

Antiviral Potential of the Silkworm Deoxynojirimycin against Hepatitis B Virus

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Over 350 million people worldwide are chronic carriers of hepatitis B virus (HBV). Chronic viral infections of the liver can progress to cirrhosis, which may ultimately lead to hepatic failure or the development of hepatocellular carcinoma. There are two antiviral drugs on the market approved for clinical management of chronic HBV infections; interferon-alpha and the nucleoside analog lamivudine. However, they showed adverse side-effects. In the rational drug design for such therapies we would like to utilize antiviral drugs that inhibit the HBV replication in the liver. Investigation of natural extracts of silkworm exhibiting antiviral potential was held in the functional HBV polymerase activity and the release of virion particle in the HepG2.2.15 cell lines. HBV-producing transgenic mouse fed with silkworm DNJ molecule was shown as an inhibitor of serum HBV particles. We could represent this DNJ molecule as an antiviral potential complementing conventional therapies after pre-clinical tests against WHBV-infected animal model, woodchuck.

Key words: Silkworm, 1-Deoxynojirimycin (1-DNJ), Priming reaction, HBV, HepG2.2.15, Transgenic mouse

Introduction

Viral hepatitis B is an infectious disease of the liver caused by Hepatitis B Virus (HBV). Worldwide more than two billion people alive today have been infected and about 350 million people are chronically infected carriers

of the HBV (WHO, 1998; Purcell, 1990). It is known that about 10 – 12% of Korean people is chronically infected with HBV. These carriers are at high risk of serious illness and death from liver cirrhosis and hepatocellular carcinoma (HCC).

Viral hepatitis B is therefore an important public health problem in Korea where treatments cost get higher at every year. Chronic hepatitis B can be treated with interferon (INF) and/or lamivudine, both of which result in viral, biochemical, and virological remission in about 30% to 50% of the cases (Liaw, 1997). Each treatment, however, even in combination therapy with a nucleoside analog and IFN- α , is associated with a high incidence of recurrence, serious side-effects, and is very expensive. Combination antiviral therapy is, thus, becoming a standard for treatment of many chronic viral diseases to prevent drug resistant mutants. This strategy may come from utilization of an antiviral that targets different components of the viral life cycle. The application of such scientific principles toward the characterization of botanical products with antiviral activity against HBV or HCV would lend scientific merit to their use as complementary or alternative medicine to conventional antiviral therapies with synthesized drugs (Blumberg, 1998; Liu *et al.*, 2001). Investigation of natural ethnopharmacologic extracts exhibiting antiviral potential may complement conventional therapies (Seeff *et al.*, 2001).

An 1-DNJ molecule has been purified from the midgut of silkworm. During clinical investigations on the treatment for hyperglycemia, it is evidently suggested that this compound possessed antiviral activity against HBV. Our objective was to demonstrate antiviral activity of 1-DNJ against HBV in two antiviral assays; HBV-pol priming reaction assay and inhibitory assay in the HepG2.2.15 cell line.

HBV specifies three glycoproteins (L, M, and S) that are derived from alternate translation of the same ORF. All three glycoproteins contain a common N-glycosylation site in the S domain. All three envelope glycoproteins

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are important in the viral life cycle. 1-DNJ molecule and its derivatives may cause the alteration of a single N-linked glycosylation site that can regulate HBV secretion (Mehta *et al.*, 1997). In this study, we designed and used to screen the antiviral effects against HBV-pol function and secretion of virion particles using the functional pol protein and the HBV-producing HepG2.2.15 cells. Furthermore, our work provides a sensitive assay for quantification of virion DNA in the HBV-producing transgenic mice, which may provide guidance for patients undergoing anti-HBV clinical tests and therapeutic application.

Our hypothesis, thus, is that if these purified compounds exhibit antiviral potential against HBV viral life cycle, 1-DNJ may serve as an efficacious therapeutic agent. Then, 1-DNJ may be directly applicable toward testing in the animal model, WHV-infected woodchuck.

Materials and Methods

Antiviral compounds

A DNJ molecule has been purified from the midgut of silk-

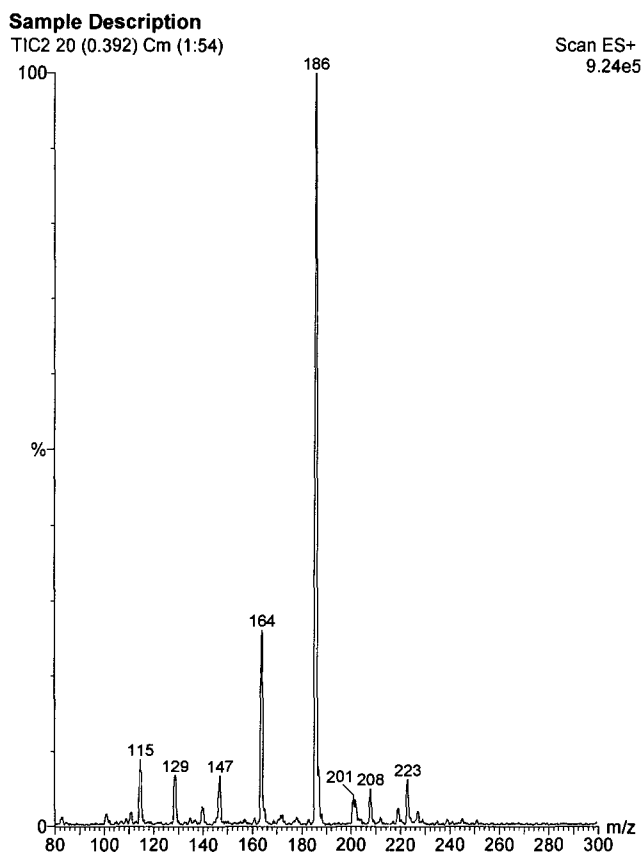


Fig. 1. Mass spectrum profile with a highly purified silkworm DNJ molecule. Typical 1-DNJ molecule is peaked at the position of MW 164.

worm, *Bombyx mori* by extraction of midgut tissues with organic solvents. This active compound was purified by ion-exchange chromatography. On the basis of Mass Spectrum analysis, purified DNJ shows one peak with molecular weight approximating 1-DNJ of 163.13 MW (Fig. 1). Freeze-dried crystals were processed by Biotopia Co., Ltd. (Whasung City, Kyonggi-do, Korea) and soluble in aqueous solutions. Lamivudine (3TC) was dissolved in H₂O to a stock concentration of 100 mM. A lyophilized form of recombinant human interferon (INF - BD, CGP 35269, 14/172/1) was dissolved in H₂O to a stock concentration of 0.5 mg/ml (1 × 10⁸ I.U./ml). Stock solutions were serially diluted in culture medium prior to *in vitro* test.

HBV-pol priming reaction

A recombinant baculovirus system (FPL-pol) expressing the HBV polymerase gene could be used for the inhibitory polymerase activity in the presence and absence of drug (Lanford *et al.*, 1995; Lanford *et al.*, 1999). Briefly, recombinant FPL-pol baculoviruses were transfected into Sf9 insect cells in the T-75 culture flask at 90% confluent culture of Sf9 cells and cultivated with or without 1-DNJ for 48 hrs at 26°C incubator. The polymerase assays were conducted with immunoprecipitated pol polypeptides still bound to the anti-FLAG affinity beads (M₂ monoclonal antibody beads; Sigma). The beads were washed according to the Lanford (1995, 1999). Following the final wash, the beads were suspended in TNM (100 mM Tris-HCl [pH 7.5], 30 mM NaCl, 10 mM MgCl₂) containing 100 μM unlabeled dATP, dGTP, and dCTP and 5 uCi of [α-³²P] TTP (3,000 Ci/mmol; NEN). Assays were routinely performed at 30°C for 30 min (Wang and Seeger, 1992; Zoulim and Seeger, 1994). After priming reaction, proteins were separated by SDS-PAGE electrophoresis as previously described (Laemmli, 1970). Gels from *in vitro* assays for pol function were stained with Coomassie blue, dried, and autoradiographed. For immunoblot analysis, proteins were electrophoretically transferred to a Fluorotrans polyvinylidene difluoride (PVDF) blotting membrane (Pall Biosupport, Glen Cove, NY). And then, membranes were processed as previously described (Lanford *et al.*, 1995).

HepG2.2.15 assay for inhibition of HBV replication

1-DNJ molecule was tested for its ability to inhibit HBV replication and its cytotoxicity as described previously (Korba and Gerin, 1992). Briefly, HepG2.2.15 cells were plated in 96 well plates and incubated together with dilutions of the compound, 1-DNJ, at 37°C and 5% CO₂ in the RPMI 1640 medium. Medium was changed and saved at day 4. Eight days after exposure to the 1-DNJ began, the supernatants were collected. The cells were lysed and

assayed for the presence of HBV-DNA by hybridization and/or PCR analysis. Both culture medium collected at day 4 and day 8, were used for PCR amplification as follows.

***In vitro* quantification of released virion by PCR**

The HepG2.2.15 cell line constitutively express HBV via an integrated HBV genome in the HepG2 cell and is used for drug evaluation (Korba and Milman, 1991) and inhibition of viral replication is quantified by molecular techniques (Korba and Gerin, 1992). Each different concentration of 1-DNJ compounds were added to the culture of HepG2.2.15 cells in the 24-well culture vessels and cultured 4 days. Culture medium were collected and continuously cultured 4 days more by adding fresh medium with same concentration of 1-DNJ. Ten ul of collected media were used for amplification of viral DNA by PCR methods. Each amplified DNA samples were analyzed on the 1.0% agarose gel electrophoresis with the control DNA, amplified with same primers.

***In vivo* treatments of transgenic mice with 1-DNJ**

Male and female HBV-transgenic mice and non-transgenic mice as controls were fed with 10 mg/kg/day of 1-DNJ (KYH-4) molecules for 15 days. These transgenic mouse were used for the experiments produced $10^5 - 10^6$ virions per ml serum (Zoulim *et al.*, 1994). At every 5 days, blood were obtained by bleeding mice through the tail vein. Mouse serum samples were analyzed in real-time PCR. We took a serum from collected blood by centrifugation at 14,000 rpm for 10 min. All DNA were extracted from 50 ul of serum with a QIAamp DNA Kit (Quiagen). We used real-time PCR for quantitative level of HBV. Amplification primer were 2005 (25-mer) 5'-TCAGCTCTGTATCGGGAA GCCTTAG-3' and 2122 (24-mer) 5'-CACCCACCCAGGTAGCTAGAG TCA-3' and we used TaqMan probe the sequence is 5'-6-FAM-CCT CAC CAT ACT GCA CTC AGG CAA-BHQ-1-3'. Sequences of primers and probes were designed against a highly conserved region in HBV genome sequences. The primer 2005 and 2122 amplified a 120 bp fragment in a core region. The amplification was performed in a 20 μ l reaction mixture containing: 3 mM MgCl₂, 200 μ M dNTP, 200 nM primer 2005, primer 2122 and probe. The real-time PCR using the Rotor-Gene 2000 processing is as below results. An incubation of 10 min at 95°C allowed the activation of the AmpliTaq Gold DNA polymerase and the denaturation of the nucleic acids. Thirty cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec and extension at 72°C for 15 sec were then carried out allowing the amplification-detection of HBV genomes (Chen *et al.*, 2001).

Results and Discussion

Inhibition of pol-primer complex formation

A recombinant baculovirus FPL-pol expresses the functional HBV polymerase transfected in the Sf9 insect cell. This expressed pol proteins may lead to the formation of functional pol complex with other cellular factors bound to the epsilon structure at the 3'-end of HBV pregenomic RNA (Lanford *et al.*, 1995). Then, they make the four polynucleotides of primer covalently bound to tyrosine residue at the TP domain of the pol protein. This reaction is called as pol priming reaction.

When we add 1-DNJ molecules into medium of the Sf9 cells infected with recombinant FPL-pol baculovirus, DNJ molecule may prevent the function of pol protein and/or cellular factors in order not to make the pol-primer complex in a dose-dependent mode (Fig. 2). It is clearly confirmed in the western-blot analysis that 1-DNJ did not interfere with the expression of pol protein. Mehta *et al.* (2001) describe the effects of the two novel anti-HBV agents, N-nonyl-DNJ (NN-DNJ) and N-nonyl-DGJ (NN-DGJ), on the inhibition of correct glycosylation of HBV surface antigen, respectively. Furthermore, it is known that N-butyldeoxyojirimycin (NB-DNJ) mediated the inhibition of HIV replication and HIV-induced syncytium formation *in vitro* (Fischer *et al.*, 1996). And also, Werr and Prange (1998) found that incorrect glycosylation of middle envelope proteins of HBV virus leads to improper assembly and secretion of virus resulted in the degradation of virion particles in the cytosol. In the concentration used in an HBV-DNA polymerase function study, 1-DNJ and its derivatives had no activity against the viral DNA polymerase. They hypothesize that the inhibitors might exert their effects by interfering with the encapsidation of the pregenomic RNA, accelerating the degradation of viral core particles or affecting the priming reaction that is required for the reverse transcription of the pregenomic RNA. Alternatively, they suggested that the inhibitors

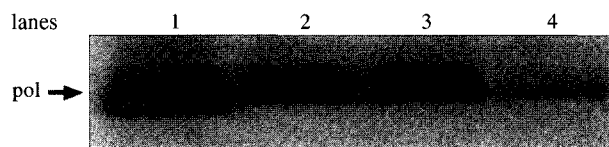


Fig. 2. Inhibition of priming reaction in the formation of pol-primer complex by addition of silkworm DNJ: (lane 1) no treatment of 1-DNJ as a control; (lane 2) 10 mM (lane 3) 20 mM (lane 4) 40 mM of 1-DNJ molecules were added into 75 ml of T-75 flask culture medium where Sf9 cells are grown and transfected with recombinant FPL-pol baculovirus, respectively. Pol arrow (\rightarrow) indicates that pol-primer complexes were labeled with α -P³²TTP banded at the position of pol protein on the SDS-PAGE gel analysis.

may inhibit interactions between viral core proteins and specific cellular factors leading to incorrect nucleocapsid assembly and stability. In *in vitro* priming reaction in the baculovirus system, however, formation of primer and elongation products of the minus strand of HBV DNA synthesis were inhibited by 1-DNJ only. In *in vitro* priming reaction contains only expressed pol and some cellular factors which experienced and suppressed with 1-DNJ during their expression in the insect cells.

Thus, we can hypothesize that 1-DNJ might affect on the interaction between pol and cellular factors which are involved in the formation of pol-primer complex and the elongation of products. It is also known that heat shock proteins, hsp60 and hsp90, are involved in the HBV DNA replication process (Hu and Seeger, 1996; Park and Jung, 2001). These cellular factors are involved in the priming reaction and elongation process in a chaperoning activity. If 1-DNJ molecule inhibits the binding of hsp90 and/or hsp60 to the pol bound to epsilon structure, pol protein may change the conformation in order not to form the pol-primer complex and elongation products. On the contrary, 1-DNJ molecule might inhibit the correct conformation of pol proteins in the priming reaction.

Inhibition of viral release out of HepG2.2.15 cells

1-DNJ and its derivatives such as NN-DNJ and NB-DNJ are known as an inhibitors of α -glucosidase, an ER early glycan processing enzyme (Saunier *et al.*, 1982). These molecules have also been shown to have antiviral activities against many enveloped viruses, including HBV, HCV, and HIV viruses (Fischer *et al.*, 1996; Zitzmann *et al.*, 1999; Mehta *et al.*, 2001). Misfolded glycoproteins of HBV surface proteins by DNJ derivatives are prevented from secretion or exit from the Golgi apparatus and undergone to proteasomes for degradation (Lu *et al.*, 1997; Mehta *et al.*, 1997; Mehta *et al.*, 2001).

In HepG2.2.15 cells, which harbor integrated HBV genomes into HepG2 cells and secrete infectious HBV particles, 1-DNJ affected HBV surface or core protein production by -glycosylation inhibition or inhibited the cellular factors involved in the HBV DNA replication by assisting HV pol protein. After prolonged addition of 1-DNJ into culture medium, released HBV virion DNA in the 10 μ l volume of culture media were amplified by PCR method. The data showed that addition of over 20 mM concentration of 1-DNJ affected the secretion of viral par-

ticles into medium (Fig. 3). At the addition of 30 mM of 1-DNJ, the cells were slightly affected on the viability, but the production of virion particle was completely blocked (Fig. 3, lanes 7 and 8).

We can hypothesize that 1-DNJ might exert their effect by interfering with the encapsidation of viral core particles or affecting the priming reaction system that is required for the reverse transcription of the pregenomic RNA. 1-DNJ molecule, KYH-4, was compared to lamivudine (3TC), serving as a positive antiviral control in HepG2.2.15 cell cultures (Korba *et al.*, 1992). No cytotoxicity was observed with 1-DNJ at the dosages used. Therefore, the highest concentration of drug tested was used to calculate a selectivity index (S.I.= CC_{50}/EC_{90}) (Table 1). Lamivudine inhibited viral-induced cell killing with a calculated EC_{50} = 0.058 μ M. The reduction in viral yields was a calculated EC_{90} = 0.18 μ M. Furthermore, this concentration was much lower than the midpoint of drug cytotoxicity, calculated as CC_{50} = 2155 μ M. The antiviral effects of 3TC have been known as a promising synthetic drug approved by FDA. However, 3TC compounds showed many unapplicable effects in the clinical trials. Compared to 3TC effects, 1-DNJ and its derivatives exhibited substantial antiviral activity against HBV produced in the HepG2.2.15 cells. Over 20 mM concentration did completely inhibit the secretion of HBV virion. These tendencies shown in Fig. 3 and Table 1 allow some comparison to be made as one of potential antiviral agents.

The antiviral activities of KYH-4 molecules from silkworm were compared in HepG2.2.15 cell cultures. Culture media was supplemented with each molecules of 30 mM at

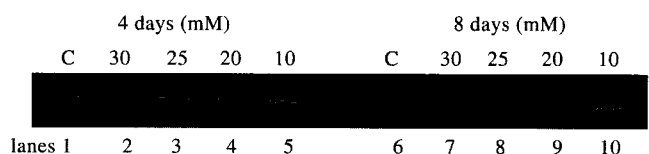


Fig. 3. Secretory viral particles in the HepG2.2.15 cell cultures were inhibited by addition of 1-DNJ molecules. Culture media was supplemented with KYH-4 (1-DNJ) with different concentration (mM) level except controls (C) at 4 days and 8 days posttreatment. Prolonged treatment of 1-DNJ with higher concentrations showed no release of virion particles. Each 10 μ l of culture media at two different periods was used for PCR amplification of HBV DNA at indicated culture periods. The PCR products were performed at the small S surface region with 670 nucleotides long.

Table 1. Antiviral Activity of drugs tested against HBV expressed in HepG2.2.15 cell lines

Antiviral	EC_{50} (μ M)	EC_{90} (μ M)	CC_{50} (μ M)	S. I. (CC_{50}/EC_{90})
3TC (Lamivudine)	0.057 \pm 0.007	0.179 \pm 0.025	2155	>12,000
1-DNJ (KYH-4)	27 \pm 2.7	75 \pm 2.7	>1,000	>13.0

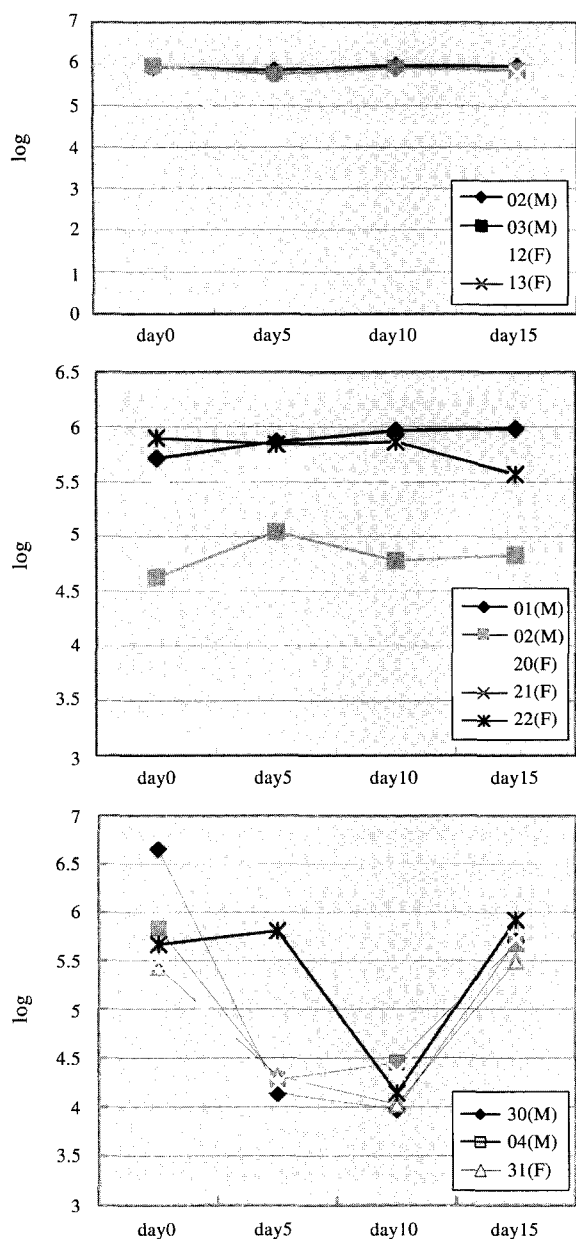


Fig. 4. Real time PCR performance of primer pair HBV DNA when $10^4 - 10^6$ copies of HBV serum virion DNA were used as template. (A) According to Materials and Methods, silkworm DNJ (KYH-4) was fed to transgenic mice with 10 mg/kg/day for 15 days. (B) control group of TG mice were used as control. (C) nucleoside analog, PMEAs, were used as standard chemical drug. In figures A, B and C, each different number of TG mouse was aused as an identified animal code. M and F stand for male and female mouse, respectively.

highest concentration and 5 mM at lowest concentration at 4 day intervals for one week. Virion nucleic acids were extracted at day 4 and 8 post-treatment. Nucleic acid amplification by PCR followed by agarose-gel electro-

phoresis analysis showed a reduction in HBV DNA fragments produced in PCR primed with 600 bp distance primers. Fig. 3A showed a panel after treatment with 3TC as a control. Fig. 3B showed panel after treatments with KYH-4 molecules from silkworm. 3TC compound showed early inhibition of HBV release, but KYH-4 represented slow inhibition of viral production, but completely blocked release of virion at 30 mM at day 8 post-treatment. We conclude that the inhibitory action of KYH-4 demonstrated against the HBV release in the HepG2.2.15 cells. This evidence supports our use of the live animal model to test the potency and efficacy of KYH-4 for the treatment of chronic HBV infections.

Based on the low cytotoxicity, furthermore, KYH-4 (1-DNJ) exhibited in vitro, we need to test the efficacy of KYH-4 in the WHV-infected woodchuck model and to show it does exhibit the effective therapy in combination with conventional drugs such as lamivudine or IFN- α .

Preliminary investigation of the efficacy of 1-DNJ in the HBV-producing transgenic mice

Four transgenic mice consistently producing non-infectious HBV viruses into blood and body fluid were orally fed and their serum samples were analyzed to evaluate the efficacy of antiviral effect of 1-DNJ in real-time PCR methods at every 5 days for 15 days. An amplified 120 bp DNA fragment in a core region were quantified and compared with non-fed mice. Among 2 sets of male and female mice, one female mouse showed 20 – 25% reduction of serum HBV titre in a short period of treatments. Three other mice showed no reduction, but might represent the reducing tendency if they were treated for a longer period.

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