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# 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

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**Abstract**

Interferon α-2b (IFN) alone or in combination with Ribavirin is approved in the United States for the treatment of chronic hepatitis C virus (HCV) infection. We have previously reported that the glucosidase inhibitor, *n*-butyl deoxynojirimycin (*n*B-DNJ) inhibits the production of infectious bovine diarrhea virus (BVDV) (Proc. Natl. Acad. Sci. 96 (1999) 11878). Since BVDV has been used as a model for HCV and grows productively in tissue culture, and IFN and glucosidase inhibitors are thought to act at different steps in the virus life cycle, it was of interest to determine the antiviral impact of combining *n*B-DNJ with IFN. Using plaque reduction and single-step growth analyses of the cytopathic BVDV strain NADL, data are presented that shows human IFN inhibited BVDV production in a dose dependent manner, with 3 IU/ml inhibiting 50% of the yield of virus (IC50) when added within 1 h post infection. Under the same conditions, the glucosidase inhibitors *n*B-DNJ and castanospermine (CST) also prevented BVDV production in a dose dependent manner with IC50s of 226 μM and 47 μM, respectively. In combination with 138 μM *n*B-DNJ the apparent IC50 for IFN was 0.056 IU/ml. This 54-fold increase in IFN potency suggests that *n*B-DNJ can synergize with IFN. Two additional independent analyses were performed to measure combination effects which demonstrated that the combined antiviral effect of *n*B-DNJ and IFN were greater than would be expected for a simple additivity. These data are consistent with an interpretation that glucosidase inhibitors and IFN have a synergistic antiviral effect in tissue culture. The relevance of these finding to treatment of HCV infection is discussed. 

*Keywords:* Interferon α-2b (IFN); *n*-Butyl deoxynojirimycin (*n*B-DNJ); Hepatitis C virus (HCV); Bovine viral diarrhea virus (BVDV)

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## 1. Introduction

In recent years, combination therapy for treatment of viral infections has become increasingly popular. The combined action of antiviral compounds that inhibit virus replication by different mechanisms often result in greater than additive antiviral activity. For example, treatment of Hepatitis C virus (HCV) infection using combinations of interferon α-2b (IFN) and ribavirin, produced a sustained response in 31–38% of patients as defined by the absence of HCV RNA in serum 24 weeks after treatment (Reichard et al., 1998; Davis et al., 1998). In contrast, IFN α-2b monotherapy produced a sustained response in only 18% of patients, while ribavirin monotherapy showed only transient reduction in ALT levels and no effect on serum HCV RNA levels (Bodenheimer et al., 1997; Reichard et al., 1998). Thus, combination therapy is often more effective than monotherapy in treating persistent viral infection and is likely to be more commonly used.

IFN is widely used in the treatment of viral infections both as monotherapy and as combination therapy (Liang, 1998; Brook et al., 1989; Hulton et al., 1992; Johnson et al., 1990; Lane et al., 1990). IFN binds to cell surface receptors and stimulates signal transduction pathways that lead to activation of cellular enzymes that repress virus replication (Samuel, 2001; Kaufman, 1999). Enzymes induced by IFN include double-stranded RNA-activated protein kinase (PKR) and RNase L which inhibit translation initiation and degrade viral RNA, respectively (Samuel, 2001; Kaufman, 1999). Many viruses have evolved mechanisms to counteract the effects of IFN by blocking the activity of these enzymes. For example, HCV E2 glycoprotein and NS5a have been shown to block PKR activity in vitro(Song et al., 1999; Gale et al., 1997, 1998; Taylor et al., 1999). Moreover, strains of HCV that are more resistant to IFN show less sequence diversity in regions of E2 and NS5a required for interaction with PKR(Gale et al., 1998; Taylor et al., 1999). The ability of viruses to counteract the effects of IFN has made it necessary to use IFN in combination with other antiviral agents for the treatment of persistent virus infection.

Recently, it has been discovered that inhibitors of host endoplasmic reticulum (ER) glucosidase are potent antiviral agents that inhibit replication of a number of viruses including bovine viral diarrhea virus (BVDV); a tissue culture surrogate of HCV (Zitzmann et al., 1999; Fisher et al., 1996b,b,a,b; Courageot et al., 2000; Schlesinger et al., 2000, 1985). Glucosidase inhibitors block the first step in glycoprocessing and interfere with glycoprotein folding. Since IFN and glucosidase inhibitors act by different mechanisms to inhibit virus replication, it was reasoned that the combined effects of these two compounds would produce greater than additive antiviral effects.

In this study, we show that human IFN used in combination with the glucosidase inhibitor *n*-butyl deoxynojirimycin (*n*B-DNJ), produced greater than additive antiviral effects against BVDV infection. In contrast, *n*B-DNJ, in combination with the chemically distinct glucosidase inhibitor, castanospermine (CST), showed only additive antiviral effects against BVDV. The results of this study suggest that *n*B-DNJ can potentiate the antiviral effects of IFN and thus may be useful as combination therapy for the treatment of HCV infection.

## 2. Materials and methods

### 2.1. Cells and viruses

Madin–Darby bovine kidney cells (ATCC-CCL22) were grown in DMEM/F12 (Gibco/BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated horse serum (Gibco/BRL). BVDV cytopathic strain NADL was kindly provided by Dr Ruben Donis, University of Nebraska. The virus stock was plaque purified three times on MDBK cell monolayers prior to large scale virus stock preparation.

### 2.2. Glucosidase inhibitors and IFN

Castanospermine (Sigma Chemical, St Louis, MO) was prepared as a concentrated stock at 100 mM in DMSO. *n*B-DNJ was provided by Searle Pharmaceuticals, (St. Louis, MO). Stocks of *n*B-

DNJ were prepared in water at 450 mM. There was no evidence of insolubility of CST or *n*B-DNJ in the stock solutions or when diluted into medium at 100 μM (CST) or 4.5 mM (*n*B-DNJ). Human IFN α-2b (PBL Biomedical Inc., New Brunswick, NJ) was diluted in MDBK media at $10^6$ IU/ml. Human IFN has been shown to inhibit BVDV replication in MDBK cells (Senssui et al., 1998; Gillespie et al., 1985). One international unit (IU) of IFN was defined as the amount of IFN that reduced vesicular stomatitis virus (VSV)-induced cytopathic effect by 50% on MDBK monolayers.

### 2.3. Plaque reduction assays

MDBK monolayers ($5 \times 10^4$ per well of a 96 well tray) were infected with 10-fold serial dilutions of BVDV strain NADL. At 1 h post-infection, the inoculum was removed and the cultures were washed twice in MDBK media. Media containing *n*B-DNJ, CST, or IFN alone or in combinations were added to the cultures. Viral plaques were counted at 48–72 h post-infection.

### 2.4. BVDV single-cycle replication

MDBK cells ($5 \times 10^4$ cells per well of a 96 well tray) were infected in triplicate at a multiplicity of infection of $>1$ PFU/cell. At 1 h post-infection, the virus inoculum was removed, and monolayers were washed twice with 100 μl of MDBK medium. MDBK medium in the presence and absence of 100 μM CST, 180 μM *n*B-DNJ, 5 IU/ml IFN, or combinations of *n*B-DNJ and IFN were added to the cultures. At 24 h post-infection the cultures were harvested and frozen at −70 °C. The cultures were thawed at 37 °C and cell debris was removed by centrifugation at $1000 \times g$ for 10 min at 4 °C. Virus yields in the supernatant fluids were measured by plaque assay.

### 2.5. Toxicity measurements

The toxicity of each compound alone or in combination was measured by MTT assay, which measures mitochondrial dehydrogenase activity (Heo et al., 1990). MDBK monolayers were treated with each compound alone or in combination for 48 h. The cells were processed for MTT activity as described (Heo et al., 1990).

In addition to MTT testing, cell number and viability were assessed by direct cell counting of confluent and subconfluent MDBK monolayers incubated with each compound alone or in combination. MDBK monolayers were seeded at 10% confluency ($0.25 \times 10^5$ cells per well of a 24 well tray) or 90% confluency ($2 \times 10^5$ cells per well of a 24 well tray) and incubated with medium supplemented with 4.5 mM *n*B-DNJ, or 4.5 IU/ml of IFN either alone or in combination. At 24, 48, and 72 h post-treatment, the cultures were trypsinized and diluted 1:4 in 1X PBS containing 0.1% trypan

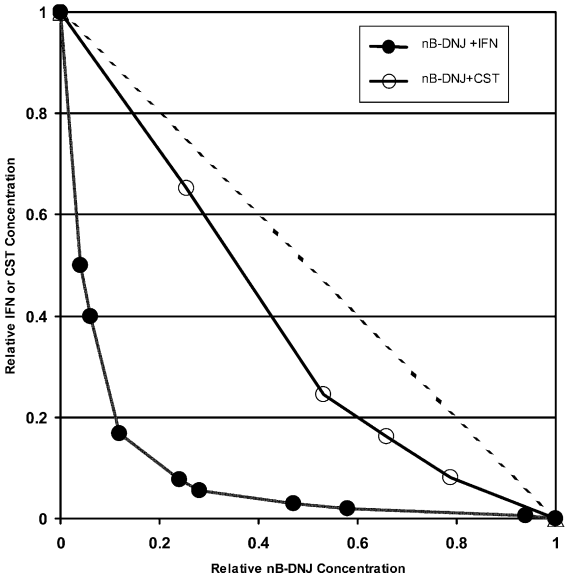


Fig. 1. Isobologram analysis of IC50 values for combinations of *n*B-DNJ and IFN or *n*B-DNJ and CST. Infected MDBK monolayers were treated with increasing concentrations of *n*B-DNJ, CST, and IFN alone or in combination. The IC50 values for *n*B-DNJ and IFN (closed circles) or *n*B-DNJ and CST (open circles) were calculated and normalized to the IC50 values of each compound alone. These normalized values were plotted as an isobologram. The dashed diagonal line represents the theoretical values for two compounds that are additive with respect to antiviral activity. The IC50 values for *n*B-DNJ, CST, and IFN alone are 240, 47 μM, and 3 IU/ml, respectively. The IC50 value for IFN in combination with 138 μM *n*B-DNJ (relative *n*B-DNJ concentration of 0.58) was 0.056 U/ml. The IC50 value for CST in combination with 126 μM *n*B-DNJ (relative *n*B-DNJ concentration of 0.53) was 11 μM.

blue. Cell numbers were determined by counting the diluted cell suspension using a hemacytometer.

### 2.6. Data analysis

Linear regression analysis was used to calculated IC50 values from virus yield or plaque reduction assays. The IC50 values were normalized to the IC50 of each compound alone and used to calculate the relative IC50 values for the drug combinations. These values were used to generate the isobologram (Fig. 1) (Poch et al., 1990).

Combination antiviral effects for IFN, *n*B-DNJ, and CST was analyzed using an independent effects model (Prichard and Shipman, 1990). In this model, 3-dimensional dose response surfaces were generated that represent a difference map between experimental and theoretical interaction values (Fig. 2).

To determine the significance of the results, the data were subjected to analysis of variance (ANOVA), and the intraclass correlation coefficient was calculated to quantitate the test to test variation of replicate antiviral assays. The error due to variance of replicates were used to calculate 95% confidence limits for the combination data.

## 3. Results

### 3.1. The effects of *n*B-DNJ, CST, and IFN on BVDV plaque formation

Glucosidase inhibitors and IFN both inhibit virus replication through interaction with host targets, however, their mechanisms of action are distinct. To better understand the combined effects of glucosidase inhibitors and IFN it was necessary to first characterize the antiviral properties of these drugs individually. Virus yield assays were used to measure the antiviral effects of each compound using doses of compounds that reflect approximate IC90 values. MDBK monolayers were infected at $>1$ pfu/cell and at 1 h post-infection, media containing *n*B-DNJ, CST, or IFN were added to the cultures. At 24 h post-infection the cultures were harvested and virus yields were measured by plaque assay (Table 1). In the presence of 180 μM *n*B-DNJ, 100 μM CST, or 5 IU/ml IFN, the 24 h BVDV yield was reduced by 11-fold, 50-fold, and 14-fold, respectively. These results demonstrate that BVDV replication is sensitive to glucosidase inhibitors and IFN when added independently.

Plaque reduction assays were performed using a range of drug concentrations to establish the concentration of each compound required to inhibit virus replication by 50% (IC50). The IC50 for *n*B-DNJ, CST and IFN was determined to be 226, 47 μM, and 3 IU/ml, respectively. In single-round 24 h virus yield assays performed at high multiplicity of infection ($>1$ PFU/cell) the IC50 for *n*B-DNJ, CST, and IFN were 112, 5 μM and 5 IU/ml. Notably, the IC50 values for CST in the plaque reduction assays and virus yield assays were substantially different. While the reason for this difference in IC50 values is unknown, this result does not alter the interpretations of combination data described below. Pre-treatment of MDBK cells with IFN did not enhance the antiviral activity in these experiments. The results of these tests demonstrate that glucosidase inhibitors and IFN are potent inhibitors of BVDV replication.

While glucosidase inhibitors and IFN are potent antiviral agents against BVDV replication, they had little effect on cell viability at the concentrations used to inhibit virus replication as measured by MTT assay and trypan blue staining. In addition, there was no evidence of decreased cell proliferation as measured by direct cell counting over a 72-h period (Table 2). Treatment of cells under the same conditions used for the plaque reduction assays with each compound alone or in combination showed no evidence of toxicity at the concentrations used to inhibit viral replication (Table 2). The concentration of CST that was cytotoxic for 50% of the cells (CC50) as measured by mitochondrial dehydrogenase activity (MTT assay, (Heo et al., 1990)) was 6500 μM. The actual CC50 for *n*B-DNJ and IFN could not be achieved at the concentrations tested. The highest concentrations used in the toxicity studies, both alone and in combination, were 9000 μM for *n*B-DNJ and 4000 IU/ml for IFN. Notably, these concentrations are well above the amounts used in the

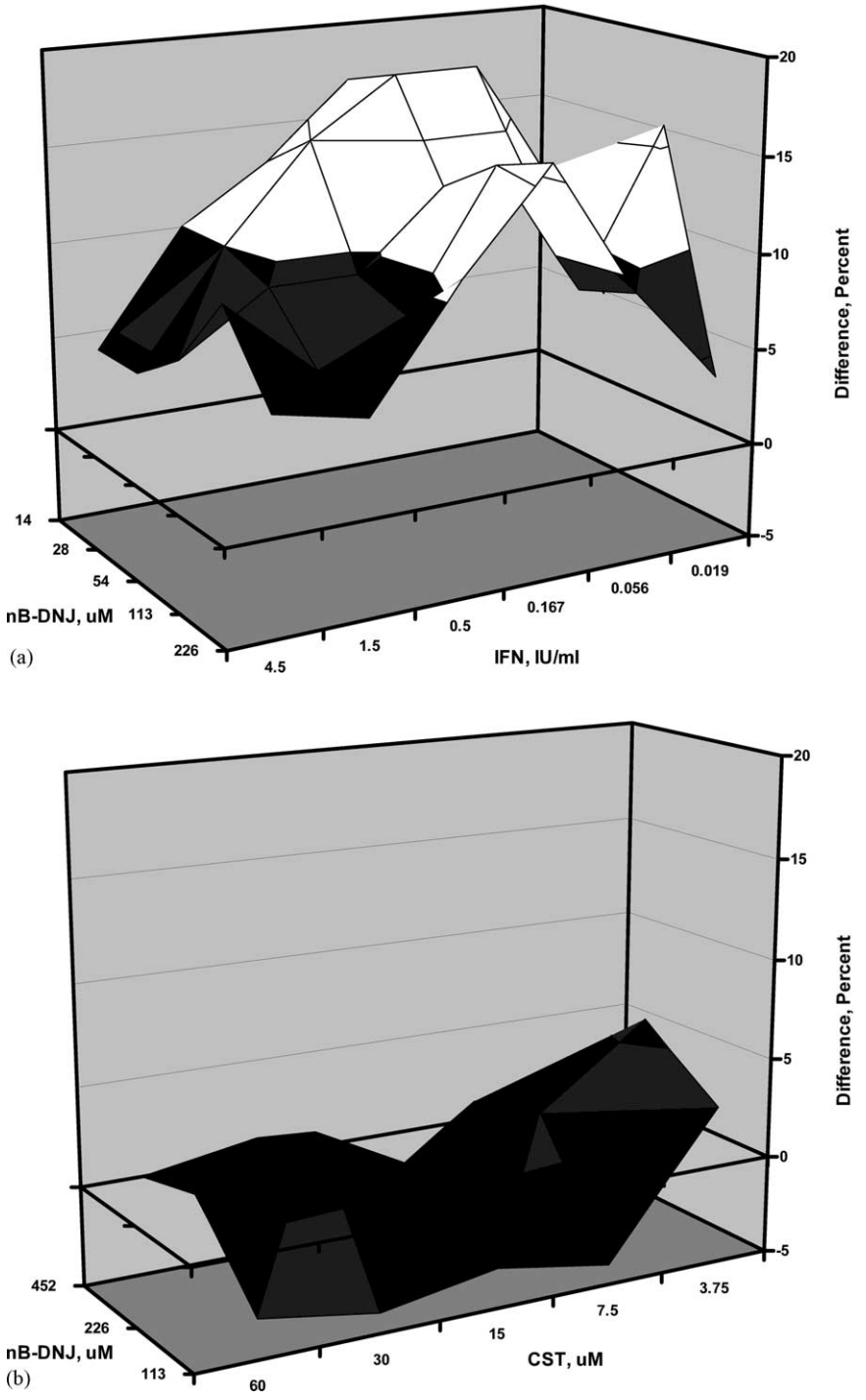
20
15
10
5
0
-5
Difference, Percent
14
28
54
nB-DNJ, uM
113
226
(a)
4.5
1.5
0.5
0.167
0.056
0.019
IFN, IU/ml
20
15
10
5
0
-5
Difference, Percent
452
226
nB-DNJ, uM
113
(b)
60
30
15
7.5
3.75
CST, uM

Fig. 2

Table 1
The effects of *n*B-DNJ, CST, and IFN on 24 h virus yields

| | No drug (PFU per ml) | Drug (PFU per ml) | Fold inhibition |
|---|---|---|---|
| *n*B-DNJ | $1.1 \times 10^6$ | $1.0 \times 10^5$ | 11 |
| IFN | $1.1 \times 10^6$ | $7.7 \times 10^4$ | 14 |
| IFN+*n*B-DNJ | $1.1 \times 10^6$ | $1.2 \times 10^4$ | 90 |
| CST | $5.0 \times 10^6$ | $1.0 \times 10^5$ | 50 |

MDBK monolayers were infected at MOI of $>1.0$ PFU/cell. At 1 h post-infection, the virus inoculum was removed and media in the presence and absence of 180 μM *n*B-DNJ, 100 μM CST, and 5 IU/ml IFN was added to the cultures. In the combination study, 180 μM *n*B-DNJ and 5 IU/ml IFN were added to the infected-cultures. Virus yields were measured at 24 h post-infection by plaque assay. The fold-inhibition represents the reduction in virus yield in the presence of antiviral compounds.

antiviral assays (Table 2). Moreover, at 400 μM of *n*B-DNJ, a concentration which reduced BVDV 24 h yields by more than 90%, there was very little difference in the amount of secreted glycoproteins eventhough greater than 95% of the secreted glycoproteins contained terminal glucose residues as measured by glycan sequencing and HPLC analysis (data not shown). This result indicates that *n*B-DNJ inhibited intracellular ER glucosidase activity without effecting glycoprotien secretion. Taken together, these results suggest that viral plaque formation is considerably more sensitive to glucosidase inhibitors and IFN than are host functions essential for cell viability.

### 3.2. Combination studies with *n*B-DNJ and IFN

To determine if combinations of *n*B-DNJ and IFN would be more potent at inhibiting BVDV replication than each compound alone, MDBK cells were infected with BVDV at $>1$ PFU/cell, and at 24 h post-infection virus yields were measured. As mentioned previously, in the presence of 180 μM *n*B-DNJ and 5 IU/ml IFN alone the 24 h virus yield was reduced by 11-fold and 14-fold, respectively (Table 1). In the presence of both *n*B-DNJ and IFN the virus yield was reduced by approximately 90-fold (Table 1). Moreover, the IC50 value for IFN alone was 3 IU/ml while in combination with 138 μM *n*B-DNJ (relative *n*B-DNJ concentration of 0.58, Fig. 1) the apparent IC50 for IFN was 0.056 IU/ml. This represents a 54-fold increase in potency of IFN in the presence of *n*B-DNJ. This increased antiviral effect suggests that *n*B-DNJ interacts with IFN to produce greater than additive antiviral activity against BVDV replication.

Interactions between antiviral compounds are most easily observed when antiviral assays are performed using concentrations of compounds at or below the IC90 values for each compound separately (Prichard and Shipman, 1990). Therefore, plaque reduction assays and sub IC90 concentrations of compounds were used to measure interactions between *n*B-DNJ and IFN. To examine the combined effects of these two compounds, the relative change in IC50 values for IFN at different fixed concentrations of *n*B-DNJ were calculated and plotted as an isobologram (Fig. 1, closed circles). The IC50 value for IFN alone was 3 IU/ml while in combination with 138 μM *n*B-DNJ (relative *n*B-DNJ concentration of 0.58, Fig. 1) the

Fig. 2. A 3-dimensional dose response analysis of the effects of combination of *n*B-DNJ and IFN or *n*B-DNJ and CST on BVDV plaque formation in MDBK cells. The combination data in Fig. 1 was reanalyzed using an independent effects model that generates a 3-dimensional dose response surface that represents a difference map between experimentally derived fractional inhibition values and calculated theoretical inhibition values. The data used to calculate IC50 values in the isobologram analysis were used to produce the 3-dimensional dose response surfaces. Statistical analysis of the data were performed as described in Section 2 and were used to calculate 95% confidence limits for the combination data. The results of the statistical analysis are shown graphically where unshaded areas indicate statistically significant synergy. (a) A 3-dimensional dose response surface showing interactions between *n*B-DNJ and IFN. (b) A 3-dimensional dose response surface for interactions between *n*B-DNJ and CST. No statistically significant synergy or antagonism was observed for *n*B-DNJ and CST.

Table 2
Toxicity measurements for *n*B-DNJ, CST, and IFN

| Compound | [a]$CC_{50}$ | [b]Cell number (S.D.) sub-confluent | [b]Cell number (S.D.) confluent |
|---|---|---|---|
| Mock | N.D. | $0.88 \times 10^6$ (0.076) | $1.41 \times 10^6$ (0.015) |
| *n*B-DNJ | >9000 μM | $0.88 \times 10^6$ (0.036) | $1.39 \times 10^6$ (0.032) |
| CST | 6500 μM | N.D. | |
| IFN | >4000 IU/ml | $0.90 \times 10^6$ (0.053) | $1.42 \times 10^6$ (0.017) |
| *n*B-DNJ+IFN | >9000 μM+4000 IU/ml | $0.88 \times 10^6$ (0.038) | $1.44 \times 10^6$ (0.038) |

[a] MDBK monolayers were treated for 72 h with *n*B-DNJ, CST, and IFN alone or in combination. The actual CC50 for *n*B-DNJ and IFN, either alone or in combination, could not be achieved. The CC50 values represent the highest concentrations of drug tested.
[b] MDBK monolayers were seeded at $0.25 \times 10^5$ cells per well (sub confluent) or $2 \times 10^5$ cells per well (confluent) in a 24 well tray and incubated with 9000 μM *n*B-DNJ or 4000 IU/ml IFN, either alone or in combination. Cells numbers were determined by direct counting after trypan blue staining. Cells were counted from three separate wells and standard deviations (S.D.) were calculated.

IC50 for IFN was 0.056 IU/ml. The diagonal dashed line in the isobologram represents the theoretical IC50 values for additivity between the two compounds. IC50 values above this line are considered antagonistic while IC50 values below this line are considered greater than additive. The isobologram analysis shows that IC50 values for all combinations of *n*B-DNJ and IFN plot well below the line of additivity, indicating a greater than additive antiviral effect (Fig. 1, closed circles). Similar results were observed with CST and IFN (data not shown). Taken together, these results suggest that *n*B-DNJ and IFN synergistically interact to inhibit BVDV replication.

Castanospermine and *n*B-DNJ are both inhibitors of ER glucosidases and likely function in the same pathway to inhibit BVDV replication. Thus, the combined effects of these two compounds should be additive with respect to inhibition of BVDV replication. To measure the combined effects of *n*B-DNJ and CST and to validate our methods of measuring synergy, plaque reduction assays were used to measure the antiviral effects of combinations of *n*B-DNJ and CST. Isobologram analysis showed that the IC50 values for combinations of *n*B-DNJ and CST deviated slightly from the theoretical line for additivity, indicating that *n*B-NDJ and CST are additive or only slightly synergistic with respect to inhibition of BVDV replication (Fig. 1, open circles). The slight deviation from additivity was much less pronounced than for the combination of *n*B-DNJ and IFN (Fig. 1, closed circles). Moreover, the calculated IC50 value for CST alone was 47 μM while in combination with 126 μM *n*B-DNJ (relative *n*B-DNJ concentration of 0.53, Fig. 1), the IC50 value for CST was 11 μM. The results of these tests suggest that the antiviral effects of *n*B-DNJ and CST are additive or slightly synergistic.

To more completely analyze the combined effects of *n*B-DNJ and IFN, and CST and *n*B-DNJ, the data were reanalyzed using an independent effects model (Prichard and Shipman, 1990). This model generates 'synergy volumes' from a 3-dimensional dose response surface that represents a difference map between experimentally derived inhibition values and calculated theoretical inhibition values. An example of this analysis is shown in Fig. 2a and b. The non-uniform surface of the dose response surface likely represents inherent variability in biological assays. Values above zero in the $x-y$ plane indicate synergistic interaction between compounds while values below zero in the $x-y$ plane indicate antagonism. As shown in Fig. 2a, combinations of IFN and *n*B-DNJ produced a dose response surface well above zero in the $x-y$ plane indicative of synergistic interactions. Similar results were observed with IFN and CST (data not shown). Combinations of CST and *n*B-DNJ produce a dose response curve that was close to or below zero in the $x-y$ plane suggesting additive or even slightly antagonistic antiviral effects (Fig. 2b). The results of these analyses are consistent with the isobologram analysis (Fig. 1) which showed that IFN and *n*B-DNJ produce greater than additive antiviral effects while CST and *n*B-

DNJ in combination are additive with respect to inhibition of BVDV replication.

To determine the significance of these results, the data were subjected to ANOVA, and the intraclass correlation coefficient was calculated to quantitate the test to test variation of replicate antiviral assays. The intraclass correlation coefficient for combinations of *n*B-DNJ and IFN was 89.8% indicating that 10.2% of the variation was within replicates samples. In contrast, a similar analysis of combinations of CST and *n*B-DNJ showed that the observed synergy and antagonism was not statistically significant. The error due to variance of replicates were used to calculate 95% confidence limits for the combination data. The results of the statistical analysis are shown graphically in Fig. 2a and b where unshaded areas of the 3-dimensional dose response surface indicate statistically significant synergy (within 95% confidence limits). In contrast, a similar analysis of combinations of *n*B-DNJ and CST did not produce statistically significant synergy or antagonism (Fig. 2b).

## 4. Discussion

This manuscript makes one simple but important point: that whereas *n*B-DNJ and CST effects are additive with respect to inhibition of BVDV replication, *n*B-DNJ and IFN effects were greater than additive. Combination studies showed that in the presence of *n*B-DNJ, the antiviral activity of IFN was increased as shown by a decrease in IC50 values. Moreover, when analyzed by two independent methods, the combination data consistently showed synergistic interaction between *n*B-DNJ and IFN. Applying these same analyses to *n*B-DNJ and CST, the combination data showed additivity, as would be expected for compounds that likely inhibit virus replication by targeting the same step in the virus life-cycle.

While CST shows greater potency against BVDV than *n*B-DNJ, derivatives of *n*B-DNJ have been developed, which contain longer alkyl side chains, that are more potent antiviral agents (Durantel et al., 2001). However, these derivatives also inhibit viral replication by an additional mechanism, unrelated to inhibition of glucosidase activity (Durantel et al., 2001; Mehta et al., 2001). Thus, to simplify the interpretation of our combination data, *n*B-DNJ was used in these studies.

The antiviral activity of glucosidase inhibitors is not due to toxicity or changes in cell proliferation. In our experiments, cell monolayers were seeded at 90% confluence and treated for 72 h with *n*B-DNJ in the presence and absence of IFN. MTT assay, trypan blue staining, and cell counting, showed no evidence of reduced cell viability or reduced cell numbers relative to untreated controls. While several studies have demonstrated that glucosidase inhibitors reduce tumor growth and have anti-angiogenic properties, these same studies showed that glucosidase inhibitors did not inhibit cell proliferation of cultured fibroblasts (Pili et al., 1995). Thus, while glucosidase inhibitors may have antiproliferative activity in certain cell or tissue types, in MDBK cells, under the conditions used in our experiments, these compounds had no measurable effect on cell proliferation and viability.

### *4.1. Possible mechanism for synergy between glucosidase inhibitors and IFN*

Virion glycoproteins play an important role in regulating the host response to viral infection. In HCV, the E2 virion glycoprotein as well as the NS5A protein, have been implicated in conferring resistance to IFN by blocking the activity of an IFN-inducible protein kinase, PKR (Song et al., 1999; Gale et al., 1997, 1998; Taylor et al., 1999). Activated PKR inhibits viral replication by phosphorylating the translation initiation factor, eif2-α, thereby inhibiting viral translation (Mathews, 1996). E2 and NS5A physically interact with PKR and prevent phosphorylation of eif2-α as measured by coimmunoprecipitation studies and GST-affinity chromatography (Gale et al., 1998; Taylor et al., 1999). Strains of HCV that are more resistant to IFN show less sequence diversity in regions of E2 and NS5a genes required for interaction with PKR suggesting that these interactions are biologically relevant (Taylor et al., 1999; Gale et al., 1998). Moreover, recent studies suggest that BVDV E2 can also inhibit human PKR activity even though there is very little

sequence conservation between the two viruses (P. Romano and R. Jordan, unpublished observation). Therefore, glucosidase inhibitors may potentiate the activity of IFN by causing accumulation of misfolded forms of E2 that can no longer interact with PKR.

### 4.2. Therapeutic implications for treatment of HCV infection

BVDV is often used as a model for HCV infection, since HCV does not replicate efficiently in cell culture and animal models for the study of HCV infection are not readily available (Zitzmann et al., 1999; Grakoui et al., 2001). Both viruses are flaviviruses and have similar genomic structures and likely share similar replication strategies (Bartenschlager and Lohmann, 2000; Donis, 1995). Antiviral agents that target host enzymes important for BVDV replication would likely inhibit replication of HCV. Thus, glucosidase inhibitors may serve as novel antiviral agents against HCV infection.

The apparent synergistic interaction of glucosidase inhibitors and IFN suggests that these compounds used in combination could be beneficial for the treatment of HCV infection. Both IFN and *n*B-DNJ have been used as monotherapy for treatment of viral infections in humans. However, high dose IFN therapy is associated with unwanted side effects such as fatigue, malaise, and myalgias (Liang et al., 2000) while *n*B-DNJ suffers from poor efficacy due to high IC50 values (Fischl et al., 1994). In a recent clinical trial using *n*B-DNJ for the treatment of HIV infection, low steady-state serum concentrations (18 μm) of *n*B-DNJ in the highest dose groups failed to illicit a significant antiviral response (Fischl et al., 1994). Combination therapy would, therefore, allow the use of lower doses of IFN and *n*B-DNJ to achieve the same level of antiviral efficacy. Our data suggests that in the presence of IFN, 18 μm of *n*B-DNJ would still produce an antiviral effect (Fig. 1). Moreover, as new generations of glucosidase inhibitors with increased potency become available, the concentration of compound required for treatment of infection can be reduced. While it is difficult to extrapolate the results of studies based in cell culture to clinical outcomes in humans, these data suggest that combinations of IFN and *n*B-DNJ might improve the efficacy and reduce the side effects associated with each compound.

The hypothesis that glucosidase inhibitors increase the potency of IFN by blocking the activity of viral proteins that counteract the IFN response suggest that combination therapy with IFN and glucosidase inhibitors would be useful in the treatment of IFN non-responder HCV patients. IFN non-responder patients often harbor strains of virus that contain gene products such as E2 or NS5a, which block the IFN response (Song et al., 1999; Gale et al., 1997, 1998; Taylor et al., 1999). Strains of HCV that require E2–PKR interaction for resistance to IFN for example, would be more susceptible to IFN in the presence of glucosidase inhibitors. Thus, combination therapy using glucosidase inhibitors and IFN may be useful in treating patients infected with IFN resistant strains of virus. This hypothesis is currently under investigation.

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