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Enhancement of antiviral activity against hepatitis C virus in vitro by interferon combination therapy

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Abstract

Alpha, beta, and gamma interferons (IFN-α, IFN-β, IFN-γ) have been shown to be effective inhibitors of HCV replication in human cell lines carrying HCV replicons. To help define the divergent cellular processes involved in the control of intracellular HCV replication by these agents, we have characterized the activity of monotherapies and combination therapies with the major types of human interferons against HCV replication in the HCV replicon-containing cell line, AVA5. IFN-α, IFN-β, and omega interferon (IFN-ω) were equally effective at inhibiting HCV replication, while IFN-γ was approximately 10-fold more potent. In kinetic experiments, IFN-β and IFN-γ inhibited HCV replication more rapidly, and for a more prolonged period following the removal of treatment, than IFN-α. Combination interferon therapies produced enhanced anti-HCV activity in most cases, and displayed a diverse range of interactions. Mixtures of IFN-α and IFN-β exhibited generally additive to slightly antagonistic interactions, IFN-α or IFN-β combined with IFN-ω were strongly antagonistic, while IFN-α/IFN-γ and IFN-β/IFN-γ combinations displayed the most enhanced and strongly synergistic antiviral effects. Simultaneous administration of interferons in the combination treatments was found to be superior to sequential administration. Ribavirin did not exhibit any selective anti-HCV activity in cell culture, consistent with in vivo monotherapies, and did not influence the effectiveness of IFN-α in combination treatments. A panel of human cytokines and immune response modifiers induced by interferon and ribavirin therapies in vivo did not demonstrate anti-HCV activity in HCV replicon-containing cultures. Combination therapy can be effectively modeled using HCV replicon technology yielding potentially more effective treatment regimens. HCV replicon technology has potential utility in designing combination therapies to significantly enhance the anti-HCV activity of IFN-α.

Keywords: Hepatitis C Virus; Combination antiviral therapy; Interferon; Cytokines; HCV replicon

1. Introduction

Hepatitis C virus (HCV) is a major public health problem with an estimated 170 million worldwide infections (Alter and Seeff, 2000; Poynard et al., 2000). Infection with HCV is usually subclinical or associated with mild symptoms. Most infected individuals are unable to eliminate the virus, and persistent infection with HCV causes chronic liver disease, including cirrhosis and hepatocellular carcinoma (Alter and Seeff, 2000; Lauer and Walker, 2001). The current standard of care for chronic HCV infection is treatment with a combination of alpha interferon (IFN-α) and ribavirin (Davis et al., 1998; McHutchinson et al., 1998; Poynard et al., 2000). Although sustained response rates with this treatment regimen have approached 50% in some studies, substantial deleterious side affects are common and not all HCV chronic carriers are candidates for this therapy, underscoring the need for new antiviral approaches.

Combination therapies offer substantial potential for improvements in efficacy by targeting multiple viral functions and/or cellular pathways, as well as for therapeutic approaches against drug-resistant strains. The improved utility

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of combination therapy with ribavirin and IFN-α attest to this approach for chronic HCV infection. Limited clinical studies with combinations of IFN-α with interferons beta (IFN-β) and gamma (IFN-γ) have reported some improved efficacy (Asahina et al., 2001; Horiike et al., 2003; Katayama et al., 2001; Watanabe et al., 2002).

The establishment of stable HCV subgenomic replicon systems (Lohmann et al., 1999; Blight et al., 2000; Gao et al., 2001; Lanford et al., 2003) in the human hepatobalstoma cell line Huh7 has provided a useful system for the development of new antiviral approaches against HCV (Dhanak et al., 2002; Llinas-Brunet et al., 2004). IFN-α, IFN-β, and IFN-γ have been shown to be effective inhibitors of HCV replication in replicon-carrying cell cultures (Lohmann et al., 1999; Blight et al., 2000; Gao et al., 2001; Cheney et al., 2002; Frese et al., 2002; Lanford et al., 2003; Tanabe et al., 2004).

Interferons are currently subdivided into two primary classes, Type I (generally represented by IFN-α, IFN-β, and omega [IFN-ω]) and Type II (represented by IFN-γ), which are distinguished by differences in respective intracellular signaling pathways although a number of other intracellular pathways are activated in common (Der et al., 1998; Stark et al., 1998; Goodbourn et al., 2000). Since different interferons most likely utilize both overlapping and unique pathways to exert observed anti-HCV activities, interferon combinations may prove to be more effective than monotherapies by simultaneously activating parallel cellular pathways to control HCV replication.

As an initial step to define the divergent cellular processes involved in the control of intracellular HCV replication, and to utilize the different intracellular antiviral pathways influenced by the various interferons, we have characterized the activity of monotherapies and combination therapies with the major types of human interferons against HCV replication in the HCV replicon-containing cell line, AVA5 (Blight et al., 2000). In this report, we establish the variety of interactions between Type I and Type II interferons with respect to their antiviral activity against HCV replication, as well as a large panel of human cytokines and immune modulators. In addition, we report, for the first time, the anti-HCV activity of IFN-ω (Hauptmann and Swetly, 1985; Flores et al., 1991).

2. Materials and methods

2.1. Cell culture and antiviral treatments

AVA5 cells (Huh7 cells containing the subgenomic HCV replicon, BB7) (Blight et al., 2000) were used for these studies. Cultures were maintained in a sub-confluent state in DMEM with glutamine, non-essential amino acids, and 10% heat-inactivated fetal bovine serum (Biofluids Inc.) as previously described (Blight et al., 2000). Stock cultures were maintained in a sub-confluent state in this culture medium with 1 mg/ml G418 (Invitrogen Inc.) (Blight et al., 2000). Cells for antiviral analysis were seeded into 24- or 48-well tissue culture plates (Nunc Inc.) and grown for 3 days in the presence of G418. G418 was then removed for the duration of the antiviral treatments to eliminate potential loss of cells due to the reduction of HCV replicon (and G418-resistance) copy number. Cultures (rapidly dividing, three to four cultures per concentration, per experiment) were treated for three consecutive days with the test compounds. Medium was replaced daily with fresh test compounds. Analysis of HCV RNA was performed 24 h following the last addition of test compounds. Toxicity analyses using neutral red dye uptake were performed as previously described (Korba and Gerin, 1992). Cultures for the toxicity analyses were seeded from the same stock cultures and maintained on separate plates under conditions identical to those used for the corresponding antiviral assays.

Interferons (PBL Biomedical Laboratories Inc.) were solubilized and/or diluted in sterile phosphate-buffered saline (PBS)/1% BSA as instructed by the manufacturer. Ribavirin (Sigma) was solubilized in 100% tissue culture grade DMSO (Sigma). Cytokines and immune response modifiers were obtained as solutions and stored at −20 °C (PBL Biomedical Laboratories Inc.; Research Diagnostics Inc.). Stock solutions were stored (−70 °C, interferons; −20 °C, ribavirin) in volumes sufficient for a single experiment and used only once. Daily aliquots of test compounds were made from the stock solutions in individual tubes and stored at the appropriate temperatures. On each day of treatment, daily aliquots of the test compounds were suspended into culture medium at room temperature, and immediately added to the cell cultures, thereby subjecting each aliquot of test compound to the same, limited, number of freeze–thaw cycles.

2.2. Combination treatments

Combination treatments were conducted as previously described (Belen'kii and Schinazi, 1994; Korba, 1996). Briefly, for each combination of agents, three concentration ratios were used that were centered upon the use of the compounds at equipotent antiviral concentrations, based on the monotherapy EC_{90} values of each compound. A dilution series (six, 3-fold concentration steps, beginning at the approximate EC_{90} values) was then generated with the concentration ratio of the two agents remaining constant in each dilution step. Separate dilution series of the corresponding monotherapies were also used in the same experiment. Analysis of drug interactions in the combination studies was determined by the use of the CALCUSYN™ program (Biosoft Inc., Cambridge, UK). This program evaluates synergy, additivity, or antagonism by the use of several methodologies, including isobolagrams, median-effect plots, and the method of Chou and Talalay with a statistical analysis employing the Monte Carlo technique to provide confidence limits and Fa–CI (fraction affected–confidence interval) plots (Belen'kii and Schinazi, 1994).

2.3. HCV RNA analysis

Whole cell RNA was extracted from cells using either RNeasy™ mini-columns (Qiagen Inc.), or Purescript RNA Purification kits (Gentra Systems Inc.). Aliquots of cellular RNA were quantitatively analyzed for HCV RNA levels using a modification of a previously described dot blot hybridization assay (Korba and Gerin, 1992). RNA samples were denatured in 10× SSC/18% deionized formaldehyde for 20 min at 80 °C, applied to nitrocellulose under vacuum, washed once with 20× SSC, baked for 15 min at 80 °C under vacuum, and hybridized against ^{32}P-labelled DNA probes. Following the denaturation step, each RNA sample was split onto two nitrocellulose membranes for hybridization with either HCV-specific or human B-actin-specific ^{32}P-labelled DNA probes (95% of the sample for HCV, 5% for B-actin). The HCV hybridization probe used was a gel-purified, 6600 bp *Hin*dIII fragment isolated from the HCV replicon source plasmid, BB7 (Blight et al., 2000). The B-actin probe was a gel-purified, 550 bp PCR product generated from AVA5 cell RNA using a commercial PCR kit (Invitrogen Inc.). Both probes were labeled with ^{32}P-dCTP using a commercial random priming procedure (Clonetech-BD Biosciences Inc.). Hybridization was performed overnight at either 47 °C (HCV), or 40 °C (B-actin), and washing was performed at either 65 °C (HCV), or 60 °C (B-actin), as previously described (Korba and Gerin, 1992). Quantitation against independently determined standards present on each hybridization membrane was achieved using a beta scanner (Packard Instruments Inc.). This procedure provides for a relatively rapid and inexpensive assay that generates robust, quantifiable signals from large numbers of cultures as small as those contained in a 48-well tissue culture plate (Fig. 1).

The mean levels of B-actin RNA present in six to eight untreated cultures contained in each experiment were used as the basis for determining the relative level of B-actin RNA in each individual sample. Levels of HCV RNA were normalized to the levels of B-actin RNA present in each individual sample. HCV RNA levels in treated cultures were then compared to the normalized mean levels of HCV RNA present in the six to eight untreated cultures contained in each experiment.

2.4. HCV protein extraction and semi-quantitative Western blot analysis

Analysis of HCV protein was performed on cultures maintained on 6-well plates. Cells were washed three times with 5 ml of ice-cold phosphate-buffered saline and lysed by lysis buffer (Protein G Immunoprecipitation kit, Sigma, including one tablet of Sigma protease inhibitor cocktail tablets for 10 ml of extraction solution). The lysate was clarified by centrifugation and collected in a fresh tube. The protein concentration in each lysate was determined using a spectrophotometric protein assay (Bio-Rad Inc.). For Western blot analysis, 20 μg of protein were loaded

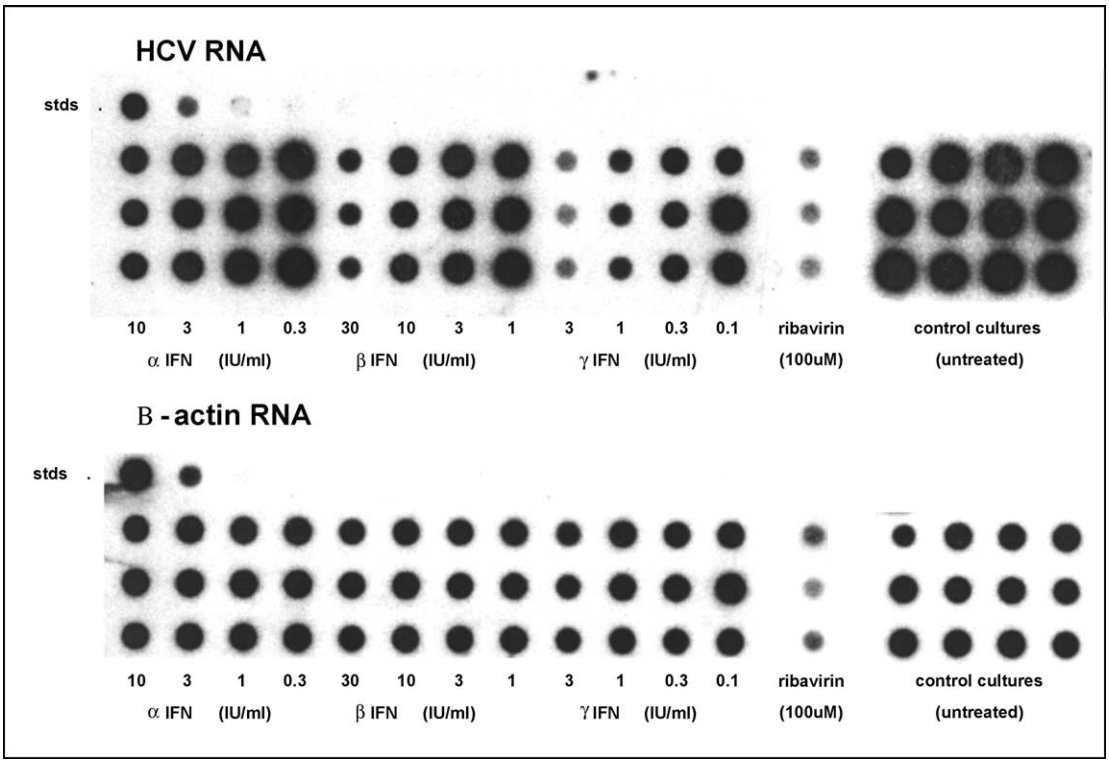

Fig. 1. Examples of dot blot hybridization assay for HCV and B-actin RNA. Each sample represents whole cell RNA obtained from a single well of cultures maintained on 48-well tissue culture plate. The HCV and B-actin panels comprise matched blotting pairs from individual RNA samples (95% of the sample for HCV, 5% for B-actin). Exposure shown: Kodak BioMax™ film, HCV (24 h), B-actin (8 h).

onto 10% Tris–glycine gels (Novex®, Invitrogen Inc.) and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen Inc.). Membranes were blocked with 5% non-fat dry milk, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and 0.1% Tween 20 (pH 7.4) for 1 h at room temperature. Blots were incubated overnight at room temperature with a 1:100 dilution of either anti-NS3 mouse monoclonal antibody, anti-NS5A mouse monoclonal antibody, anti-NS5A/B goat polyclonal antibody (for detection of NS5B) (Virostat Inc.), anti-NS4A mouse monoclonal antibody (1:500 dilution), or anti-NS4 mouse monoclonal antibody (for detection of NS4B, 1:250 dilution) (Virogen Inc.). Membranes were then washed four times for 5 min with wash buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 2 mM KH_2PO_4, and 0.1% Tween 20). Membranes exposed to anti-NS3, NS4A, NS4, or NS5A antibodies were incubated with [^{125}I] goat anti-mouse IgG (Perkin-Elmer Inc.) for 4 h at room temperature. For the detection of NS5B, the membranes were incubated with rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories Inc.) for 4 h at room temperature. Then, after being washed four times for 5 min with wash buffer, the membranes were incubated with [^{125}I] goat anti-rabbit IgG (Perkin-Elmer Inc.) for 4 h at room temperature. After incubation with [^{125}I]-labeled antibodies, membranes were washed four times for 5 min with wash buffer. Semi-quantitation of HCV proteins versus the levels in untreated cells and/or at the 'zero' time point present on each membrane was achieved using a beta scanner (Packard Instruments Inc.).

3. Results

3.1. Effect of ribavirin, and human interferons and cytokines on HCV replication in AVA cells

All of the major classes of human interferons displayed selective anti-HCV activity in AVA5 cells (Table 1). Dose response analyses in several experiments demonstrated that recombinant human interferons alpha B2 (IFN-α), beta (IFN-β), and omega (IFN-ω) exhibited similar anti-HCV potencies. Recombinant human Type I (αA/D) interferon ('universal interferon') was considerably less potent than IFN-α, while recombinant human gamma interferon (IFN-γ) was approximately 10–20 times as effective as IFN-α. Human leukocyte interferon (a mixture of all sub-species of alpha interferon of which IFN-α is a minor component by content) was considerably less effective (Table 1). Several sub-species of alpha interferon which collectively comprise leukocyte interferon (other than IFN-α) displayed, at best, only modest anti-HCV potencies (Table 1).

Ribavirin displayed no selective anti-HCV activity in AVA5 cells (Table 1 and Fig. 1), consistent with clinical observations that ribavirin monotherapy does not affect HCV viremia, despite improving HCV-induced disease (Dusheiko et al., 1996; Poynard et al., 2000). Ribavirin and IFN-α enhance the production/induction of several cytokines though various metabolic cascades (Der et al., 1998; Stark et al., 1998; Tam et al., 1999a, 1999b). An evaluation of 22 human cytokines and immune response modifiers in AVA5 cells, including those representing components of Type I and Type II responses, did not demonstrate direct anti-HCV activity in AVA5 cells (Table 1). All of these agents were used at levels that were at or near the maximum of the range routinely used to assess the activity of these molecules on their natural target cells (according to the manufacturers' specifications).

3.2. Kinetics and extended duration of interferon therapy

An analysis of the kinetics of the inhibition of HCV replication showed that IFN-α achieved a maximal antiviral effect after approximately 48–60 h of continuous treatment with concentrations that reduce HCV replication by 10-fold or less (1–10 IU/ml) after 72 h of treatment (Fig. 2). Maximal antiviral effectiveness with IFN-β and IFN-γ were achieved more rapidly (24–48 h) with equivalent effective doses (Fig. 2). At higher doses however, these differences became less distinguishable.

To further characterize potential differences in the kinetics of the antiviral effects of the different interferons, cultures were exposed to interferon for only 24 h (after 24 h, the culture medium was changed and replaced with medium containing no interferon) (Fig. 2). The same overall patterns of HCV RNA inhibition were observed following treatment with a single 24-h dose of interferon as were observed for cultures treated for 72 h. Interestingly, 24 h of interferon treatment with IFN-β and IFN-γ were essentially as effective as 72 h of therapy, while 24 h of αIFN treatment was not as effective as 72 h (Table 2 and Fig. 2). Previous studies have indicated that maximal inhibition of HCV RNA production appears to occur after approximately 48 h of therapy with 100 IU/ml IFN-α (Gao et al., 2001; Lohmann et al., 1999).

3.3. Combination treatment with ribavirin and IFN-α

Combination therapy with ribavirin and IFN-α is currently the most effective therapy for chronic HCV infection (Davis et al., 1998; McHutchinson et al., 1998; Poynard et al., 2000). Although ribavirin monotherapy exhibited no selective suppression of HCV replication in our studies (Table 1, Fig. 1), it was of interest to determine if combination therapy with IFN-α would produce an enhanced antiviral effect in AVA5 cells as it does in HCV-infected individuals (Davis et al., 1998; McHutchinson et al., 1998). For these experiments, ribavirin was used at a constant dose of 20 μM, the highest concentration that routinely displayed no apparent cytotoxicity in monotherapy, and IFN-α was used at concentrations ranging from 1.0 to 30 IU/ml. Ribavirin did not enhance the anti-HCV activity, or alter the toxicity profile, of IFN-α in AVA5 cells (Table 1).

Table 1
Relative potency of human cytokines and alpha interferons against HCV replication in AVA5 cell cultures

Treatment	CC_{50}	EC_{50}	EC_{90}	S.I. (CC_{50}/EC_{50})
IFN-α	>10000[a]	2.5 ± 0.2	10 ± 0.8	>4000
IFN-β	>10000	2.7 ± 0.2	12 ± 0.9	>3703
IFN-γ	>10000	0.194 ± 0.039	0.671 ± 0.088	>51546
IFN-ω	>10000	1.9 ± 0.2	11 ± 1.1	>5263
Ribavirin	22 ± 1.8[b]	>100[a,b]	>100[a,b]	–
IFN-α + ribavirin[c]	>10000[c]	2.2 ± 0.3[c]	9.7 ± 1.0[c]	>4546
type I IFN (αA/D)	>10000	5.8 ± 1.0	46 ± 5.2	>1724
Lymphocyte IFN	>10000	83 ± 14	455 ± 66	>125
IFN-α A	>1000	7.2 ± 1.8	21 ± 1.5	>138
IFN-α C	>1000	>1000	>1000	–
IFN-α D	>1000	>1000	>1000	–
IFN-α F	>1000	>1000	>1000	–
IFN-α G	>1000	530 ± 97	1303 ± 288	>1.9
IFN-α H2	>1000	127 ± 16	1429 ± 124	>7.9
IFN-α I	>1000	124 ± 25	844 ± 102	>8.1
IFN-α J1	>1000	545 ± 32	1348 ± 805	>1.8
IFN-α K	>1000	456 ± 91	1072 ± 215	>2.2
IFN-α WA	>1000	>1000	>1000	–
IFN-α 4b	>1000	615 ± 105	1711 ± 416	>1.6
Thymosin α-1	>1000	>1000	>1000	–
TNF-α	>200	>200		>200
TNF-β	>200	>200	>200	–
IL-1a	>100	>100	>100	–
IL-1b	>100	>100	>100	–
IL-2	>100	>100	>100	–
IL-3	>100	>100	>100	–
IL-4	>100	>100	>100	–
IL-5	>100	>100	>100	–
IL-6	>100	>100	>100	–
IL-7	>100	>100	>100	–
IL-8	>100	>100	>100	–
IL-8 (72 a.a.)	>100	>100	>100	–
IL-10	>100	>100	>100	–
IL-11	>100	>100	>100	–
IL-12	>100	>100	>100	–
IL-13	>100	>100	>100	–
IL-15	>100	>100	>100	–
IL-16	>100	>100	>100	–
IL-16 (121 a.a.)	>100	>100	>100	–
IL-17	>100	>100	>100	–
IL-18	>200	>200	>200	–
IL-18 + IL-12	>200	>200	>200	–

Values presented (± standard deviations [S.D.]) were calculated by linear regression analysis using data combined from all treated cultures. EC_{50}, EC_{90}, drug concentration at which a 2- or 10-fold depression of HCV RNA (relative to the average levels in untreated cultures), respectively, was observed. CC_{50}, drug concentration at which a 2-fold lower level of neutral red dye uptake (relative to the average levels in untreated cultures) was observed. Concentrations for αIFN are expressed as 'IU/ml'; ribavirin is expressed in 'μM'; values for TNF-α, TNF-β, and the interleukins are expressed as 'ng/ml'.

[a] No cytotoxic or antiviral effect at highest indicated concentration.

[b] The concentrations for ribavirin are expressed in 'μM'.

[c] Since ribavirin displayed no selective anti-HCV activity, this combination performed with a constant concentration of 20 μM ribavirin (highest dose that demonstrated 100% neutral red uptake versus controls) and varying concentrations of IFN-α. CC_{50}, EC_{50}, EC_{90} values for this combination are expressed in IU/ml IFN-α.

3.4. Interferon combinations

All of the interferon combinations examined were effective against HCV replication in AVA5 cells (Table 3), and most demonstrated enhanced potency over the corresponding monotherapies. However, the range of interactions between the interferons in the various mixtures varied dramatically. The most potent and favorable interactions observed were between IFN-α and IFN-γ (Table 3 and Fig. 3B). Moderate to strong synergism was observed at all combinations between IFN-α and IFN-γ. Approximately the same degree of favorable interaction was observed at all concentration ratios of the two interferons, as well as over the entire dose ranges used for these different combinations (Fig. 3B). The combinations of IFN-β with IFN-γ, and IFN-ω with IFN-γ were also generally synergistic, although the interactions between the interferons in these combinations appeared to be generally less favorable than those observed between IFN-

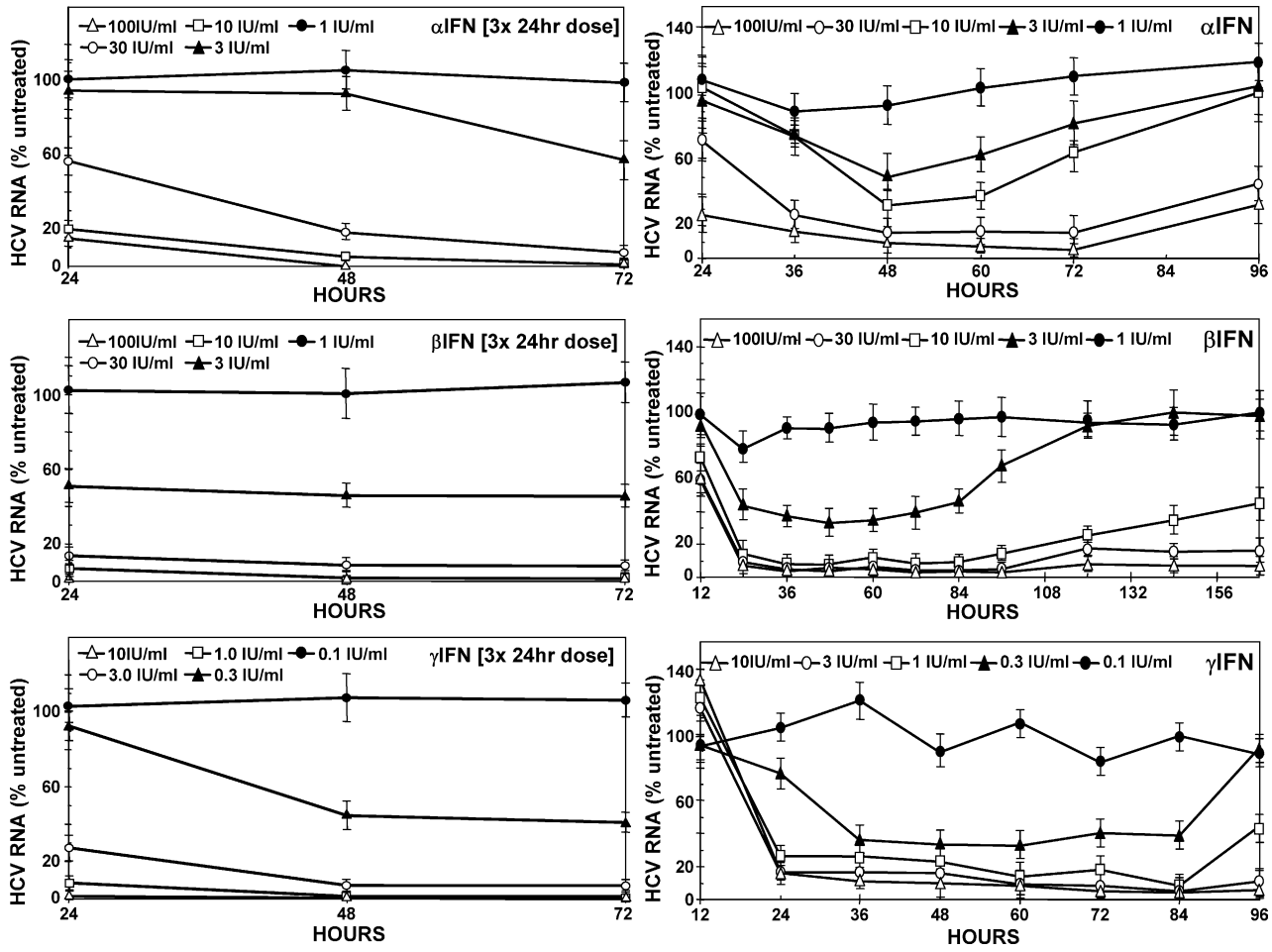

Fig. 2. Kinetics of the inhibition of HCV replication in AVA5 cells following interferon treatment. AVA5 cultures were exposed continuously to interferons as outlined in the standard assay procedures in Section 2 (left-hand panels). Alternatively (right-hand panels), AVA5 cells were exposed to interferons only during the first 24 h of the study period. After 24 h, the culture medium was removed and replaced with medium lacking interferons.

α and IFN-γ (Table 3). By contrast, combinations of IFN-α and IFN-ω, as well as IFN-β and IFN-ω, exhibited primarily antagonistic interactions (Table 3 and Fig. 3C).

The interactions between IFN-α and IFN-β were considerably more complex than that observed for the combinations discussed earlier (Table 3 and Fig. 3A). Unlike the relatively simple types of interactions observed for the combinations of IFN-α + IFN-γ or IFN-α + IFN-γ, the relative degree of favorable or unfavorable interactions between IFN-α and IFN-β was dependent on both the relative concentrations of the two interferons in the combination treatments as well as the total dose of interferon. Overall, combinations of IFN-α and IFN-β exhibited additive interactions, but as the relative concentration of IFN-β to IFN-α increased, the interactions became increasing less favorable (Table 3 and Fig. 3A). As the total dose of interferon in these combination treatments increased, less favorable interactions were observed (Fig. 3A). This is illustrated by the fact that slightly synergistic or additive interactions were observed at the 50% effective concentrations (EC_{50}) of an individual combination, but at the 90% effective concentrations (EC_{90}) primarily additive or antagonistic interactions were observed for the same mixture.

Combinations of IFN-α and either IFN-β or IFN-γ have been used in a sequential treatment regimen in limited clinical trials to treat chronic HCV infection (Asahina et al., 2001; Horiike et al., 2003; Katayama et al., 2001; Watanabe et al., 2002). For this type of therapy, a short (2-week) period of IFN-β or IFN-γ preceded a longer (22-week) period of IFN-α treatment. This type of treatment scheme was modeled in culture by treating cells for 1 day with one interferon (at a fixed dose), followed by 3 days of continuous treatment with a second interferon (in a dilution series). For these studies, the first interferon was removed after 24 h, and the second interferon was administered in three daily doses (replacing the medium with fresh interferon each 24 h).

In all cases, sequential administration of interferon combinations did not enhance the anti-HCV activity of the corresponding monotherapies to the same degree as did the simultaneous administration of interferons (Table 4). For example, sequential administration of the combination of IFN-α and IFN-β proved to be generally antagonistic irrespective of which interferon was used first, or any variances in the relative doses of each interferon (Table 4). Overall, favorable interactions were still generally observed for IFN-α + IFN-γ

Table 2
Relative potency of single and multiple doses of human interferons against HCV replication in AVA5 cell cultures

Treatment	EC_{50} (IU/ml)	EC_{90} (IU/ml)
Assayed after 72 h of treatment		
IFN-α single dose	9.8 ± 0.9	36 ± 5.5
IFN-α multiple dose	2.6 ± 0.3	9.5 ± 0.8
IFN-β single dose	2.6 ± 0.2	12 ± 2.8
IFN-β multiple dose	2.5 ± 0.3	9.8 ± 1.0
IFN-γ single dose	0.248 ± 0.029	2.3 ± 0.4
IFN-γ multiple dose	0.285 ± 0.035	2.8 ± 0.4
Assayed after 96 h of treatment		
IFN-α single dose	14 ± 0.8	80 ± 9.0
IFN-α multiple dose	2.3 ± 0.2	9.1 ± 0.7
IFN-β single dose	2.8 ± 0.2	11 ± 1.8
IFN-β multiple dose	2.1 ± 0.3	10 ± 1.2
IFN-γ single dose	0.285 ± 0.039	2.8 ± 0.4
IFN-γ multiple dose	0.205 ± 0.030	2.1 ± 0.3

Values presented (± standard deviations [S.D.]) were calculated by linear regression analysis using data combined from all treated cultures. EC_{50}, EC_{90}, drug concentration at which a 2- or 10-fold depression of HCV RNA (relative to the average levels in untreated cultures), respectively, was observed. For the single-dose treatments, interferon was present in the culture medium during only the first 24 h of the study period. After 24 h, medium was removed and replaced with fresh medium lacking interferon. For the multiple-dose treatments, interferons were added daily as described in Section 2 for the routine assay procedure. HCV RNA levels were measured after either 3 or 4 days of treatment.

or IFN-β + IFN-γ combinations, especially at lower overall interferon levels (e.g. the EC_{50} values) (Table 4).

3.5. *Effect of interferon treatments on HCV protein levels*

The rate of the decline in the steady-state levels of various HCV proteins under different interferon treatments was determined by semi-quantitative Western blot analysis (Table 5). HCV proteins in treated cells declined in parallel to that observed for HCV RNA in the same experiments. HCV RNA appeared to generally decline slightly more rapidly than HCV proteins, but following the initial lag periods, the relative rates of the loss of HCV RNA and proteins were essentially parallel. No normalization for differences in the apparent relative half-lives of HCV RNA or proteins (Lohmann et al., 1999; Blight et al., 2000) were made for these analyses.

4. Discussion

In this report, we describe the enhancement of anti-HCV activity by the use of combinations of various human interferons in a subgenomic HCV replicon system. This report demonstrates that not all anti-HCV agents can be used effectively in combination and reinforces that care must be taken in the detailed design of such therapies. The anti-HCV activity of human IFN-ω (Hauptmann and Swetly, 1985; Flores et al., 1991), several sub-species of human alpha interferons, and several human cytokines and immune response modifiers are also described for the first time. The relative potencies of IFN-α and IFN-β interferons in these experiments were consistent with previous reports in HCV replicon systems (Lohmann et al., 1999; Blight et al., 2000; Gao et al., 2001; Frese et al., 2002). The relative potency of IFN-γ observed in these studies was consistent with a previous report in HCV replicons (Cheney et al., 2002), although in another study, IFN-γ was considerably less potent, approximating the relative potency of IFN-α (Frese et al., 2002). The types of interactions observed between IFN-α and IFN-β or IFN-γ in these cell culture studies are consistent with those reported in clinical trials using combinations of these agents (Asahina et al., 2001; Horiike et al., 2003; Katayama et al., 2001; Watanabe et al., 2002). In those studies, combinations of IFN-α with IFN-γ provided substantial antiviral enhancement, while combinations of IFN-α with IFN-β provided, at best, only a moderate enhancement over IFN-α monotherapy or none at all.

The key studies described in this report were performed primarily under treatment conditions designed to reduce HCV replication by a limited amount (10–20-fold or less). This was critical for the accurate discrimination of differences in the relative ability of the various interferons to interfere with HCV replication. As shown in the kinetic studies, treatments with levels of interferons much higher than the EC_{90}–EC_{95} values eliminated many of the observed differences between the interferons. In addition, by controlling for toxicity/extraction efficiency in each culture used for these studies, non-selective (e.g. cytotoxic) antiviral responses, such as those induced by ribavirin, were eliminated.

The variety of interferons which efficiently inhibit HCV replication indicates that diverse sets of cellular processes are capable of controlling this viral infection. These agents represent components of both Type I and Type II host immune responses, and utilize several different intracellular signaling and antiviral response pathways (Der et al., 1998; Stark et al., 1998; Goodbourn et al., 2000). The complexity of the interactions observed, especially for combinations of IFN-α and IFN-β, illustrates that substantial consideration must be give to not only the relative proportions of interferons in combination treatment, but also the total dose of interferon delivered.

A substantial range of interactions was observed between the between the various interferons in the combination treatments. While the exact mechanisms responsible for these interactions remain to be elucidated, the overall patterns of interactions are not surprising. The most favorable interactions and strongest enhancement of anti-HCV activity was the result of mixing Type I and Type II interferons (e.g. IFN-α and IFN-γ). Given the divergence of the intracellular signaling and response pathways involved with IFN-α and IFN-γ (Stark et al., 1998; Goodbourn et al., 2000), the results of this study suggest that it is possible to simultaneously activate multiple host antiviral response mechanisms.

Table 3
Relative potency of human interferon monotherapy and combination therapy against HCV replication in AVA5 cell cultures

Treatment	CC_{50} (IU/ml)	EC_{50} (IU/ml)	EC_{90} (IU/ml)	S.I. (CC_{50}/EC_{50})	Type of interaction.
IFN-α	>10000[a]	2.5 ± 0.2	10 ± 0.8	>4000	
IFN-β	>10000	2.7 ± 0.2	12 ± 0.9	>3703	
IFN-γ	>10000	0.194 ± 0.039	0.671 ± 0.088	>51546	
IFN-ω	>10000	1.9 ± 0.2	11 ± 1.1	>5263	
IFN-α + IFN-β @ 1:1	>10000	0.194 ± 0.034	0.829 ± 0.092	>51546	Synergistic
IFN-α + IFN-β @ 1:3	>10000	0.235 ± 0.023	3.1 ± 0.3	>42553	Additive
IFN-α + IFN-β @ 1:10	>10000	0.710 ± 0.060	7.5 ± 0.6	>14085	Additive to antagonistic
IFN-α + IFN-γ @ 30:1	>10000	0.065 ± 0.005	0.522 ± 0.037	>153846	Synergistic
IFN-α + IFN-γ @ 10:1	>10000	0.095 ± 0.012	0.829 ± 0.108	>105263	Synergistic
IFN-α + IFN-γ @ 3:1	>10000	0.483 ± 0.066	1.7 ± 0.2	>20703	Synergistic
IFN-α + IFN-γ @ 1:1	>10000	0.929 ± 0.104	3.0 ± 0.5	>10764	Synergistic to additive
IFN-α + IFN-ω @ 3:1	>10000	0.538 ± 0.085	13 ± 2.5	>18578	Antagonistic
IFN-α + IFN-ω @ 1:1	>10000	0.864 ± 0.103	9.1 ± 1.1	>11574	Antagonistic
IFN-α + IFN-ω @ 1:3	>10000	2.1 ± 0.3	9.5 ± 1.2	>4761	Antagonistic
IFN-β + IFN-γ @ 30:1	>10000	0.185 ± 0.019	0.297 ± 0.031	>54054	Synergistic
IFN-β + IFN-γ @ 10:1	>10000	0.278 ± 0.020	0.903 ± 0.065	>35971	Synergistic
IFN-β + IFN-γ @ 3:1	>10000	0.895 ± 0.056	2.7 ± 0.2	>11173	Additive
IFN-β + IFN-ω @ 3:1	>10000	2.6 ± 0.4	10 ± 0.7	>3846	Antagonistic
IFN-β + IFN-ω @ 1:1	>10000	0.792 ± 0.080	2.7 ± 0.1	>12626	Additive to antagonistic
IFN-β + IFN-ω @ 1:3	>10000	0.780 ± 0.075	2.6 ± 0.3	>12820	Additive to antagonistic
IFN-ω + IFN-γ @ 30:1	>10000	0.264 ± 0.042	0.817 ± 0.060	>37879	Synergistic
IFN-ω + IFN-γ @ 10:1	>10000	0.621 ± 0.088	1.1 ± 0.2	>16103	Synergistic to additive
IFN-ω + IFN-γ @ 3:1	>10000	0.754 ± 0.075	2.6 ± 0.3	>13262	Additive

Values presented (± standard deviations [S.D.]) were calculated by linear regression analysis using data combined from all treated cultures, and represent the apparent potency of the first interferon listed in the combinations. CC_{50}, drug concentration at which a 2-fold depression of neutral red dye uptake relative to the average levels in untreated cultures) was observed. EC_{50}, EC_{90}, drug concentration at which a 2- or 10-fold depression of HCV RNA (relative to the average levels in untreated cultures), respectively, was observed. CC_{50}, drug concentration at which a 2-fold lower level of neutral red dye uptake (relative to the average levels in untreated cultures) was observed. S.I., selectivity index (CC_{50}/EC_{50}). 'Type of interaction', an overall summary of the analysis of the interaction between the interferons in the combination treatments as determined by Combostat™. In some cases, the interactions within a given treatment changed over the dilution series (see Fig. 3 for examples) which is indicated by a range of interactions as opposed to a single class (e.g. 'additive to antagonistic' vs. 'additive').

[a] No cytotoxic or antiviral effect at highest indicated concentration.

Generally unfavorable interactions were observed for mixtures of two Type I interferons (e.g. IFN-α and IFN-β). This was especially evident when one interferon was used at a much higher effective antiviral dose relative to the second interferon, or when higher total concentrations of the interferons were used. Given the overlap in intracellular pathways and cellular receptors (Der et al., 1998; Stark et al., 1998; Goodbourn et al., 2000), either treatment condition could be expected to potentially result in a saturation of interactions with cellular processes and/or receptors, producing a functional excess of interferon that would dilute apparent antiviral potencies. The high degree of antagonism observed between IFN-ω and the other Type I interferons may be due to the observations that IFN-ω binds to cellular α/βIFN receptors, and has been shown to efficiently block the binding of IFN-α (Flores et al., 1991).

Some of the interactions observed in the combination studies may have been due to the varied durability of the antiviral effects induced by the different interferons. Suppression of HCV replication by IFN-β or IFN-γ was induced more rapidly and lasted for a more substantial period of time following the removal of treatment than that observed for IFN-α. When a second interferon was used in a sequential treatment mode, it may not have been possible to induce further reductions in virus replication as the cellular pathways involved may have already been preprogrammed by the response to the first interferon. When two interferons were present simultaneously at the beginning of treatment, there would be, in theory, a greater chance for each agent to independently influence host responses.

Ribavirin displayed no selective anti-HCV activity in AVA5 cells, consistent with clinical observations that ribavirin monotherapy does not affect HCV viremia, although it does lead to improvement in HCV-induced disease (Dusheiko et al., 1996; Davis et al., 1998; McHutchinson et al., 1998; Poynard et al., 2000). In several previous reports, ribavirin in replicon-based culture assays had a very low selectivity index (< 2.0) (Frese et al., 2002; Saito et al., 2003; Zhou et al., 2003; Tanabe et al., 2004). Because our studies normalized HCV RNA levels to B-actin RNA levels to account for variances in sample RNA content, the nearly equivalent decline in B-actin RNA induced by the cytotoxic activity of ribavirin negated any apparent loss in 'raw' (e.g. 'non-normalized') HCV RNA levels (see Fig. 1). Such

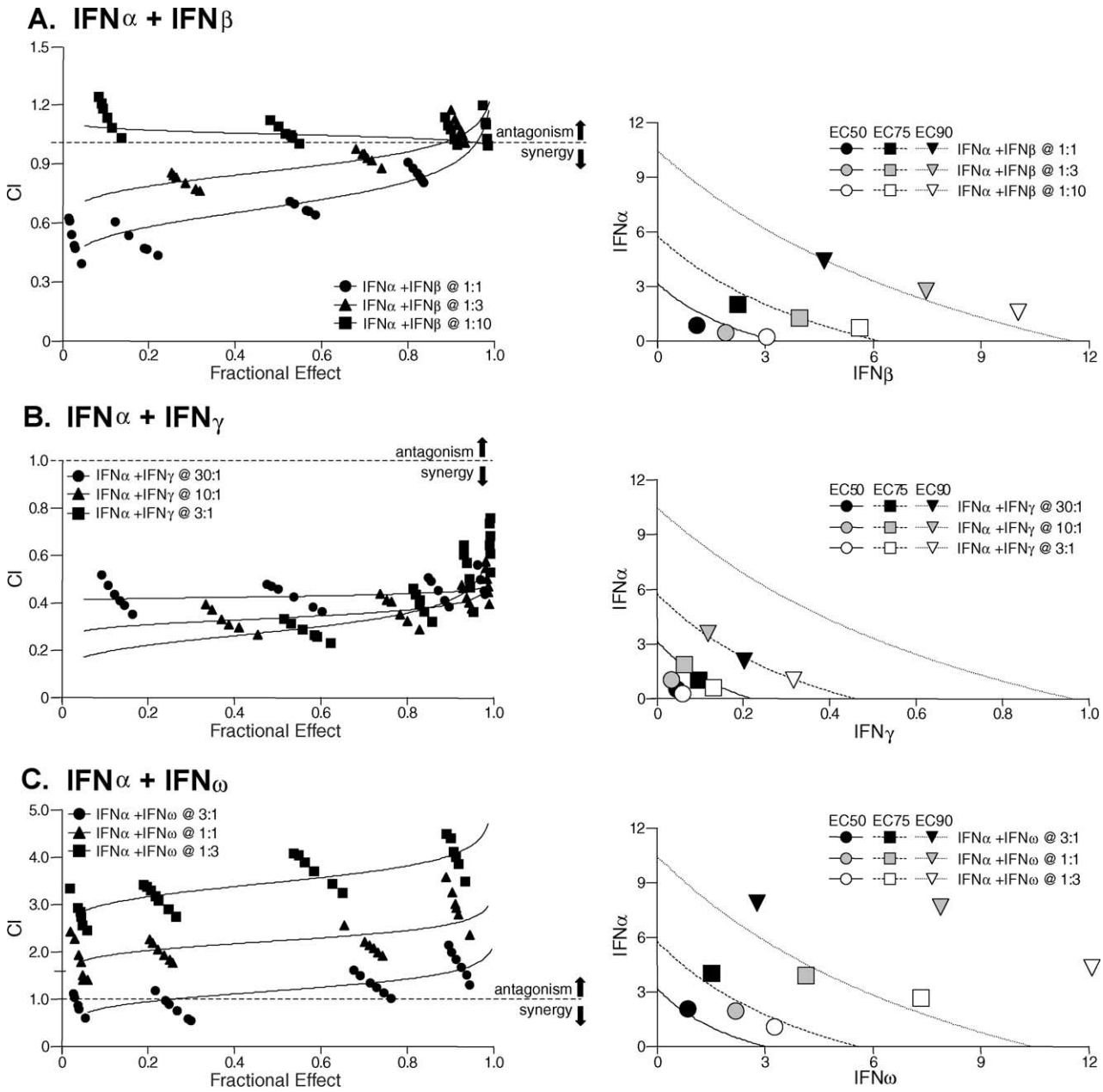

Fig. 3. Examples of the analysis of interactions between interferons in combination treatments (Combostat™, Biosoft Inc.). Two types of evaluations are presented. For each combination of interferons, three different concentration ratios are displayed. The left-hand graphs present CI–Fa (combination index–fraction (of virus) affected) plots (Belen'kii and Schinazi, 1994). For these plots, a combination index (CI) greater than 1.0 indicates antagonism and a CI less than 1.0 indicates synergism. Evaluations of synergy, additivity (summation), or antagonism at different levels of virus inhibition (e.g. 5% (Fa = 0.5) to 99% (Fa = 0.99)) are provided by the plotted lines and points. The right-hand column of the figure presents isobolograms. For these plots, EC50, EC75, and EC90 (50%, 75%, and 90% effective antiviral concentrations) values for the combination treatments are displayed as single points. Three lines radiating out from the axis denote the expected (e.g. additive) EC50, EC75, and EC90 values for drug combinations as calculated from the monotherapies. EC50, EC75, and EC90 values for the combinations that plot to the left (e.g. less than) of the corresponding lines indicate antagonism, and values plotting to the right (e.g. greater than) of the corresponding lines indicate synergy.

a pattern in our assay is equated with a 'false-positive' reading.

In our studies, ribavirin did not affect the anti-HCV profile of IFN-α. While this may not be surprising given the lack of selective efficacy for this compound, this observation may be due to the use of ribavirin at a relative low dose (chosen since it was the highest completely non-cytotoxic dose in these studies). One previous report has indicated a modest enhancement of the anti-HCV activity of IFN-α by ribavirin (Tanabe et al., 2004). The concentration of ribavirin used in those studies was several fold higher than used in the current report, and was sufficient to induce a low (but not statistically significant) level of toxicity based on the data presented in that report.

Table 4
Relative potency of sequential addition of human interferons against HCV replication in AVA5 cell cultures

Treatment [day 1 (dose), days 2–4]	72 h of treatment		96 h of treatment		Type of interaction
	EC_{50} (IU/ml)	EC_{90} (IU/ml)	EC_{50} (IU/ml)	EC_{90} (IU/ml)	
IFN-α	1.9 ± 0.3	8.9 ± 0.6	1.8 ± 0.1	8.8 ± 1.3	
IFN-β	2.0 ± 0.1	8.0 ± 0.4	1.8 ± 0.2	8.5 ± 0.4	
IFN-α (10 IU/ml), IFN-β	1.8 ± 0.2	11 ± 1.5	0.4 ± 0.1	10 ± 1.2	Antagonistic
IFN-α (3.0 IU/ml), IFN-β	1.8 ± 0.3	12 ± 1.0	0.6 ± 0.1	10 ± 1.2	Antagonistic
IFN-α (1.0 IU/ml), IFN-β	2.3 ± 0.2	9.0 ± 0.6	1.8 ± 0.2	9.6 ± 0.5	Antagonistic
IFN-β (10 IU/ml), IFN-α	1.7 ± 0.2	13 ± 1.8	1.9 ± 0.4	17 ± 1.2	Antagonistic
IFN-β (3.0 IU/ml), IFN-α	2.2 ± 0.3	13 ± 0.5	2.6 ± 0.1	13 ± 0.8	Antagonistic
IFN-β (1.0 IU/ml), IFN-α	2.0 ± 0.2	8.7 ± 1.0	2.0 ± 0.3	8.6 ± 0.6	Antagonistic
IFN-α	2.5 ± 0.2	9.5 ± 0.9	2.0 ± 0.2	8.3 ± 0.9	
IFN-γ	0.2 ± 0.03	0.9 ± 0.1	0.2 ± 0.02	0.9 ± 0.1	
IFN-α (10 IU/ml), IFN-γ	0.04 ± 0.003	0.4 ± 0.08	0.05 ± 0.003	0.4 ± 0.05	Additive
IFN-α (3.0 IU/ml), IFN-γ	0.08 ± 0.01	0.6 ± 0.06	0.02 ± 0.007	0.6 ± 0.07	Additive
IFN-α (1.0 IU/ml), IFN-γ	0.2 ± 0.03	0.8 ± 0.1	0.05 ± 0.004	0.8 ± 0.1	Additive to antagonistic
IFN-γ (1.0 IU/ml), IFN-α	0.5 ± 0.02	4.3 ± 0.4	0.5 ± 0.07	4.1 ± 0.7	Synergistic to additive
IFN-γ (0.3 IU/ml), IFN-α	0.7 ± 0.08	6.6 ± 0.8	0.3 ± 0.04	6.2 ± 0.5	Synergistic to additive
IFN-γ (0.1 IU/ml), IFN-α	1.5 ± 0.3	7.9 ± 0.9	1.1 ± 0.1	7.5 ± 0.9	Additive to antagonistic
IFN-β	2.3 ± 0.1	8.7 ± 0.8	2.2 ± 0.1	8.6 ± 0.8	
IFN-γ	0.2 ± 0.03	0.9 ± 0.1	0.2 ± 0.05	0.9 ± 0.04	
IFN-β (10 IU/ml), IFN-γ	0.03 ± 0.002	0.5 ± 0.06	0.04 ± 0.01	0.4 ± 0.06	Additive
IFN-β (3.0 IU/ml), IFN-γ	0.06 ± 0.01	0.4 ± 0.08	0.04 ± 0.06	0.6 ± 0.05	Additive
IFN-β (1.0 IU/ml), IFN-γ	0.2 ± 0.03	0.6 ± 0.07	0.2 ± 0.03	0.7 ± 0.08	Additive to antagonistic
IFN-γ (1.0 IU/ml), IFN-β	0.8 ± 0.04	5.7 ± 0.2	0.3 ± 0.05	4.4 ± 0.2	Synergistic to additive
IFN-γ (0.3 IU/ml), IFN-β	0.9 ± 0.05	6.3 ± 0.5	0.3 ± 0.04	4.3 ± 0.4	Synergistic to additive
IFN-γ (0.1 IU/ml), IFN-β	1.7 ± 0.2	7.8 ± 0.9	1.6 ± 0.1	7.2 ± 0.7	Additive to antagonistic

Values presented (± standard deviations [S.D.]) were calculated by linear regression analysis using data combined from all treated cultures, and represent the apparent potency of the second interferon listed in the combinations. EC_{50}, EC_{90}, drug concentration at which a 2- or 10-fold depression of HCV RNA (relative to the average levels in untreated cultures), respectively, was observed. CC_{50}, drug concentration at which a 2-fold lower level of neutral red dye uptake (relative to the average levels in untreated cultures) was observed. Analysis of the overall interaction between the interferons in the combination treatments was determined by analysis using Combostat™.

Table 5
Effect of treatment on the levels of HCV proteins in AVA5 cells

Treatment	Control at indicated time point (%)											
	HCV RNA				NS3				NS5A			
	12 h	24 h	48 h	72 h	12 h	24 h	48 h	72 h	12 h	24 h	48 h	72 h
IFN-α (10 IU/ml)	102 ± 11	83 ± 8	14 ± 2	9 ± 1	107 ± 12	109 ± 10	62 ± 7	28 ± 3	125 ± 14	129 ± 10	64 ± 5	34 ± 3
IFN-β (10 IU/ml)	88 ± 7	35 ± 4	6 ± 1	6 ± 1	94 ± 10	74 ± 8	45 ± 4	15 ± 1	108 ± 11	69 ± 7	47 ± 5	19 ± 2
IFN-γ (1.0 IU/ml)	78 ± 6	44 ± 3	10 ± 1	8 ± 1	93 ± 9	68 ± 7	31 ± 4	21 ± 2	121 ± 10	91 ± 9	39 ± 4	24 ± 2
IFN-ω (10 IU/ml)	74 ± 8	45 ± 5	7 ± 1	7 ± 1	87 ± 9	62 ± 6	40 ± 3	16 ± 1	114 ± 12	76 ± 8	44 ± 4	21 ± 2
IFN-α + IFN-β @ 1:1	97 ± 10	45 ± 5	10 ± 1	3 ± 1	94 ± 9	62 ± 6	37 ± 4	8 ± 1	84 ± 9	61 ± 6	39 ± 4	7 ± 1
IFN-α + IFN-γ @ 10:1	66 ± 7	26 ± 3	3 ± 1	1 ± 1	84 ± 8	60 ± 7	13 ± 1	1 ± 1	116 ± 12	78 ± 8	19 ± 2	3 ± 1
IFN-α + IFN-ω @ 1:1	96 ± 8	61 ± 5	17 ± 2	11 ± 1	86 ± 9	62 ± 7	30 ± 3	15 ± 1	97 ± 10	90 ± 10	47 ± 5	14 ± 1

Treatment	Control at indicated time point (%)											
	NS5B				NS4A				NS4B			
	12 h	24 h	48 h	72 h	12 h	24 h	48 h	72 h	12 h	24 h	48 h	72 h
IFN-α (10 IU/ml)	128 ± 13	117 ± 12	60 ± 7	28 ± 3	94 ± 10	96 ± 10	60 ± 7	21 ± 2	97 ± 8	101 ± 12	58 ± 6	18 ± 2
IFN-β (10 IU/ml)	93 ± 8	61 ± 7	43 ± 5	26 ± 2	95 ± 9	70 ± 8	35 ± 3	14 ± 1	98 ± 10	58 ± 6	30 ± 3	12 ± 1
IFN-γ (1.0 IU/ml)	119 ± 12	81 ± 8	45 ± 4	25 ± 3	92 ± 9	59 ± 6	34 ± 3	20 ± 2	102 ± 11	67 ± 7	26 ± 2	15 ± 1
IFN-ω (10 IU/ml)	89 ± 9	56 ± 6	42 ± 4	21 ± 2	105 ± 10	60 ± 6	37 ± 4	19 ± 2	108 ± 11	66 ± 6	32 ± 3	20 ± 2
IFN-α + IFN-β @ 1:1	109 ± 10	75 ± 8	53 ± 5	10 ± 1	96 ± 9	59 ± 6	25 ± 3	8 ± 1	99 ± 10	51 ± 6	21 ± 2	9 ± 1
IFN-α + IFN-γ @ 10:1	115 ± 11	75 ± 7	19 ± 2	5 ± 1	111 ± 12	62 ± 7	14 ± 1	6 ± 1	102 ± 10	59 ± 6	20 ± 3	7 ± 1
IFN-α + IFN-ω @ 1:1	113 ± 11	84 ± 7	51 ± 6	19 ± 2	94 ± 10	79 ± 8	44 ± 5	16 ± 2	110 ± 12	72 ± 7	40 ± 5	17 ± 2

Levels of HCV RNA and proteins are expressed relative to the mean levels detected in untreated cultures at each time point indicated. Values presented are means of duplicate determinations in two experiments (four cultures total) ± standard deviations.

The lack of anti-HCV activity by human cytokines and immune response modifiers in the HCV replicon system, including those representing components of both Type I and Type II immune responses activated by ribavirin and interferons (Der et al., 1998; Stark et al., 1998; Tam et al., 1999a, 1999b; Goodbourn et al., 2000), indicates that these molecules most likely act indirectly to exert observed anti-HCV effects in HCV-infected patients. Alternatively, it can be postulated that AVA5 cells (and/or the Huh7 parental cell line) lack the appropriate receptors, or have deficiencies in the intracellular response pathways, associated with these molecules.

The identification of the cellular processes altered in response to the various interferons has the potential to identify critical elements necessary for the intracellular maintenance of HCV. Some progress in the analysis of host gene expression in HCV replicon-containing cells treated with interferons and immune response modifiers has been reported that are consistent with generally accepted pathways of cellular responses to viral infections, including HCV (Davis et al., 1998; Stark et al., 1998; Goodbourn et al., 2000; Bigger et al., 2001; Cheney et al., 2002; Frese et al., 2001; Pflugheber et al., 2002; Su et al., 2002; Lanford et al., 2003). More effort in this area is obviously needed and the extension of these types of analyses to cells treated with combinations of agents that produce a variety of drug interactions should further assist in determining the factors that are critical for the maintenance/control of HCV replication. HCV replicon systems constitute a useful system to explore issues related to combination treatment which are expected to be required for long-term control of chronic hepatitis C virus infection.

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