does not depend only on its antiviral capacity. Its cytotoxicity also has to be taken into account, since the selectivity index (CC50/EC50) of a drug may prevent the use of optimal dose to reach a more potent antiviral effect [25]. Interestingly, the present study in comparison of the cytotoxic effects of FNC, lamivudine and adefovir in HepG2 cells indicated that FNC had similar cytotoxicity to lamivudine, whereas adefovir was relatively more cytotoxic. Consequently, in combination with the antiviral capacity of the compounds tested, it will be interesting to evaluate the pharmacodynamics of FNC in vivo.

In conclusion, the potent inhibitory activity of FNC against wild-type and lamivudine-resistant HBV isolates and its favourable cytotoxicity profile deserves further investigation. This compound may be a new alternative candidate for the treatment of chronic hepatitis B patients who have developed resistance to lamivudine. Furthermore, FNC might also be valuable for combination therapies with approved nucleoside/nucleotide analogues to prevent or delay the emergence of drug-resistant mutants in chronically HBV-infected patients [26].

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Disclosure statement

All authors declare no competing interests.

References


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