Background: New drugs are needed to combat HBV infection. We investigated the anti-HBV activity of the deoxycytidine analogue FNC, which has anticancer activity and has been found to inhibit HCV replication.

Methods: In this study, a human hepatoma HepG2.2.15 cell culture system and duck HBV (DHBV) infection model were used as the in vitro and in vivo models to evaluate the anti-HBV activity of FNC.

Results: In the cell model, FNC effectively suppressed the secretion of the HBV antigens in a dose-dependent manner, with 50% effective concentration values of 0.037 μM for hepatitis B surface antigen and 0.044 μM for hepatitis B e antigen on day 9. Consistent with the HBV antigen reduction, FNC also reduced the HBV DNA level by 92.31% and 93.90% intracellularly and extracellularly, respectively. DHBV DNA levels were markedly reduced after treatment with the FNC at 0.5, 1.0 and 2.0 mg/kg•day dosages. The inhibition rate of FNC at the dose of 2.0 mg/kg•day reached 91.68% and 81.96%, in duck serum and liver, respectively, on day 10. Furthermore, significant liver histology restoration after FNC treatment was observed, as evaluated by the histopathological analysis.

Conclusions: FNC can evidently inhibit the replication of HBV in the HepG2.2.15 cell line in vitro and inhibits DHBV replication in ducks in vivo. It could be potentially developed into a new anti-HBV drug.

Introduction

HBV infection remains a major public health problem worldwide. Globally, HBV is one of the most prevalent blood-borne viruses causing infectious disease. It is estimated that 2 billion people worldwide have been infected with HBV and 400 million people are chronic carriers [1–3]. Chronic HBV infection is a serious clinical problem and a major cause of severe liver-related morbidity and premature mortality. Several antiviral drugs, including interferon and five nucleoside/nucleotide analogues, are currently used to treat chronic hepatitis B [2–4]. Yet the efficacy of treatments can be affected by a number of factors, including frequent side effects, drug resistance, requirement of frequent and large doses of drugs, and most importantly, viral rebound with exacerbation of liver pathology after cessation of therapy [1,5,6]. In light of these problems, it is an urgent task for researchers to look for novel, safe and effective anti-HBV drugs.

FNC, 2′-deoxy-2′-β-fluoro-4′-azidocytidine, is a novel cytidine analogue. It is an excellent substrate for deoxycytidine kinase and is phosphorylated with higher efficiency than deoxycytidine [7]. It is designed to address the above problems based on the fundamentals of both organic chemistry and biochemistry, as well as previous findings of a relationship between biological activity and the structure of nucleoside derivatives [8]. Recent studies have demonstrated that FNC is a highly potent and selective inhibitor of HCV replication with a mean ± SD 50% effective concentration (EC50) of 24 ±3 nM [9]. As compared with other nucleoside/nucleotide analogues, FNC, a moderate inhibitor of NS5B (HCV-RNA-dependent RNA polymerase), possesses high selectivity for RNA templates, differentiated resistance profiles, high potency for the inhibition of HCV replication, exceptionally high phosphorylation efficiency in human target cells, and potential for achieving safety

Original article

Antiviral activity of FNC, 2′-deoxy-2′-β-fluoro-4′-azidocytidine, against human and duck HBV replication

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and tolerability profiles [9]. In addition, FNC inhibits cell proliferation, induces G1 and S phase arrest and promotes apoptosis in a number of human cancer cell lines [5,7]. To explore new potential clinical indications of FNC, in this study we have focused on its anti-HBV activity. Based on previous studies, we used HepG2.2.15 cell line and duck HBV (DHBV)-infected duck models to observe and evaluate the in vitro and in vivo anti-HBV activity of FNC. In vitro experiments revealed that FNC reduces the levels of the HBV antigens and DNA in the HBV-transfected liver cell line. More importantly, in vivo antiviral activity of FNC in DHBV-infected ducks was also observed.

Methods

Drugs and reagents in experiment

Lamivudine (3TC) was purchased from GlaxoSmithKline (Suzhou, PR China) and served as the positive control. FNC was designed and synthesized in a laboratory (Department of Chemistry, Zhengzhou University, Zhengzhou, PR China) with purity of 98.5%. The 10 mM stock solution in distilled water was prepared and then further diluted to the desired concentrations for in vitro experiments. For in vivo experiments in ducks, the drug solutions were administered orally once a day.

RPMI 1640 medium was obtained from Solarbio Science & Technology Co., Ltd. (Beijing, PR China). Fetal bovine serum, G-418 and MTT were purchased from Sigma (St Louis, MO, USA). Hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) enzyme immunoassay (ELISA) kits were purchased from Kehua Gen (HBsAg) and hepatitis B e antigen (HBeAg) enzyme immunoassay (ELISA) kits were purchased from Kehua Bio-engineering Corporation, (Shanghai, PR China). The DNA Extraction Kit was obtained from Omega (Bio-Tek, Norcross, GA, USA).

Cell culture and treatment

HepG2.2.15 (clonal cells derived from human hepatoma cell line HepG2) cells were provided by the Wuhan Institute of Virology (Wuhan, PR China). The HepG2.2.15 cell line was maintained in our laboratory in RPMI 1640 medium supplemented with 10% fetal bovine serum, 200 µg/ml G418 at 37°C in a humidified atmosphere containing 5% CO2.

Cytotoxicity (MTT) assay

MTT assays were used to detect the survival rates of HepG2.2.15 cells [10-13]. Cells were seeded in 96-well culture plates at a density of 2×10^4 cells/ml (100 µl/well) and cultured at 37°C for 24 h. The culture medium was then removed and replaced with fresh medium supplemented with various concentrations (cells were incubated in culture medium supplemented with 3.34, 16.72, 83.6, 419.2 and 2,096 µM in 96-well plates for 9 days) of FNC (4 wells/concentration). Cells incubated without drug were used as a negative control. After 9 days of culture, the cytotoxic effect of FNC was evaluated by MTT assay. By the end of the incubation, 20 µl of MTT solution (5 mg/ml) was added to each well, and incubation was allowed to continue for another 4 h at 37°C. Finally, 200 µl of DMSO was added to each well to solubilize the formazan. The absorbance (A) at 490 nm was measured by using an automatic plate reader (168-1000XC; BioRad, Hercules, CA, USA). The survival ratio (%) of HepG2.2.15 cells was calculated using Equation 1:

\[
\text{Survival rate} \, (\%) = \frac{A_{490} \text{ of experimental group}}{A_{490} \text{ of negative control}} \times 100\%.
\] (1)

Then, the 50% cytotoxic concentration (CC50) was calculated according to the Reed–Muench method [10,14].

Detection of HBsAg and HBeAg

The HepG2.2.15 cells were plated at a density of 2×10^4 cells per well on 24-well cell culture plates and routinely cultured. FNC solutions with different concentrations (0.04, 0.2 and 1.0 µM) were supplemented to the medium in triplicate for 24 h after cells were plated. Cells incubated with 3TC were used as a positive control (20 µM). After incubation with FNC for 3, 6 or 9 days, the supernatants and cells were collected, and then the supernatants were used for HBsAg or HBeAg assays immediately. Concentrations of HBsAg and HBeAg were quantified by the commercial ELISA kit (Kehua Bio-engineering Corporation) according to the manufacturer’s protocol. The optical density (OD) value at 450/630 nm was measured by using an automatic plate reader. Data were calculated as percentage of the control using Equation 2 [9,15]:

\[
\text{Suppression rate} \, (\%) = \frac{[\text{OD}_{\text{control}} - \text{OD}_{\text{drug}}]}{\text{OD}_{\text{control}}} \times 100\%.
\] (2)

Quantification of HBV DNA by fluorescent quantitative PCR

To further confirm the antiviral activity of FNC in HepG2.2.15 cells, the extracellular and intracellular HBV DNA levels were evaluated by fluorescent quantitative (FQ)-PCR. Viral DNA was extracted from the culture supernatant and cells, and then real-time quantitative PCR was performed in Light-Cycler 1.5 (Roche, Mannheim, Germany) using the HBV Fluorescent Quantitative PCR Detection Kit (Piji Biotechnology Development, Shenzhen, PR China) according to the manufacturer’s protocol. The cycling programme was as follows: after an initial denaturation (95°C for 2 min), the samples were subjected to 40 cycles of denaturation (94°C for 5 s) and annealing/extension (each at 56°C for 45 s). HBV DNA
was quantified using a standard curve. The inhibition ratio was calculated according to Equation 3 [10,16]:

\[
\text{Inhibition ratio} = \left( \frac{\text{HBV DNA concentration of negative control} - \text{HBV DNA concentration of experimental group}}{\text{HBV DNA concentration of negative control}} \right) \times 100\%
\] (3)

Animals
Young ducks (males and females were not distinguished) were Ma Ya from the Hubei province. All animals were treated according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health.

DHBV infection and drug treatment experiment
Each duck, aged 1 day, was injected into its tibial vein with 0.2 ml of serum from ducks with positive DHBV serology on day 3. The drug treatment experiment was carried out 7 days after ducks were infected with DHBV. The DHBV-positive ducks were randomly divided into five groups with 16 ducks in each group. FNC in different concentrations and 3TC control were given orally to DHBV-infected ducks, respectively. Five groups were observed: FNC 0.5 mg/kg•day, FNC 1.0 mg/kg•day, FNC 2.0 mg/kg•day and 3TC 20 mg/kg•day as a positive control. Normal saline was used as a mock treatment for the negative control group. The drugs were given once daily for 10 days continuously. The blood was drawn from the leg vein of all ducks before treatment, after medicating for 5 days and 10 days, and after withdrawal of the drug for 3 days. The serum samples and livers were separated and stored at -80°C.

Measurement of DHBV DNA by FQ-PCR
DHBV DNA was measured on day 0, days 5 and 10 during treatment, and day 13, that is day 3 after cessation of treatment on day 10 by FQ-PCR. For DHBV DNA, the DNA was extracted from serum using a DNA Extraction Kit (Bio-Tek), and FQ-PCR was performed in Light-cycler 1.5 (Roche) using SYBR Green I.

A pair of primers was designed based on the sequences from a previously published report [17], and used for amplifying the genome of DHBV, and the amplified PCR fragments were then cloned into pMD-18T. Based on the conserved sequences of DHBV S gene, another pair of primers for real-time PCR were designed and used to amplify the recombinant plasmid for constructing the standard curves. Meanwhile, the specificity, sensitivity and repeatability of the assay were tested. A rapid and specific SYBR Green I real-time PCR assay was established to detect DHBV. DHBV DNA from the serum and liver of experimentally infected ducklings was detected by this assay at different time points as indicated [15].

Histopathological examination of duck liver
The DHBV-positive ducks were treated with FNC and 3TC (intragastric administration) once daily for 10 days. On day 13, each duckling was laparotomized to obtain the liver immediately after collecting blood from the leg vein. Fragments of the duckling livers were fixed in formalin, embedded in paraffin, sectioned at 5 μm, stained with haematoxylin and eosin, and examined by light microscopy. The degrees of hepatocytic necrosis, degeneration and inflammation were assessed.

Statistical analysis
Results are means ±sd of the indicated number of independent experiments. Statistical significance was determined using analysis of variance. P-values <0.05 were considered statistically significant.

Results
Cytotoxicity of FNC
The growth of the HepG2.2.15 cells in the presence of various concentrations of FNC was examined. The results from the MTT test showed that FNC inhibited the growth of HepG2.2.15 cells with CC50 of 850.73 μM (Figure 1), indicating that FNC causes little toxicity on HepG2.2.15 cells. The cytotoxicity of FNC was measured to determine the treatment concentrations in the HepG2.2.15 cell culture system.

Effect of FNC on HBsAg and HBeAg in HepG2.2.15 cell line in vitro
To investigate the effect of FNC on the production of HBsAg and HBeAg by HepG2.2.15 cells, the supernatant was collected after treatment with different concentrations of FNC at different times, and the titre of HBsAg and HBeAg was determined by ELISA kits (Kehua Bio-engineering Corporation). Treatment of HepG2.2.15 cells with FNC at various concentrations for 3, 6 or 9 days resulted in a significant reduction of HBsAg and HBeAg secretion in a dose-dependent manner, with EC50 values of 0.33 μM for HBsAg on day 3. After treatment for 6 or 9 days, progressive reductions in HBsAg secretion were observed (Figure 2A). FNC inhibited HBeAg secretion with potencies similar to that for HBsAg inhibition; in addition, the time course of the inhibitory effect of FNC was the same as for HBsAg, with an EC50 value of 0.044 μM on day 9 (Figure 2B). At 1.0 μM, the inhibition rates of FNC on HBsAg and HBeAg in the HepG2.2.15 cells were 86.27% and 85.12% on day 9, respectively. 3TC-treated (20 μM) groups had 34.39% and 18.88% inhibition on day 9. These results indicated that FNC was more potent than 3TC for inhibiting both HBsAg and HBeAg secretion.
Effect of FNC on HBV DNA replication

Consistent with the inhibitory effects on HBsAg and HBeAg secretion, treatment of HepG 2.2.15 cells with FNC at various concentrations for 3, 6 or 9 days resulted in the reduction of intracellular and extracellular HBV DNA levels, as compared with a control group (Figure 3). The mean inhibition percentage of viral DNA levels with FNC at the dosages of 0.04, 0.2 and 1.0 μM was 81.93%, 83.02% and 92.31% intracellularly and 91.99%, 92.18% and 93.90% extracellularly, respectively, on day 9. The inhibition rates of 3TC (20 μM) on intracellular and extracellular HBV DNA levels were 77.81% and 91.60%, respectively, on day 9. The results showed that FNC had a stronger inhibitory effect on HBV DNA in HepG2.2.15 cell line at a low concentration extracellularly and intracellularly than 3TC.

Inhibitory effect of FNC on DHBV replication in vivo

The in vivo anti-HBV activity of FNC was investigated in DHBV-infected ducks. Significant adverse effects were not observed, either in treated birds or in controls. Serum DHBV DNA levels were measured by FQ-PCR. As shown in Figure 4A, with the exception of the control group, serum levels of DHBV DNA of each group decreased to different extents after treatment with FNC and 3TC on days 5 and 10, respectively. Among these, the mean inhibition percentage of viral DNA levels with FNC at the dosages of 0.5, 1.0 and 2.0 mg/kg•day was 50.14%, 71.30% and 73.67% for day 5 and 75.26%, 83.67% and 91.68% for day 10, respectively. FNC significantly reduced serum DHBV DNA levels in a time- and dose-dependent manner. 3TC-treated (20 mg/kg•day) groups resulted in 69.60% and 89.84% inhibition on days 5 and 10, respectively. Following withdrawal of the drug for...
3 days, the inhibition rates of FNC (2.0 mg/kg•day) and 3TC (20 mg/kg•day) on the DHBV DNA were 83.17% and 82.08%, respectively. This shows that the levels of serum DHBV DNA had a lower degree of rebound after withdrawal of the drug for 3 days. FNC had a stronger inhibitory effect on DHBV DNA at a low concentration in vivo than 3TC.

To further confirm the in vivo anti-HBV effect of FNC in ducks, DHBV DNA levels were examined by FQ-PCR in livers obtained at days 5, 10 and 13. Consistent...
with the inhibitory effect on serum DHBV DNA level, the FNC-treated ducklings showed a marked decrease in liver DHBV DNA levels for all three dose levels in a time- and dose-dependent manner. The mean inhibition percentage of viral DNA levels with FNC at the dosage of 2.0 mg/kg•day and 3TC at 20 mg/kg•day was 81.96% and 78.10% on day 10, respectively; however, the rebound of DHBV DNA level in livers was to a much higher degree as compared with that in duck serum (Figure 4B).

Histopathological examination of duck livers

Typical photographs of liver sections by light microscopy are shown in Figure 5. Histopathological profiles of the liver from the control group ducklings revealed necrosis, inflammatory cell infiltration and massive degeneration of the hepatic cytoplasm. The protective effect of FNC was confirmed by histopathological examinations. It also shows that histopathological evaluation of the liver revealed significant improvement by FNC at 2.0 mg/kg. It is worth noting that FNC in the high dosage group of FNC (2.0 mg/kg) resulted in more significant improvements than that with 3TC at 20 mg/kg.

Discussion

Despite the availability of an effective vaccine against HBV, the prevalence of chronic infection has not significantly decreased. Individuals with chronic hepatitis B not only suffer a wide range of symptoms associated with hepatitis but are at significant risk for the development of cirrhosis and hepatocellular carcinoma [18–23]. Currently, there are mainly two arms of therapy to manage chronic active hepatitis B: one is direct antiviral therapy to inhibit replication of HBV, and the other is indirect immunomodulatory therapy to enhance cellular immunity to destroy the virus-infected hepatocytes. However, since the immunomodulatory therapy with interferon-α has limited efficacy and has many other limitations, direct antiviral therapy draws more interest. Nucleoside analogues have played an important role in the antiviral therapy of hepatitis B. However, drug resistance, serious side effects and viral mutants may appear after a long-term treatment with drugs [16]. Every year, millions of people are diagnosed with acute or chronic infection [24]. Thus, it is still important to search for more effective agents against HBV, especially for reagents with an improved therapeutic index or more effective anti-HBV activities with relatively low cellular toxicities [14,16,25–27].

To date, there is no report on the anti-HBV activity of FNC. At present, the HepG2.2.15 cell line and DHBV-infected ducks are the two main models used for evaluating the anti-HBV effect of drugs [28]. In the present study, we confirmed the in vitro inhibitory effect of FNC on HBV antigen secretion in HBV-transfected HepG2.2.15 cells. Our results indicated that FNC could alleviate the toxicity to the HepG2.2.15 cell line, as its CC₅₀ was 850.7 μM (Figure 1). At 1.0 μM, the inhibition rates of FNC on HBsAg and HBeAg in the HepG2.2.15 cells were 86.27% (Figure 2A) and 85.12% (Figure 2B) on day 9, and more potent than 3TC-treated (20 μM) groups, with inhibition rates of 34.39% (Figure 2A) and 18.88% (Figure 2B), respectively, on day 9. FNC exhibited a potent inhibitory activity on HBsAg and HBeAg secretion, with treatment ECₐ₀ values of 0.037 μM and 0.044 μM on day 9. These results showed that the FNC inhibitory effect on HBsAg and HBeAg secretion was better than that of 3TC. Therefore, it is worth noting that the potency of FNC was higher than 3TC to the HepG2.2.15 cell line.

One rational approach to the development of drugs for the treatment of HBV infection in patients is to identify those compounds that specifically inhibit HBV DNA replication [3,29]. In our current research, the quantity of HBV DNA released in the cell supernatant and intracellularly was assayed by FQ-PCR. The inhibition rates of FNC (1.0 μM) and 3TC (20 μM) on the intracellular and extracellular HBV DNA were 92.31% and 77.81%, and 93.90% and 91.60%, on day 9, respectively (Figure 3). It was found that FNC effectively suppressed HBV DNA level, indicating that FNC has lower cytototoxicity and a higher anti-HBV effect than 3TC in vitro.

In contrast to the in vitro analysis of antiviral activity described above, evidence from the in vivo investigation may be regarded as more convincing. At present, the DHBV infection model is an important reference animal model to evaluate antiviral agents against HBV infection and viral replication activity [6,16,30]. Furthermore, the present study demonstrated that FNC had potent anti-HBV activity in vivo. The in vivo anti-HBV activity was investigated in DHBV-infected ducks [31]. DHBV was assayed by SYBR Green I FQ-PCR. The real-time PCR assay was rapid, highly specific and had a broad linear detecting range (7.02×10⁸ to approximately 7.02×10⁴ copies/μl; R²=1.00), with 105% PCR amplification efficiency. It had a detection threshold of 70 copies of plasmid DNA and was highly sensitive [15]. The results of our study showed that FNC was effective in suppressing DHBV replication in DHBV-infected duck models. In ducklings, the anti-DHBV activity of FNC was time- and dose-dependent with all three daily dosage levels (0.5, 1.0 and 2.0 mg/kg), which was considerably effective in inhibiting DHBV replication. After treatment for 5 and 10 days, as well as the withdrawal of the drug for 3 days, the mean percentage inhibition of viral DNA levels with FNC (2.0 mg/kg•day) and 3TC (20 mg/kg•day) were 79.67%, 91.68% and 83.17%,
and 69.60%, 89.84% and 82.08%, in duck serum, respectively (Figure 4A). It was shown that the duck serum DHBV DNA levels in the high dosage group of FNC (2.0 mg/kg) had no significant difference as compared with the 3TC group (20 mg/kg). Consistent with the 3TC group, the levels of serum DHBV DNA had a lower degree of relapse in the high dosage group of FNC (2.0 mg/kg) after withdrawal of the drug for 3 days. In duck liver, the 10-day treatment with FNC at all doses, particularly 2.0 mg/kg, significantly improved livers. There were significant differences in every dosage group of FNC on day 10. The mean inhibition percentage of viral DNA levels with FNC at the dosages of 0.5, 1.0 and 2.0 mg/kg • day was 52.68%.

Figure 5. Histopathological changes in duck livers

Ducks were treated with FNC at (A) 0, (B) 0.5, (C) 1.0 and (D) 2.0 mg/kg or with (E) 3TC at 20 mg/kg once a day for 10 days. Liver sections were stained with haematoxylin and eosin, and examined by light microscopy. Representative photographs are presented (magnification ×400).
69.62% and 81.96%, respectively, for day 10 (Figure 4B). In addition, FNC at 2.0 mg/kg resulted in better liver improvements better than with 3TC at 20 mg/kg. The in vitro anti-HBV activity was confirmed by histopathological improvement. From these results we concluded that FNC effectively inhibits DHBV with low cytoxicity in vitro.

In conclusion, we provided the first evidence that FNC can efficiently inhibit the HBV DNA replication and the expression of HBsAg and HBeAg in HepG2.2.15 cells and inhibit the DHBV DNA replication in vitro. Data suggested that FNC was effective and low toxic in vitro and in vivo. Further investigation is warranted to develop FNC as a potential alternative or complementary anti-HBV agent.

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Disclosure statement

The authors declare no competing interests.

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