The Journal of Microbiology, October 2007, p. 431-440
Vol. 45, No. 5

Natural Iminosugar Derivatives of 1-Deoxynojirimycin Inhibit Glycosylation of Hepatitis Viral Envelope Proteins

James R. Jacob[1§], Keith Mansfield[2], Jung Eun You[3], Bud C. Tennant[1], and Young Ho Kim[3*]

[1]*Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, NY 14853, USA*
[2]*Division of Primate Resources, New England Regional Primate Research Center, Harvard Medical School, MA 01772, USA*
[3]*Department of Life Sciences, The University of Suwon, Kyonggi-do 445-743, Republic of Korea*

(Received June 15, 2007 / Accepted August 9, 2007)

A silkworm (*Bombyx mori* L.) extract known to contain naturally occurring iminosugars, including 1-deoxynojirimycin (1-DNJ) derived from the mulberry tree (*Morus alba* L.), was evaluated in surrogate HCV and HBV *in vitro* assays. Antiviral activity of the silkworm extract and one of its purified constituents, 1-DNJ, was demonstrated against bovine viral diarrhea virus (BVDV) and GB virus-B (GBV-B), both members of the Flaviviridae family, and against woodchuck hepatitis virus (WHV) and hepatitis B virus (HBV), both members of the Hepadnaviridae family of viruses. The silkworm extract exhibited a 1,300 fold greater antiviral effect against BVDV in comparison to purified 1-DNJ. Glycoprotein processing of BVDV envelope proteins was disrupted upon treatment with the naturally derived components. The glycosylation of the WHV envelope proteins was affected largely by treatment with the silkworm extract than with purified 1-DNJ as well. The mechanism of action for this therapy may lie in the generation of defective particles that are unable to initiate the next cycle of infection as demonstrated by inhibition of GBV-B *in vitro*. We postulate that the five constituent iminosugars present in the silkworm extract contribute, in a synergistic manner, toward the antiviral effects observed for the inhibition of intact maturation of hepatitis viral particles and may complement conventional therapies. These results indicate that pre-clinical testing of the natural silkworm extract with regards to the efficacy of treatment against viral hepatitis infections can be evaluated in the respective animal models, in preparation for clinical trials in humans.

Keywords: silkworm, iminosugar, 1-deoxynojirimycin, hepatitis virus, glycosylation inhibition

Worldwide, over 300 million people are chronic carriers of HBV and over 100 million people are chronic carriers of HCV (Hoofnagle and Di Bisceglie, 1997; Di Bisceglie and Bacon, 1999). Based upon seroprevalence data, it has been estimated that 10-15% of infected individuals in an endemic region may have concurrent HBV/HCV infections (Liaw, 2001). Chronic viral hepatitis infections can progress to cirrhosis, which may ultimately lead to hepatic failure or the development of a hepatocellular carcinoma (Crespo *et al.*, 1997). In North America, over 10,000 individuals die from complications of chronic viral liver disease each year (Chisari and Ferrari, 1997). In Korea, about 8% of the population is reported as chronic carriers of HBV, whereas less than 1% of the population is infected with HCV. On a worldwide basis, there is an urgent need for effective, accessible, and affordable treatments for chronic viral liver disease.

Several antiviral drugs on the market have been approved for the therapy of chronic viral hepatitis infections. The nucleoside analog Lamivudine has been approved for the treatment of chronic HBV infections (Jarvis and Faulds, 1999). However, there is a problem with the development of drug resistant HBV mutants after prolonged treatment. The drug Adefovir dipivoxil, recently was approved by the FDA, shows activity against the Lamivudine-resistance mutants (Tsiang *et al.*, 1999; Jacob *et al.*, 2004). IFN-α has been used to treat chronic HCV infections. However, a beneficial response to IFN-α treatment is observed in only 30% of patients, and treatment is not without adverse side effects (McHutchinson *et al.*, 1998). Conjugated forms of IFN-α are now used for therapy, and have increased the sustained viral response rate to over 50% (Cavalletto *et al.*, 2000). However, resistance of some HCV genotypes during long-term IFN-α therapy is still a problem. The nucleoside analog ribavirin has been used in conjunction with IFN-α to increase the response rate, especially in IFN-α non-responders (Schvarcz *et al.*, 1995; McHutchison *et al.*, 1998; Look *et al.*, 1999). However, whether used as monotherapy or in combination, adverse side effects are associated with the various drugs and better therapeutic regimens are still needed for afflicted individuals. The use of alternative therapies to complement existing antivirals for chronic viral infections is generally recognized (Liang, 1999; Strader and Zimmerman, 2000; Seeff *et al.*, 2001; Martin and Ernest, 2003). Clinical studies on the effects of such treatments in patients with chronic liver disease are limited in scope and often lack appropriate control groups for comparison (Liang, 1999; Strader and Zimmerman, 2000; Seeff *et al.*, 2001; Liu *et al.*, 2002; Martin and Ernest, 2003). This lack of scientific evidence on the therapeutic benefit, and of any associated toxicity, contributes to the

§ This author contributed equally to this work
* To whom correspondence should be addressed.
(Tel) 82-31-222-7632; (Fax) 82-31-220-2484
(E-mail) kimyh@suwon.ac.kr

widespread belief that the use of natural products is "safe and effective". We have developed surrogate culture models as preliminary screening tools to identify candidate antiviral compounds for testing *in vivo*. We can examine both cellular and viral processes as they respond to treatment *in vitro*, showing the likelihood of therapeutic success in the respective animal model. The application of sound scientific principles toward the evaluation of ethnopharmacologic therapies in relevant animal models for human diseases will provide critical pre-clinical data (Schuppan *et al.*, 1999; Seeff *et al.*, 2001).

In the rational drug design for antiviral therapies, one ideally would like to identify a drug that targets different components of the viral life cycle. Several enveloped viruses bud via the endoplasmic reticulum (ER), and N-linked glycans on the newly synthesized proteins need to be processed prior to interaction with the ER chaperones that have a role in the proper folding of mature glycoproteins. Thus, improper glycosylation can induce misfolding of the viral envelope proteins, inhibiting maturation of the enveloped viruses (Block *et al.*, 1994; Wu *et al.*, 2002). Iminosugars are five or six member ring sugar analogues, with nitrogen replacing the oxygen in the ring structure, which exhibit anti-glycosidase activity (Asano, 2003). Evidence has accumulated that iminosugar derivatives exert antiviral effects against several human viral pathogens including HIV, HBV, Dengue and Japanese Encephalitis viruses (Durantel *et al.*, 2001). While changes in anti-glycosidase activity are observed among iminosugar derivatives, a lack of correlation with the antiviral activity of the compounds suggests other mechanisms may be responsible for the antiviral effects (Jacob, 1995; Mehta *et al.*, 2002).

The piperidine alkaloid 1,5-dideoxy-1,5-imino-D-glucitol (1-DNJ) is an isosteric analogue of the iminosugar D-glucopyranose with functional hydroxyl groups (Asano *et al.*, 1994). 1-DNJ and other polyhydoxylated alkaloids are potent α-glycosidase inhibitors. These chemical moieties were actually synthesized before there were shown to be naturally occurring products. Natural 1-DNJ and derivatives are extracted from the Korean mulberry plant (*Morus* sp.) with organic solvents (Asano *et al.*, 2001). Five of the 18 alkaloids are concentrated in the midgut of silkworms (*Bombyx mori* L.) allowed to feed on mulberry leaves. The constituent compounds of mulberry extracts have been isolated by ion-exchange column chromatography and the structures have been identified (Asano *et al.*, 2001). The primary component in the silkworm extract is 1-DNJ, representing approximately 0.2-0.4% of the total extract.

Mulberry leaves have been used in traditional Chinese medicine for the treatment of diabetes and are also used as a nutraceutical food in Korean and Kampo medicines (Asano *et al.*, 2001). The isolation of 1-DNJ from the mulberry root bark led to the knowledge that it is one of several active compounds responsible for lowering blood glucose levels (Asano *et al.*, 1994), similar to the chemically synthesized 1-DNJ used as an anti-hyperglycemic in non-insulin dependent diabetes (Joubert *et al.*, 1985; Jacob, 1995). Silkworm extracts that are enriched 2.7 fold for 1-DNJ also show anti-hyperglycemic effects in animal models for diabetes (Kimura *et al.*, 1995). Based on similar mechanisms of action for the natural derived and synthetic 1-DNJ, and that the synthetic derivatives of 1-DNJ demonstrate antiviral effects *in vitro* (Block *et al.*, 1994; Zitzmann *et al.*, 1999) we propose that a processed silkworm extract employed in Korean and Kampo medicines may be efficacious against both HBV and HCV. The objective of this study was to evaluate the antiviral potential of this silkworm extract in surrogate HBV and HCV *in vitro* assays as a preliminary step to pre-clinical testing in the respective animal models.

Materials and Methods

Silkworm extract and 1-DNJ

The silkworm extract is manufactured according to a patented process entitled "Manufacturing methods of silkworm powder as a hyperglycemia depressant containing effective molecules", covered in Korea (KP 151731:1998), Japan (JP 2757937:1998), and China (CP ZL95115559.8:2002). Essentially, each capsule contains approximately 300 mg of lyophilized powder harvested by rapid freezing of the fifth instar larvae of the silkworm. The constituent iminosugars of the extracts have been isolated by ion-exchange column chromatography and the structures were identified by 1D and 2D NMR spectra and high resolution FAB-MS analysis (Asano *et al.*, 2001). 1-DNJ, fagomine (1,2-dideoxynojiri-mycin), 3-epi-fagomine, a furanose analogue 1,4-dideoxy-1,4-D-arabinitol, and 1,4-dideoxy-1,4-imino-(2-*O*-β-glucopyranosyl)-D-arabinitol are present in both the mulberry plant and silkworm extracts (Asano *et al.*, 2001). Freeze-dried crystals of 1-DNJ purified from the silkworm extracts were processed by Biotopia Co., Ltd. (Korea). Purified preparations show a single peak on HPLC chromatography. The structure of the compound with a calculated M.W. of 164 daltons was identified and confirmed as 1-DNJ by NMR and LC-MS. In our assays, these crystals were dissolved at a concentration of 1 M in phosphate buffered saline (PBS), and were stored at -20°C. Based upon *in vitro* studies, the purified 1-DNJ loses activity in PBS solution after 5 months. A 10% solution of the silkworm extract was prepared by dissolving the powder in DMSO overnight at 4°C followed by clarification of insoluble material. A starting dilution of 1:50 was made to reduce the toxicity of DMSO during the *in vitro* assays. After 3 months in DMSO solution, no measurable loss of antiviral activity has been observed.

Standard antiviral controls

Ribavirin is a synthetic non-interferon-inducing, broad-spectrum antiviral nucleoside that has been used as a therapeutic treatment for chronic HCV infections in humans (Schvarcz *et al.*, 1995). A lyophilized form of this compound (Virazole®; Viratek Inc., USA) was dissolved in H_2O to produce a stock concentration of 100 mM. INFα is a type I interferon that inhibits viral replication and cellular proliferation, modulates the immune response, and has been used in conjunction with ribavirin for the treatment of chronic HCV infections in humans (Vilcek and Sen, 1996). A lyophilized form of recombinant human interferon (rHu*B*/*D*αIFN; Novatis, Basel, Switzerland) was dissolved in H_20 to a stock concentration of 0.5 mg/ml (1×10^8 I.U./ml). Stock solutions were serially diluted in culture medium prior to *in vitro* testing.

BVDV assay

An immortalized bovine uterine cell line (NCL), which was generated via immortalization of primary bovine uterine cells with the SV40 large T antigen oncogene, and was permissive to both non- and cyto-pathogenic isolates of BVDV, was used in the development of this assay (Dobrinski *et al.*, 1999). Cell lines were maintained in growth medium containing 10% bovine serum negative for BVDV contamination (gamma irradiated for virus) and tested negative for an antibody against BVDV. A quantitative measure of cell numbers was obtained by reading the absorbance of methylene blue uptake by viable cells. Three days after infection of cells with cytopathogenic BVDV and antiviral treatment, cultures were rinsed with PBS followed by fixation in Hank's balanced salt solution containing 1.25% glutaraldehyde and 0.06% methylene blue, for 1 h at 37°C. The plates were rinsed in several volumes of H_2O, air dried, and subsequent elution of the methylene blue stain from fixed cells by incubation in a solution of PBS containing 50% ethanol and 1% acetic acid. The absorbance of the methylene blue in solution was measured using an ELISA plate reader (BioTek, USA) with a light filter setting at 630 nm. The concentration at which BVDV-induced cell killing was reduced 50% (EC_{50}), the yield of BVDV was reduced 90% (EC_{90}), and at which the drug killed 50% of the uninfected cells (CC_{50}) were determined by regression analysis (SigmaPlot 8.0; SPSS Scientific, USA). Untreated, uninfected cells served as controls for calculating cytotoxicity. Untreated, BVDV-infected cells served as controls for calculating viral-induced cell killing. In all experiments, a calculation of the cell number for each experimental data point was expressed as the average of cells in three wells per experimental treatment. In some experiments, BVDV proteins were metabolically labeled, as described below, and immune precipitated using polyclonal hyper-immune cow serum or monoclonal antibodies specific to the BVDV envelope glycoprotein gp48 E0 (MAb 15.c.2) and gp53 E2 (Mab10.11.2), as previously documented (Corapi *et al.*, 1990).

2-Drug combination assay

The median-effect principle described by Chou and Talalay (Chou and Talalay, 1984) was used to examine the dose-effect of combined drug therapies (calculated with Calcusyn 1.1.4, Biosoft, UK). Dose-response curves generated for individual drugs were used to calculate the EC_{50}. The drugs were tested in combination at 0.25×, 0.5×, 1× and 2× the EC_{50} and analyzed by linear-regression. Combination Index (CI) values were calculated under conditions of mutually non-exclusive drugs; assuming the mechanism of inhibition targeted different viral components. CI values indicated synergism (CI<1), additive effect (CI=1), or antagonism (CI>1) of the drug combination based on measurements of inhibiting BVDV-induced cell killing.

Primary cell cultures

Hepatocytes were isolated by standard collagenase perfusion protocols (Stephensen *et al.*, 1991; Jacob *et al.*, 1994). Cell viability, based on trypan blue exclusion, exceeded 80% for freshly isolated cells. Hepatocytes were seeded at a viable cell density of $5\times10^5/cm^2$ onto culture plates pre-treated with rat-tail collagen. Primary hepatocytes isolated from marmosets (*Callithrix jacchus*) and woodchucks (*Marmota monax*) have been maintained for greater than 4 months as stationary cultures expressing fully differentiated qualities in a growth medium consisting of Williams Media E (WME), supplemented with fetal bovine serum, 10 mM HEPES, 100 μg/ml gentamycin, glucagon (2 μg/ml), and an insulin, selenium, transferrin mix (each at 5 μg/ml). Primary hepatocytes adapt to culture within the first 48 h of plating, and were subsequently incubated in the presence of the test compounds on day 3 post-plating. Medium and fresh drug were changed at 2-day intervals.

GBV-B assay

Marmoset hepatocytes isolated from a GBV-B infected animal were grown in the presence of the antiviral drugs for 6 days. Uninfected hepatocytes were isolated from normal animals and 1 day post-plating were inoculated with GBV-B infectious serum (10 μl of 2×10^8 ge/ml) for 18 h, followed by drug treatment. At the end of antiviral treatment, supernatant fluids were processed for GBV virion RNA (QIAamp Viral RNA isolation kit, Qiagen, Germany) and cellular RNA was extracted from the cultures (Trizol Reagent, Invitrogen, USA) after performing the cytotoxicity assays. A total of 0.2 μg of cellular RNA and a fraction of supernatant representing 75 μl were analyzed by real time RT-PCR (TaqMan System, Applied Biosystems, USA). The reaction conditions and primers have been previously reported (Beames *et al.*, 2000). Basically, one-step RT-PCR amplification utilized the following primer pairs and probe-forward; 5'-AAC-GAG-CAA-AGC-GCA-AAG-TC-3', reverse; 5'-CAT-CAT-GGA-TAC-CAG-CAA-TTT-TGT-3', and probe; 6Fam-AGC-GCG-ATG-CTC-GGC-CTC-GTA-Tamra. The primer pairs and fluorescence reporter probe for the sequence of analysis were synthesized commercially (Applied BioSystems). An internal reference standard employed primers and a probe for the 18S ribosome to normalize the total cellular RNA between samples during RT-PCR. Regression analysis was performed using a Regression Wizard library of equations that best fit the collected data (SigmaPlot 8.0). Statistical analysis was performed on the data for the viral genome equivalents (GE)/ml or the GE/μg of the cellular RNA versus the compound concentration to calculate the effective antiviral concentrations. To control for interassay variation (i.e. level of viremia, status of cultures) GBV viral RNA levels were expressed relative to untreated controls from all assay plates to allow for a comparison between the antiviral effects of the positive antiviral controls ribavirin and IFN-α, and 1-DNJ.

WHV assay

Woodchuck (*Marmota monax*) hepatocytes isolated from chronic WHV-infected animals were grown in the presence of the antiviral drugs for 6 days. The nedium and fresh drug were changed at 2-day intervals. Radioisotope labeling of cell cultures with [^{35}S] met/cys (Trans-label, ICN) commenced 18 h prior to harvest at the designated time point by methods previously descried (Jacob *et al.*, 1994). WHsAg was immune precipitated from radiolabeled culture media and cell extracts using a rabbit polyclonal antiserum against purified WHsAg particles. Proteins were separated by 10%

SDS-PAGE, and were analyzed by autofluorography. The size and distribution of the WHsAg polypeptides detected by these methods were in accord with published reports (Tolle *et al.*, 1998).

HBV assay

The HepG2.2.15 cell line constitutively expresses HBV via an integrated HBV genome, and is used extensively for drug evaluation (Korba and Gerin, 1992). Cultures of HepG2.2.15 cells were supplemented with increasing concentrations (10-30 mM) of 1-DNJ purified from silkworm extracts. On days 4 and 8 of treatment, the presence of HBV in the culture media (10 ul) was determined by PCR compared to the untreated control cells.

Cytotoxicity

At the end of antiviral treatment, prior to RNA extraction, the cytotoxicity of the antiviral compound was evaluated using a CellTiter Assay (Promega) which is based on conversion of a methanethiosulfinate (MTS) substrate by the mitochondria of viable cells. Antiviral testing in primary hepatocytes has determined that the optimal time point to assess cytotoxicity relative to antiviral effects is day 6 of treatment (9 days post-plating). Values obtained from three sets of untreated control cells in wells were set as 100% of the total viable cells. A decrease in the MTS values in the treated cells versus the untreated cells indicated cytotoxicity. Residual MTS reagent was removed after a 1 h incubation period, the cultures were washed 3× with PBS and were then processed for intracellular virus as described above.

Results and Discussion

Silkworm extract exhibits anti-BVDV activity

Two drugs approved by the FDA for clinical use against HCV are effective against related members of the virus family Flaviviridae, BVDV and GBV-B, in surrogate assays (Zitzmann *et al.*, 1999; Beames *et al.*, 2000). By analogy, other compounds tested in these systems that exhibit similar or greater antiviral activity may also inhibit HCV. Attesting to the antiviral potential of the silkworm extract containing 1-DNJ against HCV, antiviral activity was demonstrated in both surrogate models. Ribavirin (Fig. 1A) inhibited viral-induced cell killing with a calculated EC_{50}=9.92 μM. The reduction in viral yields was calculated as EC_{90}=26.3 μM. However, this concentration was greater than the midpoint

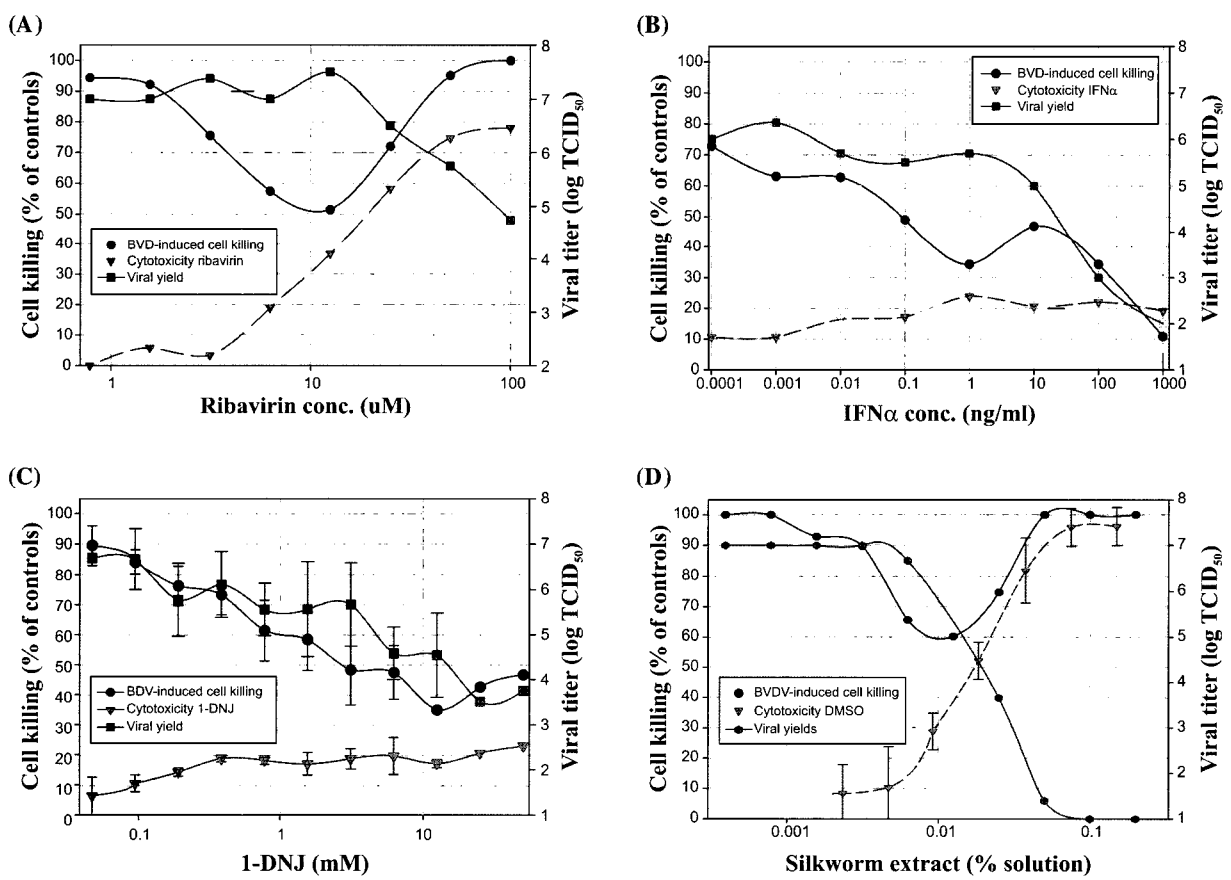

Fig. 1. The dose-effect of antiviral treatments versus the BVDV infected cells. Cultures were grown in the presence of increasing drug concentrations (X-axis); (A) ribavirin, (B) IFN-α, (C) 1-DNJ, (D) silkworm extract. Virus in the culture media and viral-induced killing was analyzed after 3 days of treatment. Titers (right axis) of secreted virus were expressed as tissue culture infectious doses (-■-) and viral-induced killing (left axis) was expressed as the percent of untreated control cultures (-●-). Cytotoxicity (left axis) of the drug treatment was based on methylene blue uptake by viable cells, expressed as a percentage of the untreated, uninfected control cultures (-▼-).

of the drug cytotoxicity, calculated as CC_{50}=18.4 μM. The antiviral effects of IFN-α were also determined in this assay (Fig. 1B). Inhibition of viral-induced cell killing was calculated as EC_{50}=0.23 ng/ml. There was a substantial reduction in viral yield with a calculated EC_{90}=0.013 ng/ml, and IFN-α did not exhibit cytotoxicty up to a concentration of 1 μg/ml. In comparison, 1-DNJ exhibited substantial antiviral activity against BVDV *in vitro* (Fig. 1C). 1-DNJ inhibited viral-induced cell killing with a calculated EC_{50}=2.96 mM and reduced progeny viral yields 3 logs, with a calculated EC_{90}=0.24 mM. Cytotoxicity was not observed up to a concentration of 50 mM. The silkworm extract dissolved in DMSO exhibited an EC_{50}=0.016% and EC_{90}=0.007% (Fig. 1D). A CC_{50}=0.031% reflects the toxicity associated with DMSO (Fig. 1D), though the toxicity of other components was not tested.

Silkworm extract in combination with IFN-α enhances antiviral effects in vitro

The synergistic effects of ribavirin and IFN-α and the iminosugar derivative NB-DNJ and IFN-α *in vitro* have been previously reported (Ouzounov *et al.*, 2002). Based upon

Table 1. Antiviral activity of the 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 μM	26.3 μM	18.4 μM	0.7
Interferon-α	0.23 ng/ml 45 IU/ml	0.013 ng/ml 2.5 IU/ml	>1000 ng/ml 2×10^5 IU/ml	80,000
1-DNJ	2.96 mM	0.24 mM	>50 mM	208
10% Silkworm extract in DMSO	0.016%	0.007%	≈*0.031%	4.4

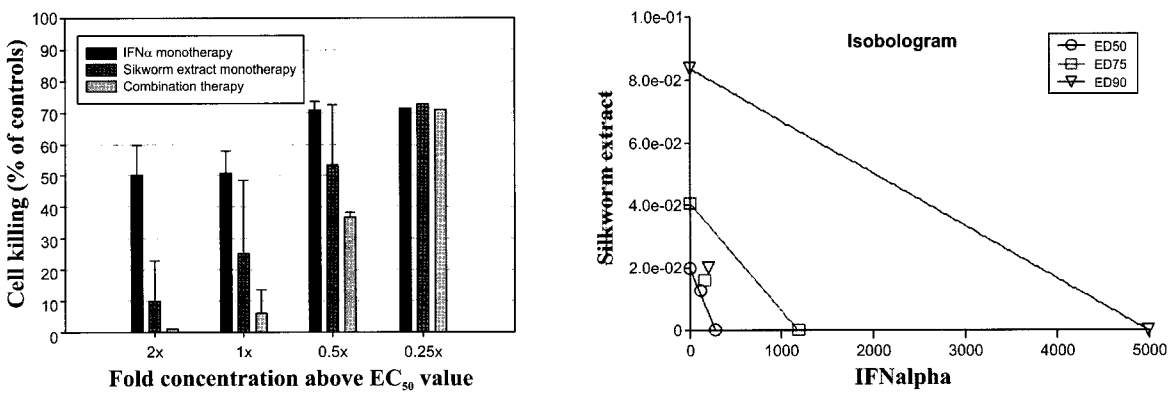

Fig. 2. The dose-effect of combined silkworm extract plus rIFN-α to reduce BVDV-induced cell killing. Concentrations 2×, 1× and 0.5× the monotherapeutic EC_{50} were tested. An isobologram showing the theoretical line of the additive effect at specific effective doses (ED). A synergistic effect is indicated when the independent experimental values (○, □, ▼) fall below the line of additive effect.

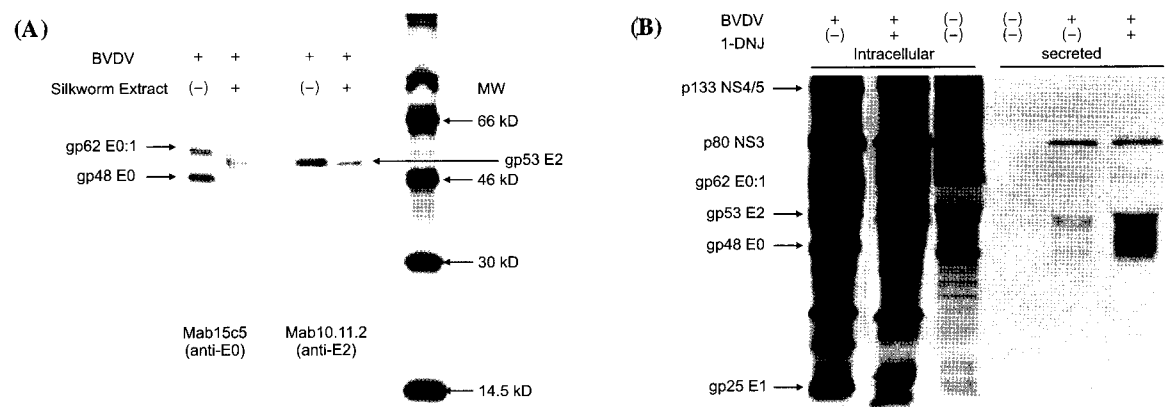

Fig. 3. (A) [^{35}S] met-labeled BVDV proteins expressed from cells treated with + or without (-) 1-DNJ, and precipitated with hyper- immune bovine serum. (B) [^{35}S] met-labeled BVDV proteins expressed in cells treated with silkworm extract, and precipitated with monoclonal antibodies Mab15c5 and 10.11.2 specific to BVDV gp48E0 and gp53E2, respectively.

the EC_{50} values in Table 1, the silkworm extract was tested in combination with rIFN-α in the BVDV assay. The combined silkworm extract plus IFN-α therapy was more beneficial than either monotherapy, as illustrated in Fig. 2. Based on these results, combination index (CI) simulations were calculated based upon the theoretical effective dose (ED) of the two drugs in combination (ED_{50}, ED_{75}, and ED_{90}). The CI values indicate synergism (CI<1), additive effect (CI≈1), or antagonism (CI>1) of the drug combination. The accompanying isobologram in Fig. 2 indicates a slight additive effect if the drugs were used at the equipotent ED_{50} ratio (CI= 1.38), and would exhibit synergism when both drugs were used at the ED_{75} (CI=0.58) or ED_{90} (CI=0.29).

1-DNJ and silkworm extract affects both expression and secretion of BVDV glycoproteins

It has been previously reported the glycosidase inhibitor NB-DNJ affects BVDV gp53E2 glycoproteins (Branza-Nitchita *et al.*, 2001). Concerning the mechanism of action for 1-DNJ and the silkworm extract, BVDV infected cell cultures subject or not to treatment were metabolically labeled and BVDV specific proteins were imaged following gel separation by autoradiography (Fig. 3). BVDV specific proteins were identified at the appropriate M.W. (Fig. 3A, lane 1 vs lane 3). Treatment with 1-DNJ altered the expression of the BVDV envelope (E) glycoproteins (gp), whether expressed as an intracellular protein (lane 1 vs lane 2) or secreted into the culture medium (lane 5 vs lane 6). Upon 1-DNJ treatment of the BVDV-infected cultures, a significant reduction was observed in the viral envelope proteins E0 and E1, as compared to the untreated cultures. The expression of the nonstructural proteins, which are not glycosylated, was not affected by 1-DNJ treatment. Treatment with silkworm extract led to an aberrant expression of gp48E0 and its precursor gp62E1:0 (Fig. 3B, lane 1 vs 2). Although it has been reported that glycosidase inhibitors affect the BVDV gp53E2 glycoprotein (Branza-Nichita *et al.*, 2001), a decrease in expression was observed in our system (Fig. 3, lane 3 vs lane 4) upon treatment with the silkworm extract.

1-DNJ inhibits GBV-B infection

Purified 1-DNJ was evaluated against positive antiviral controls in persistently GBV-B infected marmoset hepatocytes (Fig. 4). A dose-effect relationship was moderately demonstrated with increasing drug concentrations for the intracellular virus. In comparison to parallel hepatocyte cultures grown in the presence of ribavirin or IFN-α, purified 1-DNJ exhibited an antiviral effect similar to IFN-α. The maximal reductions in intracellular GBV-B titers were 1.41, 0.9, and 0.52 logs for IFN-α, 1-DNJ, and ribavirin, respectively. The EC_{90} values to reduce intracellular viral replication could be calculated for ribavirin, IFN-α and 1-DNJ (Table 2). Based upon the calculated EC_{90} to reduce intracellular viral replication and the CC_{50} for each drug, the selectivity index ($S.I.=CC_{50}/EC_{90}$) indicated 1-DNJ as a more favorable agent than either ribavirin or IFN-α. Based on results from the BVDV assay, we believe the mechanism of antiviral action for the silkworm extract, containing 1-DNJ, lies in disrupting viral glycoprotein processing resulting in the production of non-infectious particles. This mechanism of action was not as pronounced for persistently infected GBV-B cultures. Data from the infectious GBV-B assay where uninfected, normal marmoset hepatocytes were inoculated *in vitro* with GBV-B infectious serum followed by

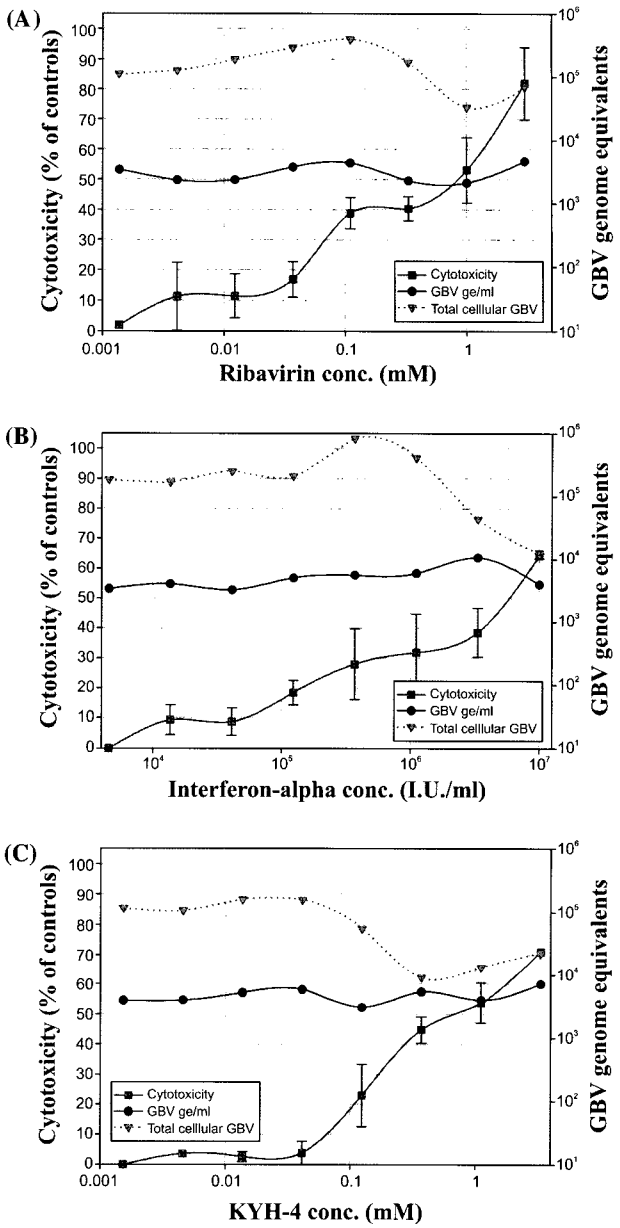

Fig. 4. The dose-effect of antiviral treatment for GBV-infected marmoset hepatocytes. Cultures were grown in the presence of increasing drug concentrations (X-axis); (A) ribavirin, (B) IFN-α, (C) 1-DNJ extract. Virus present in the culture media and cellular RNA were extracted after 6 days of antiviral treatment. GBV-B genome equivalents (GE) were quantified by RT-PCR using GBV-B specific primers in relation to a standard curve for rGBV-B RNA. The titers (right axis) of the secreted virus are expressed as GE/ml (-●-) and titers of intracellular virus expressed as GE/μg cell RNA (-▼-). The cytotoxicity (left axis) of drug treatment was based on the conversion of MTS to formazan by viable cells and expressed as a percentage of untreated control values (-■-).

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

Antiviral Drug	EC_{90}[a] 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-α	*n.c.*	3.39×10^6 IU/ml	4.8×10^6 IU/ml	1.42
1-DNJ	*n.c.*	1.37 mM	5.67 mM	4.14

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

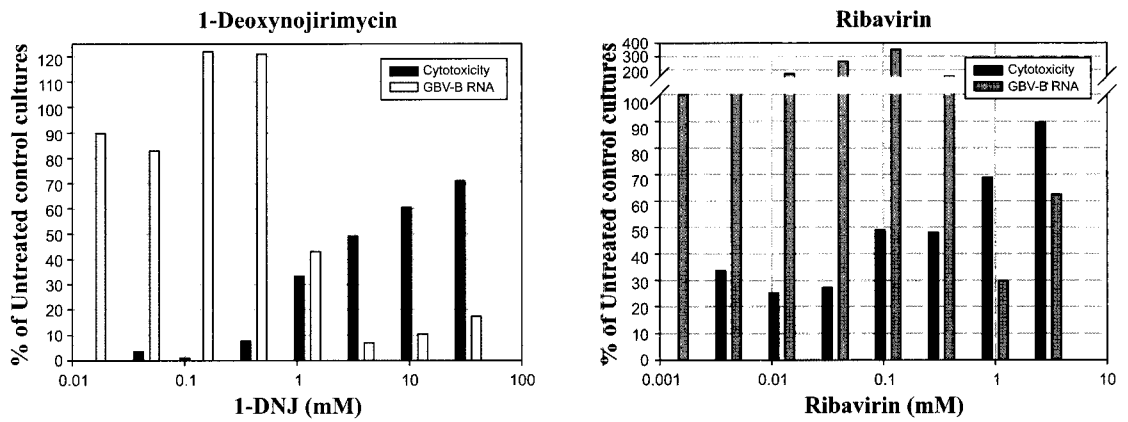

Fig. 5. The dose-effect of purified 1-DNJ and ribavirin on marmoset hepatocytes inoculated with GBV-B. At 24 h post-infection, cultures were grown in the presence of drug and total RNA was extracted after 5 days of treatment. GBV-B genome equivalents were quantified by RT-PCR; the data was normalized to the 18S ribosome content, and was expressed as a percent of the untreated control cultures. Cytotoxicity was expressed as a percent of the untreated controls based on a conversion of the MTS substrate by the mitochondria of the viable cells.

drug treatment exhibited a dose-response toward reducing the amount of GBV-B detected in cellular RNA extracts, as compared to the untreated controls (Fig. 5). In cultures treated with ribavirin, diminution of GBV-B was not evident until cytotoxicity was a factor. This result implies that upon 1-DNJ treatment, additional rounds of infection were not initiated. This was more apparent when compared to cultures treated with ribavirin, where the virus was not impeded. The antiviral mechanism attributed to ribavirin, a nucleoside analog, is purported to induce errors in replicating viral RNA (Lanford *et al.*, 2001). Particles with mutated genomes may still be packaged and may be able to infect naïve cells. Either drug did not affect the amount of virus secreted into the culture medium, again implying that glycosidase inhibition affects the viral attachment/penetration phase, and not replication.

1-DNJ and silkworm extract affect the glycosylation of WHV surface antigens

Synthetic DNJ derivatives have been investigated for their capacity to inhibit glycosylation of HBV surface envelope proteins (Block *et al.*, 1994; Lu *et al.*, 1995; Mehta *et al.*, 2002). Based on the chemical constituents of the silkworm extract we ascertained whether the natural derived products inhibited glycosylation of the surface antigens (WHsAg) of the related virus WHV. An evaluation of anti-glycosidase therapy on WHV-infected hepatocytes showed significant changes in the expression of WHsAg known to contain small (sm), middle (mid), and large (lg) WHs polypeptides (Fig. 6). Treatment with 1-DNJ altered the intracellular levels of gly-

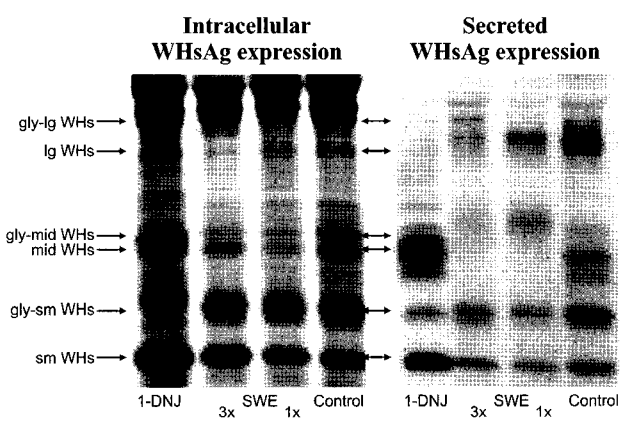

Fig. 6. WHsAg expressed from WHV-infected hepatocytes treated with 1-DNJ or silkworm extract [SWE 3×/1×]. [^{35}S] met-labeled WHV-infected hepatocytes were precipitated with rabbit anti-WHsAg marked as small (sm), middle (mid) or large(lg) in the non- or glycosylated (gly) form.

cosylated (gly)-smWHs and gly-midWHs (Fig. 6, lane 1 vs lane 4). The silkworm extract prevented intracellular accumulation of the gly-midWHs and both forms of the lgWHs (lanes 2-3 vs lane 4). Treatment with 1-DNJ affected secretion of WHsAg; suppressed expression of the gly-smWHs, increased the gly-midWHs content, and suppressed the level of lgWHs in comparison to the untreated cells (Fig. 6, lane 5 vs lane 8). Treatment with the silkworm extract increased the secretion of the glycosylated forms of midWHs and

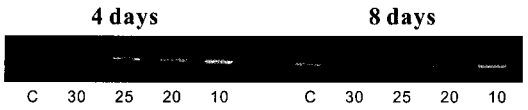

Fig. 7. Inhibition of the release of viral particles from HepG2.2.15 cells expressing HBV after treatment with purified 1-DNJ from silkworm extracts.

lgWHs, and suppressed non-glycosylated midWHs (Fig. 6, lanes 6-7 vs lane 8). The size and distribution of the WHs polypeptides detected by these methods are in accord with a previously published report (Tolle *et al.*, 1998).

1-DNJ suppresses the secretion of HBV particles

The results presented above suggest that secretion of WHsAg occurs after refinement of the intracellular glycosylated WHsAg. It is possible that the hyper-glycosylated WHsAg will not assemble with DNA-containing core particles. 1-DNJ was evaluated for its capacity to suppress HBV in the HepG2.2.15 system (Fig. 7). After treatment of cells with 1-DNJ, the release of virus particles was inhibited at concentrations >25 mM, specifically after 8 days of treatment. These results demonstrated that the naturally derived 1-DNJ inhibited HBV maturation in a dose and time dependent manner. Similar results were reported from studies on synthetic DNJ derivatives as antiviral agents against HBV (Lu *et al.*, 1995). The results of that study led to a search for more potent viral inhibitors, culminating in the evaluation of NN-DNJ in the woodchuck model (Block *et al.*, 1998). In this study, we began evaluating a nutraceutical product known to contain the active antiviral compound 1-DNJ. The mechanism of antiviral action, inhibiting glycosylation of the viral envelope glycoproteins, was demonstrated upon treatment with purified 1-DNJ and the antiviral effects were potentiated upon treatment with the silkworm extract. A selectivity index was calculated using the effective antiviral concentration relative to its cytotoxicity. Based on this result, the S.I. for IFN-α favors it over other drugs as a therapeutic agent (Table 1). An S.I. >208 for synthetic 1-DNJ has been reported previously (Durantel *et al.*, 2001). The S.I. of 4.4 for the silkworm extract was six fold greater than that for ribavirin. There was also a closer association between reducing viral loads and inhibiting BVDV-induced cell killing using the silkworm extract than there was with treatment with ribavirin. This finding indicated that the silkworm extract may be more effective than ribavirin, either as monotherapy or in combination with IFN-α. A 1,000 fold greater antiviral effect was observed upon testing the whole silkworm extract in comparison to the purified 1-DNJ suggesting synergistic effects of the constituent compounds present. This result is based on the effect for BVDV where the EC_{50} against viral-induced cell killing for 1-DNJ was a calculated as 2.96 mM (485 μg/ml). The EC_{50} for the silkworm extract was 0.016%. We assume the silkworm extract contains approximately 0.3% 1-DNJ, and if this were the only active compound present in the extract, it is present at a concentration of 0.48 μg/ml or 2.93 μM. The silkworm extract also shows a 187 fold greater effect on the reduction of viral yields (EC_{90}) than the purified 1-DNJ, based on similar calculations of 0.21 μg/ml verses 39.4 μg/ml, respectively. Although admittedly it is difficult to make a comparison between *in vitro* data and *in vivo* effects, based on the pharmacologic properties of ribavirin and IFN-α during therapy *in vivo* (Khakoo *et al.*, 1998; Jen *et al.*, 2002) we can translate our *in vitro* results to estimate the minimal therapeutic levels required for preclinical studies. An International Unit (I.U.) of interferon activity is defined as the effective concentration against Vesicular Stomatitis Virus in MDBK cells (Pestka, 1986). The EC_{90}=of 0.012 ng/ml calculated for rIFN-α in the BVDV assay (Table 1) converts to 2.3 I.U./ml, comparable to the international standard. The suggested therapeutic dose in humans is 3×10^6 I.U. administered 3× weekly, which results in short lived maximum serum-concentrations of 10-30 I.U./ml (Khakoo). This roughly approximates the 45 I.U. (0.23 ng/ml) required to inhibit viral-induced cell killing (EC_{50}) in the BVDV assay (Table 1). In reference to ribavirin, a steady-state serum concentration of approximately 2,200 ng/ml (9 μM) can be attained when administered at 600 mg/day over 4 weeks of therapy (Khakoo *et al.*, 1998). This approximates the 9.92 μM (2400 ng/ml) concentration required to inhibit viral-induced cell killing in the BVDV assay (Table 1). If the estimations made above hold true, a minimum 1-DNJ serum concentration of approximating 0.5 μg/ml should be attained upon dosing with the silkworm extract to elicit an antiviral response. Relevant to the above calculations, it has been reported the derivative N-nonyl-DNJ (NN-DNJ; M.W. 289 daltons) exhibits an EC_{50}=12.5 μM in the BVDV assays (Durantel *et al.*, 2001). Based on our calculations, this would equate to an effective serum concentration of 3.6 μg/ml. The identical compound was administered to WHV- infected woodchucks (25-50 mg/kg/day oral dose) and serum concentrations of 1.5-4.5 μg/ml, measured by HPLC methodology, were effective in reducing viral loads after 4 weeks of therapy (Block *et al.*, 1998). Although NN-DNJ is 10 fold more potent than 1-DNJ (Durantel *et al.*, 2001), the synergistic action of the compounds in the silkworm extract, as discussed above, suggest lower serum concentrations of 1-DNJ may be required upon dosing with the silkworm extract to elicit the similar desired antiviral effects.

Evaluation of ethnopharmacologic therapies (traditional Chinese and Kampo medicines, Indian and Korean medicinal plants) exhibiting antiviral potential may lead to their development as alternative or complementary medicine for conventional antiviral therapies in clinical use (Liang, 1999; Schuppan *et al.*, 1999; Seeff *et al.*, 2001; Jassin and Naji, 2003). Investigations into the constituent components of the ethnopharmacologic extracst that exhibit antiviral activity may lead to the discovery of new classes of compounds for the treatment of chronic viral hepatitis infections (Schuppan *et al.*, 1999; Seeff *et al.*, 2001; Jassim and Naji, 2003).

Acknowledgements

This work was partially supported by a grant from the BioGreen 21 Program, Rural Development Administration, Republic of Korea.

References

Asano, N. 2003. Glycosidase inhibitors: update and perspectives on practical use. *Glycobiology* 13, 93R-104R.

Asano, N., K. Oseki, H. Kizu, and K. Matsui. 1994. Nitrogen-in-the-ring pyranoses and furanoses: structural basis of inhibition of mammalian glycosidases. *J. Med. Chem.* 37, 3701-3706.

Asano, N., K. Oseki, E. Tomioka, H. Kizu, and K. Matsui. 1994. N-containing sugars from *Morus alba* and their glycosidase inhibitory activities. *Carbohydr. Res.* 259, 243-255.

Asano, N., T. Yamashita, K. Yasuda, K. Ikeda, H. Kizu, Y. Kameda, A. Kato, R.J. Nash, H.S. Lee, and K.S. Ryu. 2001. Polyhydroxylated alkaloids isolated from Mulberry trees (*Morus alba* L.) and silkworms (*Bombyx mori* L.). *J. Agric. Food Chem.* 49, 4208-4213.

Beames, B., D. Chavez, B. Guerra, L. Notvall, K.M. Brasky, and R.E. Lanford. 2000. Development of a primary tamarin hepatocyte culture system for GB virus-B: a surrogate model for hepatitis C virus. *J. Virol.* 74, 11764-11772.

Block, T.M., X. Lu, A.S. Mehta, B.S. Blumberg, B.C. Tennant, M. Ebling, B. Korba, D.M. Lansky, G.S. Jacob, and R.A. Dwek. 1998. Treatment of chronic hepadnavirus infection in a woodchuck animal model with an inhibitor of protein folding and trafficking. *Nature Med.* 4, 610-614.

Block, T.M., X. Lu, F.M. Platt, G.R. Foster, W.H. Gerlich, B.S. Blumber, and R.A. Dwek. 1994. Secretion of hepatitis B virus is inhibited by the imino sugar N-butyldeoxynojirimycin. *Proc. Natl. Acad. Sci. USA* 91, 2235-2239.

Branza-Nichita, N., D. Durantel, S. Carrouee-Durantel, R.A. Dwek, and N. Zitzmann. 2001. Antiviral effects of N-butyldeoxynojirimycin against bovine viral diarrhea virus correlates with misfolding of E2 envelope proteins and impairment of their association into E1-E2 heterodimers. *J. Virol.* 75, 3527-3536.

Cavalletto, L., L. Chemello, C. Donada, P. Casarin, F. Belussi, E. Bernardinello, F. Marino, P. Pontisso, A. Gatta, and A. Alberti. 2000. The pattern of response to interferon alpha predicts sustained response to a 6-month alpha-INF and ribavirin retreatment for chronic hepatitis C. *J. Hepatol.* 33, 128-134.

Chisari, F.V. and C. Ferrari. 1997. Viral hepatitis, p. 745-778. *In* N. Nathanson (ed.), Viral pathogenesis. Lippincott-Raven Publishers, Philadelphia, PA, USA.

Chou, T.C. and P. Talalay. 1984. Quantitative Analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Reg.* 22, 27-55.

Corapi, W.V., R.O. Donis, and E.J. Dubovi. 1990. Characterization of a panel of monoclonal antibodies and their use in the study of the antigenic diversity of bovine viral diarrhea virus. *Am. J. Vet. Res.* 51, 1388-1394.

Crespo, J., J.L. Lozano, B. Carte, B. De Las Heras, F. De La Cruz, and F. Pons-Romero. 1997. Viral replication in patients with concomitant hepatitis B and C virus infections. *Eur. J. Clin. Infect. Dis.* 16, 445-451.

Di Bisceglie, A.M. and B.R. Bacon. 1999. The unmet challenges of hepatitis C. *Scientific American* 281, 80-85.

Dobrinski, I., J.R. Jacob, B.C. Tennant, and B. Ball. 1999. Generation of an equine oviductal epithelial cell line for the study of sperm-oviduct interaction. *Theriogenology* 52, 875-885.

Durantel, D., N. Branza-Nichita, S. Carrouee-Durantel, T.D. Butters, R.A. Dwek, and N. Zitzmann. 2001. Study of the mechanism of antiviral action of iminosugar derivatives against bovine viral diarrhea virus. *J. Virol.* 75, 8987-8998.

Hoofnagle, J.H. and A.M. Di Bisceglie. 1997. The treatment of chronic viral hepatitis. *New Engl. J. Med.* 336, 337-356.

Jacob, G.S. 1995. Gylcosylation inhibitors in biology and medicine. *Curr. Opin. Struct. Bool.* 5, 605-611.

Jacob, J.R., R.H. Liu, C. Roneker, F. Noronha, J.H. Hotchkiss, and B.C. Tennant. 1994. Characterization and immortalization of woodchuck hepatocytes isolated from normal and hepadnavirus infected woodchucks (*Marmota monax*). *Exp. Cell Res.* 212, 42-48.

Jacob, J.R., I. Toshkov, B.E. Korba, P.J. Cote, W. DeLaney, J.L. Gerin, and B.C. Tennant. 2004. Suppression of lamivudine-resistant B-domain mutants by adefovir dipivoxil in the woodchuck hepatitis virus model. *Antiviral Res.* In press.

Jarvis, B. and D. Faulds. 1999. Lamivudine. A review of its therapeutic potential in chronic hepatitis B. *Drugs* 58, 101-141.

Jassim, S.A.A. and M.A. Naji. 2003. Novel antiviral agents: a medicinal plant perspective. *J. Appl. Micro.* 95, 412-427.

Jen, J., M. Laughlin, C. Chung, S. Heft, M.B. Affrime, S.K. Gupta, P. Glue, and G. Hajian. 2002. Ribavirin dosing in chronic hepatitis C: Application of population pharmacokinetic-pharmacodynamic models. *Clin. Pharmacol. Therapeutics* 72, 349-361.

Joubert, P.H., C.P. Venter, H.F. Joubert, and I. Hillebrand. 1985. The effect of 1-deoxynojirimycin derivative on post-pradial blood glucose and insulin levels in healthy black and white volunteers. *Eur. J. Clin. Pharmacol.* 28, 705-708.

Khakoo, S., P.G. Glue, L. Grellier, B. Wells, A. Bell, C. Dash, I. Murray-Lyon, D. Lypnyj, B. Flannery, K. Walters, and G.M. Dusheiko. 1998. Ribavirin and interferon alfa-2b in chronic hepatitis C: assessment of possible pharmacokinetic and pharmacodynamic interactions. *Br. J. Clin. Pharmacol.* 46, 563-570.

Kimura, M., F.J. Chen, N. Nakashima, I. Kimura, N. Asano, and S. Koya. 1995. Antihyperglycemic effects of N-containing sugars derived from Mulberry leaves in streptozocin-induced diabetic mice. *J. Trad. Med.* 12, 214-219.

Korba, B.E. and J.L. Gerin. 1992. Use of a standardized cell culture assay to assess activities of nucleoside analogs against hepatitis B virus replication. *Antiviral Res.* 19, 55-70.

Lanford, R.E., D. Chavez, B. Guerra, J.Y.N. Lau, Z. Hong, K.M. Brasky, and B. Beames. 2001. Ribavirin induces error-prone replication of GB virus B in primary tamarin hepatocytes. *J. Virol.* 75, 8074-8081.

Liang, T.J. 1999. Complementary and alternative medicine: The roots of healing. *Gastroenterology* 117, 1041.

Liaw, Y.F. 2001. Concurrent hepatitis B and C virus infection: is hepatitis C virus stronger? *J. Gastroenterol. Hepatol.* 16, 597-598.

Liu, J., L. Kjaergard, and C. Glund. 2002. Misuse of randomization: a review of Chinese randomized trials of herbal medicines for chronic hepatitis B. *Amer. J. Chinese Med.* 30, 173-176.

Look, M.P., A. Gerard, G.S. Rao, T. Sudhop, H.P. Fischer, T. Sauerbruch, and U. Spengler. 1999. Interferon/antioxidant combination therapy for chronic hepatitis C-a controlled pilot trial. *Antiviral Res.* 43, 113-122.

Lu, X., A. Mehta, R. Dwek, T. Butters, and T. Block. 1995. Evidence that N-linked glycosylation is necessary for hepatitis B virus secretion. *Virol.* 213, 660-665.

Martin, K.W. and E. Ernest. 2003. Antiviral agents from plants and herbs; a systemic review. *Antivir. Ther.* 8, 77-90.

McHutchison, J.G., S.C. Gordon, E.R. Schiff, M.L. Shiffman, W.M. Lee, V.K. Rustgi, Z.D. Goodman, M.H. Ling, S. Cort, and J.K. Albrecht. 1998. Interferon alpha 2a alone or in combination with ribavirin as initial treatment for chronic hepatitis C. *New England J. Med.* 339, 1485-1492.

Mehta, A., B. Conyers, D.L.J. Tyrrell, K.A. Walters, G.A. Tipples, R. Dwek, and T.M. Block. 2002. Structure-activity relationship of a new class of anti-hepatitis B virus agents. *Antimicro. Agents Chemo.* 46, 4004-4008.

Ouzounzov, S., A.D. Menta, T.M. Block, and R. Jordan. 2002. The combination of interferon α-2b and *n*-butyl deoxynojirimycin has a greater than additive antiviral effect upon production of infectious bovine viral diarrhea virus (BVDV) *in vitro*: implications for hepatitis C virus (HCV) therapy. *Antiviral Res.* 55,

425-435.

Pestka, S. 1986. Interferon standards and general abbreviations. *Meth. Enzymol.* 119, 14-23.

Schuppan, D., J.D. Jia, B. Brinhaus, and E.G. Hann, 1999. Herbal products for liver diseases: A therapeutic challenge for the new millennium. *Hepatology* 30, 1099-1104.

Schvarcz, R., Z.B. Yun, A. Sonnerborg, and O. Weiland. 1995. Combined treatment with interferon alpha-2b and ribavirin for chronic hepatitis C in patients with a previous non-response or non-sustained response to interferon alone. *J. Med. Virol.* 46, 43-47.

Seeff, L.B., K.L. Lindsay, B.R. Bacon, T.F. Kresina, and J.H. Hoofnagle. 2001. Complementary and alternative medicine in chronic liver disease. *Hepatology* 34, 595-603.

Stephensen, C.B., J.R. Jacob, R.J. Montali, R.J. Montali, E.D. Armes, M.J. Buchmeler, E. Muchmore, K.V. Holmes, and R.E. Lanford. 1991. Isolation of an arenavirus from a marmoset with callitrichid hepatitis and its serologic association with disease. *J. Virol.* 65, 3995-4000.

Strader, D.B. and H.J. Zimmerman. 2000. Complementary and alternative medicine in Hepatitis C, p. 363-386. *In* T.J. Liang and J.H. Hoofnagle (ed.), Biomedical Research Reports; Hepatitis C. Academic Press, New York, NY, USA.

Tolle, T.K., D. Glebe, M. Linder, D. Linder, S. Schmitt, R. Geyer, and W.H. Gerlich. 1998. Structure and glycosylation patterns of surface proteins from woodchuck hepatitis virus. *J. Virol.* 72, 9978-9985.

Tsiang, M., J.F. Roonex, J.J. Toole, and G.S. Gibbs. 1999. Biphasic clearance kinetics of hepatitis B virus from patients during adefovir dipivoxil therapy. *Hepatology* 29, 1863-1869.

Vilcek, J. and G.C. Sen. 1996. Interferons and other cytokines, p. 375-399. *In* B.N. Fields, D.M. Knipe, and P.M. Howley (ed.), Fields Virology. Lippincott-Raven Publishers, Philadelphia, PA, USA

Wu, S.F., C.J. Lee, C.L. Liao, R.A. Dwek, N. Zitzmann, and Y.L. Lin. 2002. Antiviral effects of an iminosugar derivative on flavivirus infections. *J. Virol.* 67, 3596-3604.

Zitzmann, N., A.S. Mehta, S. Carrouee, T.D. Butters, F.M. Platt, J. McCauley, B.S. Blumberg, R.A. Dwek, and T.M. Block. 1999. Imino sugars inhibit the formation and secretion of bovine viral diarrhea virus, a pestivirus model of hepatitis C virus: implications for the development of broad spectrum anti-hepatitis virus agents. *Proc. Natl. Acad. Sci. USA* 96, 11878-11882.