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Deoxynojirimycin extracted from the Korean Mulberry Plant and Silkworm Exhibits Antiviral Activity in Surrogate Hepatitis C Virus Assays

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Abstract

Over 100 million people worldwide are chronic carriers of hepatitis C virus (HCV)(1). 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 a limited number of antiviral drugs on the market approved for clinical management of chronic HCV infections; interferon-alpha (IFNα) and the nucleoside analog ribavirin. However, whether used as monotherapy or in combination, adverse side-effects are associated with each drug and better therapeutic regimens are needed. In the rational drug design for such therapies one ideally would like to utilize antiviral drugs that targets different components of the viral life cycle. Investigation of natural ethnopharmacologic extracts (Traditional Chinese Medicine, Kampoo Medicine, Korean and Indian medicinal plants) exhibiting antiviral potential may complement conventional therapies (2).

Mulberry trees (Morus alba L.) are cultivated in China, Korea, and Japan. Their leaves have been used to feed silkworms (Bombyx mori L.) to produce the natural silk. The mulberry leaves have been used traditionally to cure and prevent diabetes. The root bark has been used for antiinflammatory, diuretic, antitussive, and antipyretic treatment in oriental medicine.

Yagi et al., found that mulberry leaves contain the 1-deoxynojirimycin (1-DNJ), which suppresses blood glucose levels (3). Currently, 18 different alkaloids including 1-DNJ have been identified in the leaves and root bark of the mulberry tree. Hikino et al., demonstrated that an extract of root bark had antihyperglycemic effects in a mouse model for hyperglycemia (4). Kimura et al., found that leaf extracts also showed a potent antihyperglycemic effect in insulin-independent diabetic mice (5). Silkworms feed on mulberry leaves may accumulate 1-DNJ in their midgut. Ryu et al., isolated the 1-DNJ compound from powdered extracts of the silkworm (6). This 1-DNJ molecule also showed a significant antihyperglycemic effect in the diabetic mouse model.

Asano et al., demonstrated that some iminosugars were potent inhibitors of alpha-glucosidase (7). DNJ and its derivatives inhibited alpha-glucosidase processing which lead to improper glycosylation of glycoproteins in the endoplasmic reticulum. The iminosugar DNJ and its N-alkylated derivatives (NN-DNJ and NB-DNJ) have been characterized for their inhibition of alpha-glucosidase, as well as, inhibitory effects on the maturation of HBV, HIV, BVDV, Dengue, YFV, Influenza viruses. Thus improper

glycosylation may induce misfolding of viral envelope proteins and may act on HCV E1-E2 dimerization by inhibiting proper glycosylation.

A DNJ (KYH-4) molecule has been purified from the midgut of silkworm. On the basis of Mass Spectrum analysis, KYH-4 shows one peak with molecular weight approximating 1-DNJ of 163.13MW. During clinical investigations on the treatment for hyperglycemia, anecdotal evidence suggested this compound, KYH-4, possessed antiviral activity against HCV. Our objective was to demonstrate antiviral activity of KYH-4 against HCV in two surrogate antiviral assays. Our hypothesis is that if this purified compound exhibits antiviral potential against related flaviviruses, KYH-4 may serve as an efficacious therapeutic agent. Results derived from this *in vitro* model are directly applicable toward testing in the small new world primate models.

Methods and Materials

Antiviral compounds. KYH-4 is derived from the Korean mulberry (*Morus sp.*) by extraction of the leaves with organic solvents. The active constituent of KYH-4 was purified by ion-exchange chromatography. Qualitative analysis on KYH-4 preparations shows a single peak by HPLC chromatography. A single compound of calculated MW 164 was identified by NMR spectroscopy. Further refinement of the purification scheme includes extraction of KYH-4 concentrated in the midgut of silkworms allowed to feed on the leaves of the Korean mulberry. Freeze-dried crystals were processed by Biotopia Co., Ltd. (Whasung City, Kyonggi-do, Korea) and soluble in aqueous solutions. Ribavirin (Virazole^{R}; Viratek, Inc., Covina, CA) was dissolved in H_2O to a stock concentration of 100 mM. A lyophilized form of recombinant human interferon (IFNαBD, CGP 35269, 14/172/1) was dissolved in H_20 to a stock concentration of 0.5 mg/ml ($1x10^8$ I.U./ml). Stock solutions were serially diluted in culture medium prior to *in vitro* testing.

Surrogate viral models. HCV is a member of the viral family Flaviviridae. Related members of this family and that have similar genomic organization and replication strategies are the flaviviruses, the pestiviruses, and the hepatitis C viruses (8). Bovine viral diarrhea virus (BVDV) is a pestivirus, and has been developed for cell-based screening assays to assess antiviral drug activity (9). GB virus B (GBV-B) is closely related to HCV, and *in vitro* assays have been developed that measure the antiviral activity against viral replication (10).

BVDV assay. An immortalized bovine uterine cells (NCL) infected with a cytopathogenic BVDV isolate was used in the development of this assay. A quantitative measure of cell numbers was obtained by reading the absorbance of methylene blue uptake by viable cells. The concentration at which BVDV-induced cell killing was reduced 50% (EC_{50}), the yield of BVDV was reduced 90% (EC_{90}), and the drug killed 50% of uninfected cells (CC_{50}) were determined by regression analysis (SigmaPlot8.0; SPSS Scientific). In all experiments a calculation of cell numbers for each experimental data point was expressed as the average of three wells per experimental treatment (dilution, titer, or antiviral concentration). Data presented in the accompanying figures represent the average of three independent

experiments.

GBV-B assay. Primary hepatocytes were isolated from marmosets (*Callithrix jaccus*) inoculated intravenously with GBV-B infectious serum. GBV-B infected marmoset hepatocytes were grown in the presence of antiviral drug for 6 days, drug and medium changed at 2 day intervals, and supernatant fluids were processed for GBV virion RNA (QIAamp Viral RNA isolation kit, Qiagen). At the end of antiviral treatment (day 6) prior to RNA extraction (Trizol Reagent, Life Technologies) the cytotoxicity of the antiviral compound was evaluated indirectly using a CellTiter Assay (Promega), based on conversion of MTS by mitochondria of viable cells. Values obtained from 3 untreated control wells were set as 100% total viable cells. Residual MTS reagent was removed after an 1 hr incubation period, cultures washed 3x with PBS and then processed for intracellular GBV-B RNA.

GBV-B quantification. One-step RT-PCR amplification utilized the following primer pair and probe; forward: AAC-GAG-CAA-AGC-GCA-AAG-TC, reverse: CAT-CAT-GGA-TAC-CAG-CAA-TTT-TGT, and probe: 6Fam-AGC-GCG-ATG-CTC-GGC-CTC-GTA-Tamra. The primer pairs and fluorescence reporter probe for the sequence of analysis are synthesized commercially (Applied BioSystems, Foster City, CA). The reaction conditions have been previously reported (10). A reference standard of GBV(+) serum has been calculated to contain $1x10^7$ GBV ge/ml in head-to-head comparison to a rGBV RNA transcript standard. RT-PCR is routinely used in our laboratory for quantifying WHV and GBV-B serum titers, and for the analysis of gene expression (TagMan system, Applied BioSystems, Seq. Dect. Sys.). Regression analysis was performed using equations that best fit the collected data (SigmaPlot8.0) and used to calculate effective antiviral concentrations.

Results

BVDV assay. Ribavirin inhibited viral-induced cell killing with a calculated EC_{50}= 9.92 uM. The reduction in viral yields was a calculated EC_{90}= 26.3 uM. However, this concentration was greater than the midpoint of drug cytotoxicity, calculated as CC_{50}= 18.4 uM. The antiviral effects of IFNα were also measured in our assay. Inhibition of viral-induced cell killing was a calculated EC_{50}= 0.23 ng/ml. There was a substantial reduction in viral yield with a calculated EC_{90}= 0.013 ng/ml, and IFNα did not exhibit cytotoxicty up to1 ug/ml. In comparison to the conventional standard drugs; KYH-4 exhibited substantial antiviral activity against BVDV *in vitro*. KYH-4 inhibited viral-induced cell killing over a broad range with a calculated EC_{50}= 2.96 mM, and at high concentrations reduced progeny viral yields 3 logs with a calculated EC_{90}= 0.24 mM. Cytotoxicity was not observed up to 50 mM. In reference to a compounds effective antiviral concentration relative to its cytotoxicity, a selectivity index was calculated (S.I.= CC_{50}/EC_{90}). The S.I. allows some comparison to be made among the potencies of the three antiviral agents (Table 1).

Table 1. Antiviral activity of drugs tested against BVDV-infected bovine uterine NCL cells.

Antiviral Drug	EC_{50} viral-induced cellkilling	EC_{90} viral yield	CC_{50} cytotoxicity	S.I. CC_{50}/EC_{90}
Ribavirin	9.92 uM	26.3 uM	18.4 uM	0.7
Interferon-α	0.23 ng/ml 45 IU/ml	0.013 ng/ml 2.5 IU/ml	>1000 ng/ml $2x10^5$ IU/ml	80,000
KYH-4	2.96 mM	0.24 mM	>50 mM	208

GBV assay. KHY-4 was also evaluated in a GBV-B assay developed at Cornell University. GBV-B infected marmoset hepatocytes were grown in the presence of the positive antiviral controls or KYH-4 for 6 days and intracellular viral replication and titers of virus secreted into the culture medium quantified. A dose-effect relationship was demonstrated with increasing drug concentrations. In comparison to parallel hepatocyte cultures grown in the presence of ribavirin or IFNα, the KYH-4 extract exhibited an antiviral effect similar to the IFN-α control. The maximal reductions in intracellular GBV-B titers were 1.41, 0.9, and 0.52 logs for IFNα, KYH-4, and ribavirin, respectively. Effective concentrations of the drug to reduce viral titers by 90% (EC_{90}) were calculated by linear regression analysis. Only the EC_{90} values to reduce intracellular viral replication could be calculated for ribavirin, IFNα and KYH-4. Based upon the calculated EC_{90} to reduce intracellular viral replication and CC_{50} for each drug, the S.I. for KYH-4 indicated a three fold greater degree of potency that either ribavirin or IFNα (Table 2).

Table 2. Antiviral effects against GBV-B infected marmoset hepatocytes *in vitro*.

Antiviral Drug	*EC_{90} viral yield	EC_{90} intracellular	CC_{50} cytotoxicity	S.I. CC_{50}/EC_{90}
Ribavirin	*n.c.*	0.37 mM	0.59 mM	1.6
Interferon-alpha	*n.c.*	$3.39x10^6$ IU/ml	$4.8x10^6$ IU/ml	1.42
KYH-4	*n.c.*	1.37 mM	5.67 mM	4.14

*n.c.- not calculated, an EC was not attained over the drug concentrations tested

Discussion

Attesting to the antiviral potential of KYH-4 against HCV, antiviral activity was demonstrated against related members of the virus family Flaviviridae; BVDV, a pestivirus, and GBV-B, which phylogenetically is very similar to the human pathogen HCV (8). The only two drugs approved by the Federal Drug Administration for clinical use against HCV were effective in both surrogate models. By analogy, other compounds tested in this system that exhibit similar or greater antiviral activity may also inhibit HCV. The antiviral potential of KYH-4 was greater than ribavirin and comparable to IFNα in our *in vitro* assays.

Based on our *in vitro* data we can show some translation to pharmacologic properties of ribavirin and IFNα during therapy *in vivo*. An International Unit (I.U.) of interferon activity is defined as the EC_{50} against Vesicular Stomatitis Virus (VSV) in MDBK cells (11). The EC_{90} = 0.012 ng/ml calculated for

IFNα in our BVDV assay (Table 1) converts to 2.3 I.U./ml, comparable to the international standards. The suggested therapeutic dose in humans is $3x10^6$ I.U. administered 3x weekly, which results in short lived maximum serum-concentrations of 10-30 I.U./ml (12). This roughly approximates the 45 I.U. (0.23 ng/ml) required to inhibit viral-induced cell killing in our BVDV assay (Table 1). In reference to ribavirin, a steady-state serum concentration of ≈2200 ng/ml (9 uM) can be attained when administered at 600 mg/day over 4 weeks of therapy (12). This approximates the 9.92 uM (2400 ng/ml) concentration required to inhibit viral-induced cell killing in our BVDV assay (Table 1). Based on our *in vitro* data the selectivity index of ribavirin shows the effective antiviral concentration is close to its cytotoxic midpoint (Table 1). This is an important facet during human therapy. A dose/effect relationship has been reported between virologic response/toxicity upon ribavirin+IFNα combination therapy. In some patients ribavirin is increased to obtain a sustained virologic response (loss of serum titers of HCV). Whether as monotherapy or in combination with IFNα, however, increasing the ribavirin dose is also accompanied with increasing toxicity and must be managed clinically through dose reduction. This illustrates the need for less toxic therapeutic regimens.

Although it is difficult to make the comparison between *in vitro* data and *in vivo* effects, if the assumptions that were made for ribavirin and IFNα based on our BVDV data hold true, we calculate that serum concentrations of KYH-4 approximating 485 ug/ml will be required to elicit an antiviral effect *in vivo*. Ribavirin has been recommended at doses approximating 16 mg/kg/day, and although the pharmacology of KYH-4 has not been defined it has been administered to Korean patients at 10mg/kg/day. Based on the low cytotoxicity KYH-4 exhibited *in vitro* we believe it may be tolerated at higher doses *in vivo*. We propose to test the efficacy of KYH-4 in the GBV-B marmoset model and show it does not adversely affect therapy in combination with conventional drugs.

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