Available online at www.sciencedirect.com
ScienceDirect
Antiviral Research 73 (2007) 118–125
Antiviral Research
www.elsevier.com/locate/antiviral

Antiviral activity of CHO-SS cell-derived human omega interferon and other human interferons against HCV RNA replicons and related viruses

Victor E. Buckwold [a,*], Jiayi Wei [b], Zhuhui Huang [b], Chunsheng Huang [b], Aysegul Nalca [b,1], Jay Wells [b,2], Julie Russell [b], Barbara Collins [b], Roger Ptak [b], William Lang [c], Curtis Scribner [c], Dennis Blanchett [c], Tom Alessi [c], Peter Langecker [c,3]

[a] *Veracity Biotechnology, LLC, 401 Rosemont Avenue, Third Floor Rosenstock Hall, Frederick, MD 21701, USA*
[b] *Southern Research Institute, 431 Aviation Way, Frederick, MD 21701, USA*
[c] *Intarcia Therapeutics, Inc., 2000 Powell Street, Suite 1640, Emeryville, CA 94608, USA*

Received 7 February 2006; accepted 11 August 2006

Abstract

The fully glycosylated human omega interferon produced from CHO-SS cells (glycosylated IFN-ω) has been shown to be well-tolerated in man and to induce a sustained virologic response in patients infected with hepatitis C virus (HCV). We examined the antiviral activity of glycosylated IFN-ω and various human IFNs (IFN-α, -β, -γ and non-glycosylated bacterial (*Escherichia coli*) recombinant IFN-ω (non-glycosylated IFN-ω)) against HCV RNA replicons and several viruses related to HCV. Since none of the IFNs displayed cytotoxicity we compared their activities based on the effective concentration of the IFN that inhibited virus growth by 50% (EC_{50}). Glycosylated IFN-ω was found to be the most potent antiviral agent of all the IFNs tested against bovine viral diarrhea virus (BVDV), yellow fever virus and West Nile virus. With HCV RNA replicons, non-glycosylated IFN-ω was comparable in activity to IFN-α while glycosylated IFN-ω was markedly more potent, indicating that glycosylation has an important effect on its activity. Drug combination analysis of glycosylated IFN-ω + ribavirin (RBV) in BVDV showed a synergy of antiviral effects similar to IFN-α + RBV, as well as a unique antagonism of RBV cytotoxic effects by glycosylated IFN-ω. Transcription factor (TF) profiling indicated that IFN-α or glycosylated IFN-ω treatment upregulated the same 17 TFs. IFN-α and glycosylated IFN-ω also upregulated 9 and 40 additional unique TFs, respectively. The differences in the expression of these TFs were modest, but statistically significantly different for eight of the TFs that were upregulated exclusively by glycosylated IFN-ω. The activation of these additional TFs by glycosylated IFN-ω might contribute to its high potency.

Keywords: Omega interferon; Antiviral; HCV RNA replicons; Drug combination analysis; Transcription factor profiling

1. Introduction

Hepatitis C virus (HCV) infection is the most common cause of chronic hepatitis in the United States and is a major risk factor for the development of liver cirrhosis (Liang et al., 2000). Infection with HCV is currently treated using a combination therapy of pegylated human interferon-α (IFN-α) and ribavirin (RBV), which leads to a sustained virologic response (SVR = absence of detectable HCV RNA in patients 6 months following cessation of therapy) in approximately 55% of all patients (Pearlman, 2004). Other antiviral therapies to treat HCV-infected patients are desperately needed.

The interferons (IFNs) were originally described as biological agents that "interfered" with viral replication (Stark et al., 1998). Human omega interferon (IFN-ω), like other IFNs, is secreted from cells in response to viral infection and it has antiviral, anti-proliferative and immunomodulatory activities (Adolf, 1995). This type I IFN has 62% amino acid identity with alpha interferon (IFNα-2) and 33% amino acid identity with beta interferon (IFN-β), respectively, but it is unrelated to the type II gamma interferon (IFN-γ) (Adolf, 1995). As a distinct IFN,

* Corresponding author. Tel.: +1 301 644 3920; fax: +1 301 644 3939.
E-mail address: vbuckwold@veracitybiotech.com (V.E. Buckwold).
[1] Current address: USAMRIID, Center for Aerobiological Sciences, 1425 Porter Street, Ft. Detrick, MD 21701, USA.
[2] Current address: USAMRIID, Virology Division, 1425 Porter Street, Ft. Detrick, MD 21701, USA.
[3] Current address: DURECT Corporation, 2 Results Way, Cupertino, CA 95014, USA.

0166-3542/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2006.08.005

IFN-ω, used by itself or in combination with ribavirin or other antiviral therapies, might therefore be beneficial for the treatment of patients who fail to respond to IFN-α or as an additional first-line treatment option.

We have found that the fully glycosylated recombinant human IFN-ω produced from Chinese hamster ovary cells adapted to serum-free growth in suspension culture (CHO-SS) (glycosylated IFN-ω) was well-tolerated in man and that it induced a SVR in patients infected with hepatitis C virus (HCV) genotypes 1–3 (Plauth et al., 2002; Gorbakov et al., 2005). Here we describe the in vitro antiviral activity of glycosylated IFN-ω and other human IFNs against several viruses related to HCV as well as against HCV RNA replicons.

2. Materials and methods

2.1. Antiviral agents

The CHO-SS cell-derived recombinant human IFN-ω is fully-glycosylated and appears to be identical to natural IFN-ω (Buckwold et al., 2006), while the *Escherichia coli*-derived bacterial recombinant human IFN-ω (non-glycosylated IFN-ω; PBL Biomedical Laboratories) is not glycosylated. The other human IFNs utilized in this work were IFN-α (IFNα-2a, Biomedical Laboratories), IFN-β (IFNβ-1a, Biogen, Inc.) and IFN-γ (R&D Systems). RBV was from Sigma.

2.2. Inhibition of viral cytopathic effects assays

The bovine viral diarrhea virus (BVDV) assay in Madin–Darby Bovine kidney cells and the yellow fever virus (YFV) 17D assay in Vero cells were performed as previously described (Buckwold et al., 2003). The West Nile virus (WNV) antiviral evaluations were performed in Vero cells using the WNV isolate NY99-35262-11 from Flamingo (CDC, Ft. Collins, CO) under BSL-3 conditions. Vero cells were grown in Dulbecco's Modified Eagle media (DMEM), 10% fetal bovine serum (FBS), 2% glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 1% penicillin-streptomycin. The WNV assay media was the same except that 2% FBS and DMEM without phenol red was used. All cell culture reagents were from Invitrogen. On the day preceding the assay, the cells were trypsinized (1% trypsin–EDTA), pelleted (1200 rpm × 10 min), counted and plated at 2×10^4 well^{-1} in Costar 96-well flat-bottom plates (Corning). The next day compounds were added to the appropriate wells. A pre-titered aliquot of virus was then added to each well in an amount determined to give 85–95% cell killing. After 6 days incubation at 37 °C in a 5% CO_2 incubator, cell viability was determined using CellTiter96 (Promega) with a Wallac Victor microplate reader (Perkin-Elmer). Data analysis was peformed as previously described (Buckwold et al., 2003).

2.3. HCV RNA replicon assays

The HCV RNA replicon antiviral evaluation assay used a Huh7 cell line that contains an HCV RNA replicon called ET (pFK I389 Lucubineo EI NS3-3′ ET = pHCV-ET) that is based on the con1 HCV-1b strain with three cell culture-adaptive mutations and a stable luciferase (Luc) reporter for which expression is directly proportional to HCV RNA replicon RNA levels (Pietschmann et al., 2002). The HCV RNA replicon-containing cell line was kept subconfluent and grown in DMEM, 10% FBS, 1% glutamine, 1% penicillin–streptomycin, 250 μg/ml G418 in a 5% CO_2 incubator at 37 °C, while the assay media was the same except using 5% FBS, DMEM without phenol red, and in the absence of G418. The cells were trypsinized and (5000 well^{-1}) plated in 200 μl of media into the inner wells of two white 96-well plates (Costar) for the Luc-based assay or into one 96-well flat-bottom plate (Costar) for the RNA-based assay. The next day the media was removed and drugs were added in 100 μl assay media to evaluate five half-log concentrations of drug with four replicates each. Ten wells without drugs per plate served as the virus controls (and/or cell controls), 12 wells were for media alone and two wells at each concentration were used for drug color controls. The plates were then returned to the CO_2 incubator for 72 h. In the Luc-based assay, one set of plates was examined for Luc expression as an indirect indication of HCV RNA replicon levels using SteadyGlo reagent (Promega) with a luminometer plate reader (Wallac 1450 Microbeta, Perkin-Elmer), while CytoTox-1 reagent (Promega) was added to the second plate and 560 nM excitation 590 nM emission fluorescence was read with a plate reader (Analyst HT, Molecular Devices) as an indication of cell numbers and cytotoxicity. HCV RNA levels were directly assessed by TaqMan RT-PCR (Martell et al., 1999) with RNA produced in vitro using T7 RNA polymerase (T7 Megascript kit, Ambion) from a pCRII-TOPO (Invitrogen) plasmid containing the cloned TaqMan amplicon (pHCVA) used as the standard. Cellular RNA was extracted from cells using QIAGEN viral RNA 96-well kits. Ribosomal RNA (rRNA) levels determined via TaqMan RT-PCR (Ribosomal RNA control reagents, PE Biosystems) were used as an indication of cell numbers in the RNA-based assay. The average Luc or HCV RNA levels relative to that of the untreated controls, and the background and drug color-corrected fluorescence or rRNA levels relative to that of the untreated controls, was then plotted as a function of drug concentration. The effective drug concentration that reduced HCV RNA replicon levels by 50% (EC_{50}) and 90% (EC_{90}), and the toxic concentration of drug that reduced cell numbers by 50% (TC_{50}) and 90% (TC_{90}) were calculated in dedicated spreadsheets by regression analysis with semilog curve fitting. Selectivity indices (SI = TC/EC) at 50% (SI_{50}) and 90% (SI_{90}) were also calculated.

2.4. Drug combination analysis

The drug combination analyses were performed using the aforementioned model systems, as described previously (Buckwold et al., 2003).

2.5. Transcription factor analysis

HCV RNA replicon-containing ET cells at 75% confluence in T25 flasks were rinsed with PBS and treated with 1,000 IU/ml human IFNα or glycosylated IFN-ω. An untreated flask of cells

served as the control. After 24 h incubation, nuclear extracts were isolated (Nuclear Extraction Kit, Panomics; Jiang et al., 2003) and TranSignal Protein/DNA Arrays I–III (Panomics) were utilized to quantify the activation of 243 TFs, as described previously (Govindarajan et al., 2003; Jiang et al., 2004). The expression levels of each TF was analyzed by comparing the density of each IFN-treated dot on the membrane relative to the untreated control dot using Quantity One v.4.5.2 software (Bio-Rad). The entire experiment was performed three times and the average ±S.D. expression level of each TF whose levels was increased or decreased greater than two-fold relative to the untreated control was determined. We used a Student's *t*-test with $\alpha = 0.05$ to identify those TFs whose expression was statistically significantly different between IFN-α or glycosylated IFN-ω treated samples relative to the untreated control.

3. Results

3.1. Effect of ribavirin and human interferons on viruses related to HCV

Table 1 summarizes the results of the evaluation of a variety of human IFNs and the positive control compound RBV against the cytopathic viruses bovine viral diarrhea virus (BVDV), yellow fever virus (YFV) and West Nile virus (WNV). None of the IFNs displayed cytotoxic effects and inhibitory concentrations of drug that caused the reduction in viable cell numbers by 50% (IC_{50} values) were not reached in any assay. In addition, glycosylated IFN-ω did not display any cytoxicity following seven days at 1000 IU/ml in human peripheral blood mononuclear cells (data not shown). As such, comparisons of drug potency were made based on the antiviral activity using the effective concentrations that reduced viral CPE formation by 50% (EC_{50} values) rather than using selectivity indices ($SI_{50} = IC_{50}/EC_{50}$).

The behavior of IFN-α and RBV against BVDV and YFV was comparable to that observed previously (Buckwold et al., 2003), as were their effects on WNV (Jordan et al., 2000; Anderson and Rahal, 2002; Morrey et al., 2002). IFN-β and IFN-γ were not effective against BVDV and effective concentrations that reduced viral CPE formation by 50% (EC_{50} values) were not reached. In terms of potency (based on EC_{50}), glycosylated IFN-ω was more active than IFN-α against BVDV. All the human IFNs tested were active against YFV with glycosylated IFN-ω and IFN-γ being the most effective, and IFN-α similar to that observed previously (Buckwold et al., 2003), and less effective. Glycosylated IFN-ω was slightly more potent than IFN-γ and more potent than IFN-α against YFV. With WNV, IFN-ω was also more potent than IFN-α.

Table 1
Antiviral activity of human IFNs and RBV against BVDV, YFV and WNV

Virus	Treatment (*n*)	Mean ± S.D.		
		EC_{50}	IC_{50}	SI_{50}[a]
BVDV	IFN-α (9)	29 ± 33 IU/ml	>5000 IU/ml[b]	>170
	IFN-β (2)	NR[c]	>5000 IU/ml[b]	<1
	IFN-γ (2)	NR[c]	>5000 IU/ml[b]	<1
	IFN-ω (9)	1.9 ± 1.2 IU/ml	>5000 IU/ml[b]	>2600
	RBV (9)	10 ± 6.3 μM	32 ± 15 μM	3.2
YFV	IFN-α (3)	150 ± 46 IU/ml	>5000 IU/ml[b]	>33
	IFN-β (1)	140 IU/ml	>5000 IU/ml[b]	>36
	IFN-γ (2)	17 ± 1.6 IU/ml	>5000 IU/ml[b]	>290
	IFN-ω (3)	8.4 ± 5.9 IU/ml	>5000 IU/ml[b]	>600
	RBV (5)	110 ± 56 μM	>410 μM[b]	>3.7
WNV	IFN-α (3)	14 ± 7.9 IU/ml	>1000 IU/ml[b]	>71
	IFN-ω (4)	2.0 ± 2.9 IU/ml	>1000 IU/ml[b]	>500
	RBV (5)	240 ± 71 μM	>410 μMb	>1.7

[a] $SI_{50} = IC_{50}/EC_{50}$.
[b] IC_{50} was not reached.
[c] EC_{50} was not reached.

3.2. Effect of human interferons on HCV RNA replicons

Table 2 shows the results of the comparison of the antiviral activities of IFN-α, glycosylated IFN-ω and non-glycosylated IFN-ω using HCV RNA replicons. The assay was performed in Huh7 cells containing the con1 HCV-1b strain HCV RNA replicon ET (Pietschmann et al., 2002) which has a luciferase (Luc) reporter whose expression is directly proportional to HCV RNA replicon RNA levels. RBV does not show antiviral activity in this assay. The antiviral activities observed were similar using the indirect Luc endpoint or the direct RNA endpoint. None of the IFNs showed any cytotoxic effects and IC_{50} was not reached in any experiment. Based on the EC50 values, glycoslyated IFN-ω was markedly more potent. The antiviral

Table 2
Antiviral activity of human IFNs against HCV RNA replicons

Endpoint	Treatment (*n*)	Mean ± S.D.				
		EC_{50} (IU/ml)	EC_{90} (IU/ml)	IC_{50} and IC_{90} (IU/ml)[a]	SI_{50}[b]	SI_{90}[c]
Luc	IFN-α (14)	0.28 ± 0.088	1.1 ± 0.33	>100[a]	>360	>91
	Glycosylated IFN-ω (10)	0.017 ± 0.019	0.030 ± 0.017	>100[a]	>5900	>3300
	Non-glycosylated IFN-ω (4)	0.13 ± 0.060	0.54 ± 0.23	>100[a]	>770	>190
RNA	IFN-α (8)	0.32 ± 0.092	1.2 ± 0.38	>100[a]	>310	>83
	Glycosylated IFN-ω (5)	0.0047 ± 0.0022	0.017 ± 0.010	>100[a]	>21000	>5900
	Non-glycolsylated IFN-ω (3)	0.20 ± 0.021	0.90 ± 0.087	>100[a]	>500	>110

[a] IC_{50} and IC_{90} were not reached.
[b] $SI_{50} = IC_{50}/EC_{50}$.
[c] $SI_{90} = IC_{90}/EC_{90}$.

activity of non-glycosylated IFN-ω was comparable to IFN-α, as previously described (Cheney et al., 2002; Okuse et al., 2005). This difference in antiviral activity between glycosylated IFN-ω and non-glycosylated IFN-ω indicates that glycosylation has an important effect on its activity.

3.3. Drug combination analysis

A drug combination analysis was then performed using glycosylated IFN-ω + RBV with BVDV. Fig. 1 shows a representative result of this experiment. We observed a synergy of antiviral activity (Fig. 1a and b) between the two drugs (synergy volume = 61 ± 20 IU μg/ml^2 %, $n = 3$) at physiologically relevant drug concentrations. We also saw a strong antagonism of antiviral effects (antagonism volume = −1100 ± 430 IU μg/ml^2 %, $n = 3$) between the drugs at high RBV concentrations not observed in vivo (Glue et al., 2000; Larrat et al., 2003; Scott and Perry, 2002; Tsubota et al., 2002), at which RBV exhibits cytotoxicity. In the analysis of the effects of combining glycosylated IFN-ω + RBV on cytotoxicity (Fig. 1c and d), we found a moderate antagonism of the cytotoxic effects of RBV by glycosylated IFN-ω (antagonism volume = −71 ± 38 IU μg/ml^2 %, $n = 3$) at physiologically relevant drug concentrations. These same effects were also apparent in preliminary experiments when YFV was employed for the drug combination analysis (data not shown).

3.4. Transcription factor analysis in HCV RNA replicons

We next examined the ability of IFN-α and glycosylated IFN-ω to activate cellular transcription factors (TFs) from HCV RNA replicon-containing cells following treatment with 1000 IU/ml human IFN-α or glycosylated IFN-ω. An untreated flask of cells served as the control. After 24 h incubation, nuclear extracts were isolated and TranSignal Protein/DNA Arrays I, II and III (Panomics) were utilized to quantify the activation of 243 TFs, as described previously (Govindarajan et al., 2003; Jiang et al., 2004). The expression levels of each TF was analyzed by comparing the density of each IFN-treated dot on the membrane relative to the untreated control dot. The entire experiment was performed three times and the average ±S.D. expression level of each TF whose levels was increased or decreased greater than two-fold relative to the untreated control was determined (Table 3). No TF showed a significant downregulation of their levels in response to these treatments. The expression of 17 TFs were increased by treatment with either IFN-α or glycosylated IFN-ω. Glycosylated IFN-ω induced the expression of an additional 40 TFs that were not induced by IFN-α; while IFN-α induced the expression of 9 TFs that were not induced by glycosylated IFN-ω.

We used a Student's *t*-test with $\alpha = 0.05$ to identify those TFs whose expression was statistically significantly different between IFN-α or glycosylated IFN-ω treated samples. Eight TFs which were modestly-induced by glycosylated IFN-ω and not IFN-α were identified: the steroid regulatory element-binding ADD1 (Guan et al., 1995), the gamma IFN promoter repressor AP2/YY1 (Ye et al., 1996), the gamma globulin promoter repressor conserved sequence-binding protein 1 (CSBP; Gumucio et al., 1992), the beta globulin gene promoter erythroid Kruppel-like factor (EKLF; Crossley et al., 1994), the homeotic gene fork head of Drosophila 8 (HFH-8)/hepatocyte nuclear factor 3 (HNF-3)/mouse fork head lung protein (LUN; Miura et al., 1998), HNF-1a (Frain et al., 1989), the interferon consensus sequence binding protein (ICSBP, Driggers et al., 1990) and the lymphocyte-enriched DNA binding protein LyF (Lo et al., 1991).

4. Discussion

In this report we describe the in vitro antiviral activity of the glycosylated IFN-ω and various human IFNs (IFN-α, -β, -γ and non-glycosylated (*E. coli*-derived) IFN-ω (non-glycosylated IFN-ω)) against HCV RNA replicons and several viruses related to HCV. IFNs are not cytotoxic compounds and IC_{50} values were not reached for any IFN in any model system. As such, drug potency comparisons were based on drug EC_{50} values. Glycosylated IFN-ω was more potent than IFN-α against BVDV, YFV and WNV (Table 1). With HCV RNA replicons, non-glycosylated IFN-ω was comparable in activity to IFN-α, while glycosylated IFN-ω was markedly more potent, indicating that glycosylation has an important effect on its activity (Table 2). In each model system that was examined, glycosylated IFN-ω was found to be the most potent antiviral agent of all the IFNs tested.

RBV monotherapy to treat HCV infection is not at all effective while IFN-α only treatment is moderately effective, but the combined use of IFN-α + RBV in patients is much more effective clinically and is currently the standard of care. The reason for this clinical synergy of antiviral activities is not completely understood and both the indirect immune-mediated and direct antiviral effects of RBV may contribute to this success (reviewed in Buckwold, 2004). Glycosylated IFN-ω + RBV also showed a clear synergy of antiviral activity in HCV-infected patients (Gorbakov et al., 2005). We conducted a drug combination analysis of glycosylated IFN-ω + ribavirin (RBV) using BVDV (Fig. 1). A synergy of the antiviral effects of the two drugs was observed which was essentially identical to what we found previously when IFN-α + RBV were examined in this model system (Buckwold et al., 2003). Interestingly, an antagonism of the cytotoxic effects of RBV by glycosylated IFN-ω was observed. This feature was not observed when IFN-α + RBV were tested (Buckwold et al., 2003). Thus the combination of glycosylated IFN-ω + RBV seems favorable both for the synergy of antiviral activities and for the antagonism of RBV drug cytotoxic effects.

Transcription factor (TF) profiling indicated that IFN-α or glycosylated IFN-ω treatment upregulated the same 17 TFs. IFN-α and glycosylated IFN-ω also upregulated 9 and 40 additional unique TFs, respectively. The differences in the expression of these TFs was modest, but statistically significantly different for eight of the TFs that were upregulated exclusively by glycosylated IFN-ω. These IFN-ω-induced TFs were the steroid regulatory element-binding ADD1 (Guan et al., 1995), the gamma IFN promoter repressor AP2/YY1 (Ye et al., 1996), the gamma globulin promoter repressor conserved sequence-binding protein 1 (CSBP; Gumucio et al., 1992), the beta

Table 3
Comparison of transcription factor activation in HCV RNA replicon-containing Huh7 ET Cells by glycosylated IFN-ω and IFN-α

Activation by	Transcription factor	Description	Glycosylated IFN-ω/control[a]	IFN-α/control[a]
Both glycosylated IFN-ω and IFN-α	HFH-3	Forkhead box I1	3.59 ± 3.96	3.13 ± 3.39
	RREB (sequence 2)[b]	Ras-responsive transcription element	3.47 ± 3.36	3.94 ± 4.61
	HBS/xbp1	HIF binding sequence (rat, as human xbp-1)	3.21 ± 3.23	3.61 ± 4.30
	PARP	Poly(ADP-ribose) synthetase/polymerase	3.16 ± 3.52	2.52 ± 5.19
	MSP1	Amyloid precursor protein (APP) regulatory element	2.85 ± 2.59	2.08 ± 3.74
	VDR (DR-3)	VDR: Vitamin D (1,25-dihydroxyvitamin D3) receptor	2.79 ± 1.68	2.43 ± 1.06
	MEF-2 (sequence 1)[b]	Myelin gene expression factor	2.66 ± 2.13	2.15 ± 4.16
	Stat4	Signal transducer and activator of transcription 4	2.60 ± 1.36	2.06 ± 1.32
	PPUR (sequence 1)[b]	Purine-rich sequences binding sequence	2.54 ± 1.15	2.15 ± 1.20
	MT-Box	Tentative new binding domain	2.30 ± 2.19	2.52 ± 1.92
	HIF-1	Hypoxia-inducible factor 1	2.29 ± 2.42	2.61 ± 2.48
	RXR (DR1)	Retinoic acid X receptor	2.29 ± 1.18	2.45 ± 1.21
	TR (DR4)	Thyroid hormone receptor	2.15 ± 1.20	2.13 ± 1.17
	MyoD	Myogenic factor D	2.15 ± 1.20	2.01 ± 1.62
	NF-κB	Nuclear factor of kappa light enhancer in B-cells	2.11 ± 1.42	2.25 ± 1.81
	Pax4	Paired box gene 4	2.08 ± 1.44	2.39 ± 2.26
	USF-1	Upstream transcription factor	2.01 ± 1.29	2.26 ± 1.80
Glycosylated IFN-ω alone	c-Rel	NF-κB p75 kDa protein	3.85 ± 4.56	1.19 ± 0.18
	E4F, ATF	E4F transcription factor 1	2.96 ± 3.11	1.38 ± 0.23
	GATA-4	GATA binding protein 4	2.88 ± 2.42	1.64 ± 1.13
	E4BP4	Nuclear factor, interleukin 3 regulated	2.81 ± 1.78	1.56 ± 0.61
	CP1, CTF, CBTF	CCAAT-box-binding transcription factor	2.73 ± 1.09	1.62 ± 0.87
	HOXD 9,10	Homeo box D9, D10	2.73 ± 0.90	1.56 ± 0.83
	LyF	LyF binding site	2.65 ± 0.42	1.55 ± 0.60
	LyF-1 (sequence 1)[b]	LyF-1 binding site	2.64 ± 0.77	1.73 ± 0.67
	ATF/CRE	ATF/CRE binding site	2.64 ± 0.97	1.27 ± 0.67
	HNF-1A	Hepatocyte nuclear factor	2.54 ± 0.59	1.18 ± 0.24
	E47	E2A enhancer binding factors E12/E47	2.52 ± 1.61	1.22 ± 0.60
	HFH-8, HNF-3, LUN	A new mouse forkhead gene named LUN	2.52 ± 0.56	1.03 ± 0.15
	ZIC	A DNA binding domain on the EBV BZLF1 promoter	2.46 ± 2.19	1.52 ± 1.29
	Pax3	Paired box gene 3	2.37 ± 2.08	1.80 ± 3.01
	RSRFC4	MADS box transcription enhancer factor 2	2.37 ± 0.49	1.54 ± 1.12
	CSBP	Conserved sequence-binding protein	2.34 ± 0.79	1.13 ± 0.40
	ATF2	ATF2	2.30 ± 1.88	1.86 ± 1.49
	MTB-Zf	*Cis* regulatory element (MTE) binding protein	2.26 ± 0.90	1.70 ± 0.73
	CREB	cAMP responsive element binding protein 1	2.26 ± 0.84	1.48 ± 0.65
	NF-Y	Nuclear Y box factor	2.23 ± 1.07	1.92 ± 1.14
	PEBP2	Polyoma enhancer binding protein	2.20 ± 0.80	1.98 ± 0.86
	AP-2	Activating enhancer binding protein	2.19 ± 0.97	1.87 ± 0.08
	MyTI	Myelin transcription factor I	2.19 ± 0.78	1.28 ± 0.28
	ADR1	ADH regulatory gene-1 binding element	2.19 ± 1.79	1.11 ± 0.22
	MAZ	MYC-associated zinc finger protein	2.18 ± 0.98	1.46 ± 1.30
	CETP/CRE	Cholesterol esterase transfer protein/response element	2.16 ± 1.67	1.13 ± 0.08
	EVI-1	Ecotropic viral integration site 1 (zinc finger oncogene)	2.13 ± 1.93	1.31 ± 0.74
	Antioxidant RE	Antioxidant responsive element	2.13 ± 1.24	1.20 ± 0.52
	AhR/Arnt	Aryl hydrocarbon receptor/nuclear translocator binding element	2.13 ± 1.39	1.11 ± 0.39
	LCR-F1	Nuclear factor (erythroid-derived 2)-like 1	2.11 ± 1.35	1.77 ± 1.16
	AIC, CBF	ApoA-I gene promoter c region, CCAAT-binding factor	2.09 ± 0.96	1.02 ± 0.13
	EKLF (sequence 1)[b]	Erythroid Kruppel-like factor gene	2.08 ± 0.36	1.14 ± 0.12
	ADD1	Sterol regulatory element binding transcription factor	2.07 ± 0.68	1.08 ± 0.37
	LR1	A 106-kDa sequence-specific DNA-binding protein	2.06 ± 0.81	1.37 ± 0.54
	Smad3/4	MADH3/4, mothers against decapentaplegic homolog 3/4	2.05 ± 0.86	1.70 ± 0.81
	NF-E1 (YY1)	YY1 transcription factor	2.04 ± 0.88	1.83 ± 0.75
	CACC	CACC binding protein	2.04 ± 1.33	1.31 ± 0.69
	Brn-3	POU4F1: POU domain, class 4, transcription factor 1	2.02 ± 0.78	1.71 ± 0.58
	ICSBP	Interferon consensus sequence binding protein	2.02 ± 0.69	1.06 ± 0.22
	AP2, YY1	Activating enhancer binding protein 2-like YY1 site	2.02 ± 0.44	0.85 ± 0.16
IFN-α alone	HBS + HAS	Hypoxia-inducible factor 1 binding sequence/ancillary sequence	1.90 ± 1.06	2.51 ± 2.10
	Pax6	Paired box gene 6	1.88 ± 0.29	2.21 ± 0.79

Table 3 (*Continued*)

Activation by	Transcription factor	Description	Glycosylated IFN-ω/control[a]	IFN-α/control[a]
	MUSF1	Amyloid precursor protein regulatory element without USF site	1.84 ± 0.53	2.32 ± 0.87
	MZF1	Zinc finger protein 42 (myeloid-specific retinoic acid-responsive)	1.70 ± 0.42	2.61 ± 1.86
	ISRE (sequence 2)[b]	Interferon-α stimulated response element	1.69 ± 1.24	2.32 ± 1.89
	NZF-3	Neural zinc finger factor 3	1.51 ± 0.30	2.29 ± 1.69
	XRE	Xenobiotic response element	1.44 ± 0.49	2.01 ± 1.65
	RREB (sequence 1)[b]	Ras-responsive transcription element	1.38 ± 0.82	2.50 ± 0.37
	XBP-1	X-box binding protein 1	1.24 ± 0.45	2.05 ± 1.55

[a] Mean ± S.D.
[b] Sequence 1 or 2 as designated by Panomics.

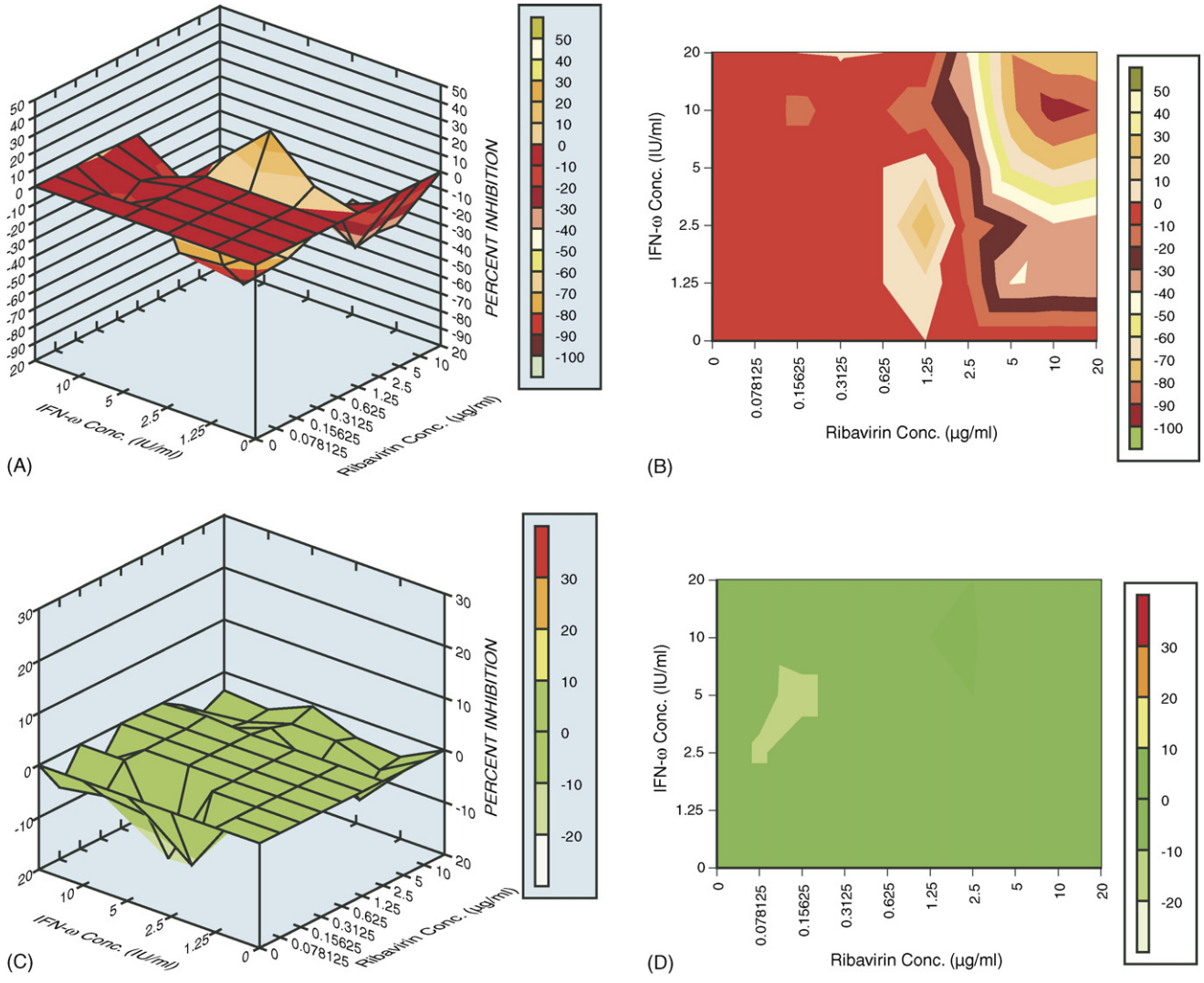

Fig. 1. Drug combination analysis of glycosylated IFN-ω + RBV using BVDV in MDBK cells. The calculated additive interactions between the drugs were subtracted from the experimentally determined values based on mean background- and drug color-corrected data to reveal the regions and corresponding drug concentrations at which synergistic or antagonistic interactions affecting antiviral activity and drug cytotoxicity occurred. Peaks of statistically significant synergy or antagonism that deviate significantly from the expected additive drug interactions derived from 95% confidence interval data are shown in the difference plots of the interactions between IFN-ω and RBV (A and C) and in the corresponding contour plots (B and D). The colors indicate the level of synergy or antagonism with the corresponding peak volumes found at each drug concentration indicated in the sidebar. The effects of the combination of IFN-ω and RBV on antiviral activity is shown in panels A and B, while the effects of the drug combination on cytotoxicity in MDBK cells are shown in panels C and D. The experiment was repeated three times with essentially identical results observed.

globulin gene promoter erythroid Kruppel-like factor (EKLF; Crossley et al., 1994), the homeotic gene fork head of Drosophila 8 (HFH-8)/hepatocyte nuclear factor 3 (HNF-3)/mouse fork head lung protein (LUN; Miura et al., 1998), HNF-1a (Frain et al., 1989), the interferon consensus sequence binding protein (ICSBP, Driggers et al., 1990) and the lymphocyte-enriched DNA binding protein LyF (Lo et al., 1991). The molecular basis of this differential regulation of host cell TFs in response to IFN-α and glycosylated IFN-ω treatment is uncertain both due to the modest nature of the activation observed and since it is unclear from these experiments whether the observed effects are transcriptional or post-transcriptional in nature. However, both IFN-α and glycosylated IFN-ω are type I IFNs that affect cells by engaging what appears to be the same cellular receptors. It is possible that structural differences may cause them to interact with their receptors in different ways since such differences are thought to explain in part, some of the significant differences observed in the clinical and biological activities of the other type I interferons (Karpusas et al., 1997; Walter, 1997). Since glycosylated IFN-ω induced the activation of several other TFs that were not induced by treatment with IFN-α, this may be in part responsible for its high potency.

Acknowledgements

This work was supported in part by Intarcia Therapeutics, Inc. and with Federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services under contract N01-AI-30047.

References

Adolf, G.R., 1995. Human omega interferon—a review. Multiple Sclerosis 1, S44–S47.

Anderson, J.F., Rahal, J.J., 2002. Efficacy of interferon alpha-2b and ribavirin against West Nile virus in vitro. Emerg. Infect. Dis. 8, 107–108.

Buckwold, V.E., 2004. Implications of finding synergistic in vitro drug-drug interactions between interferon-α and ribavirin for the treatment of hepatitis C virus. J. Antimicrob. Chemother. 53, 413–414.

Buckwold, V.E., Wei, J., Wenzel-Mathers, M., Russell, J., 2003. Synergistic in vitro interactions between alpha interferon and ribavirin against bovine viral diarrhea virus and yellow fever virus as surrogate models of hepatitis C virus replication. Antimicrob. Agents Chemother. 47, 2293–2298.

Buckwold, V.E., Lang, W., Scribner, C., Blanchett, D., Alessi, T., Langecker, P., 2006. Safety pharmacology, toxicology and pharmacokinetic assessment of recombinant human omega interferon produced from CHO-SS cells. Basic Clin. Pharmacol. Toxicol. 99, 62–70.

Cheney, I.W., Lai, V.C.H., Zhong, W., Brodhag, T., Dempsey, S., Lim, C., Hong, Z., Lau, J.Y.N., Tam, R.C., 2002. Comparative analysis of anti-hepatitis C virus activity and gene expression mediated by alpha, beta, and gamma interferons. J. Virol. 76, 11148–11154.

Crossley, M., Tsang, A.P., Bieker, J.J., Orkin, S.H., 1994. Regulation of the erythroid Kruppel-like factor (EKLF) gene promoter by the erythroid transcription factor GATA-1. J. Biol. Chem. 269, 15440–15444.

Driggers, P.H., Ennist, D.L., Gleason, S.L., Mak, W.H., Marks, M.S., Levi, B.Z., Flanagan, J.R., Appella, E., Ozato, K., 1990. An interferon gamma-regulated protein that binds the interferon-inducible enhancer element of major histocompatibility complex class I genes. Proc. Natl. Acad. Sci. USA 87, 3743–3747.

Frain, M., Swart, G., Monaci, P., Nicosia, A., Stampfli, S., Frank, R., Cortese, R., 1989. The liver-specific transcription factor LF-B1 contains a highly diverged homeobox DNA binding domain. Cell 59, 145–157.

Glue, P., Rouzier-Panis, R., Raffanel, C., Sabo, R., Gupta, S.K., Salfi, M., Jacobs, S., Clement, R.P., the Hepatitis C Intervention Therapy Group, 2000. A dose-ranging study of pegylated interferon alfa-2b and ribavirin in chronic hepatitis C. Hepatology 32, 647–653.

Gorbakov, V., Kim, H., Oronsky, B., Lang, W., 2005. HCV RNA results from a phase II, randomized, open-label study of omega interferon (IFN) with or without ribavirin in IFN-naïve genotype 1 chronic HCV patients. AASLD Meeting, 67651 (Abstract).

Govindarajan, B., Bai, X., Cynthia Cohen, C., Zhong, H., Kilroy, S., Louis, G., Moses, M., Arbiser, J.K., 2003. Malignant transformation of melanocytes to melanoma by constitutive activation of MAP kinase kinase signaling. J. Biol. Chem. 278, 9790–9795.

Guan, G., Jiang, G., Koch, R.L., Shechter, I., 1995. Molecular cloning and functional analysis of the promoter of the human squalene synthase gene. J. Biol. Chem. 270, 21958–21965.

Gumucio, D.L., Heilstedt-Williamson, H., Gray, T.A., Tarle, S.A., Shelton, D.A., Tagle, D.A., Slightom, J.L., Goodman, M., Collins, F.S., 1992. Phylogenetic footprinting reveals a nuclear protein which binds to silencer sequences in the human gamma and epsilon globin genes. Mol. Cell. Biol. 12, 4919–4929.

Jiang, X., Norman, M., Li, X., 2003. Use of an array technology for profiling and comparing transcription factors activated by TNFalpha and PMA in HeLa cells. Biochim. Biophys. Acta 1642, 1–8.

Jiang, X., Norman, M., Roth, L., Li, X., 2004. Protein-DNA array-based identification of transcription factor activities regulated by interaction with the glucocorticoid receptor. J. Biol. Chem. 279, 38480–38485.

Jordan, I., Briese, T., Fischer, N., Yiu-Lau, J., Lipkin, W.I., 2000. Ribavirin inhibits West Nile virus replication and cytopathic effect in neural cells. J. Infect. Dis. 182, 1214–1217.

Karpusas, M., Nolte, M., Benton, C.B., Meier, W., Lipscomb, W.N., Goelz, S., 1997. The crystal structure of human interferon beta at 2.2-A resolution. Proc. Natl. Acad. Sci. USA 94, 11813–11818.

Larrat, S., Stanke-Labesque, F., Plages, A., Zarski, J.-P., Bessard, G., Souvignet, C., 2003. Ribavirin quantification in combination treatment of chronic hepatitis C. Antimicrob. Agents Chemother. 47, 124–129.

Liang, T.J., Rehermann, B., Seeff, L.B., Hoofnagle, J.H., 2000. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann. Int. Med. 132, 296–305.

Lo, K., Landau, N.R., Smale, S.T., 1991. LyF-1, a transcriptional regulator that interacts with a novel class of promoters for lymphocyte-specific genes. Mol. Cell. Biol. 11, 5229–5243.

Martell, M., Gómez, J., Esteban, J.I., Sauleda, S., Quer, J., Cabot, B., Esteban, R., Guardia, J., 1999. High-throughput real-time reverse transcription-PCR quantitation of heaptitis C virus RNA. J. Clin. Microbiol. 37, 327–332.

Morrey, J.D., Smee, D.F., Sidwell, R.W., Tseng, C., 2002. Identification of active antiviral compounds against a New York isolate of West Nile virus. Antiviral Res. 55, 107–116.

Miura, N., Kakinuma, H., Sato, M., Aiba, N., Terada, K., Sugiyama, T., 1998. Mouse forkhead (winged helix) gene LUN encodes a transactivator that acts in the lung. Genomics 50, 346–356.

Okuse, C., Rinaudo, J.A., Farrar, K., Wells, F., Korba, B.E., 2005. Enhancement of antiviral activity against hepatitis C virus in vitro by interferon combination therapy. Antiviral Res. 65, 23–34.

Pearlman, B.L., 2004. Hepatitis C treatment update. Am. J. Med. 117, 344–352.

Pietschmann, T., Lohmann, V., Kaul, A., Krieger, N., Rinck, G., Rutter, G., Strand, D., Bartenschlager, R., 2002. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J. Virol. 76, 4008–4021.

Plauth, M., Jetschmann, J.-U., Meisel, H., Langecker, P.J., Moran, M., Lang, W., Blanchett, D., Brack, J., Von Wussow, P., 2002. Open-label study of omega interferon in previously untreated HCV-infected patients. In: Proceedings of the 37th Annual Meeting EASL (Abstract 208).

Scott, L.J., Perry, C.M., 2002. Interferon-α-2b plus ribavirin. A review of its use in the management of chronic hepatitis C. Drugs 62, 507–556.

Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., Schreiber, R.D., 1998. How cells respond to interferons. Ann. Rev. Biochem. 67, 227–264.

Tsubota, A., Akuta, N., Suzuki, F., Suzuki, Y., Someya, T., Kobayashi, M., Arase, Y., Saitoh, S., Ikeda, K., Kumada, H., 2002. Viral dynamics and pharmacokinetics in combined interferon alfa-2b and ribavirin therapy for

patients infected with hepatitis C virus of genotype 1b and high pretreatment viral load. Intervirology 45, 33–42.

Walter, M.R., 1997. Three-dimensional models of interferon-alpha subtypes IFN-con1, IFN-alpha8, and IFN-alpha1 derived from the crystal structure of IFN-alpha2b. Semin. Oncol. 24, 52–62.

Ye, J., Cippitelli, M., Dorman, L., Ortaldo, J.R., Young, H.A., 1996. The nuclear factor YY1 suppresses the human gamma interferon promoter through two mechanisms: inhibition of AP1 binding and activation of a silencer element. Mol. Cell. Biol. 16, 4744–4753.