NOTES

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

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Monotherapy of hepatitis C virus infection with either alpha interferon or ribavirin alone is rather ineffective, while the use of the two antivirals together is much more efficacious. In vitro drug-drug combination analysis utilizing related members of the family *Flaviviridae*, bovine viral diarrhea virus and yellow fever virus, revealed significant direct synergistic interactions between these drugs' antiviral activities that might explain this clinical observation.

Hepatitis C virus (HCV) is the most common cause of chronic hepatitis in the United States and represents a major risk factor for the development of liver cirrhosis and hepatocellular carcinoma (3, 19). Currently, HCV infections are treated with a combination therapy of alpha interferon (IFN- α) and the purine nucleoside analogue ribavirin (RBV; 1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide). Monotherapy of HCV-infected patients with RBV does not reduce viral loads or lead to sustained virologic responses (SVR) (4, 10, 11), while monotherapy utilizing IFN- α leads to SVR in approximately 20% of chronically infected HCV patients (16). The combination of IFN- α and RBV is much more effective clinically than either drug alone, leading to rates of SVR of approximately 40% (16).

The mechanism of action of IFN- α as an effective agent against HCV is uncertain, but both direct and immune-mediated mechanisms may be involved (5, 17, 24). It has been hypothesized that RBV may act indirectly as an antiviral agent against HCV through an enhancement of the immune response and/or act directly against HCV through its action as an IMP dehydrogenase inhibitor (40), as an inhibitor of the HCV RNA-dependent RNA polymerase NS5B (27), or as an RNA mutagen (8, 9, 21, 22, 35; reviewed in reference 14). Recently it was demonstrated that RBV has activity against HCV RNA replicons (22) and against HCV that was produced in a full-length binary expression system (7). Both of these studies found evidence that the antiviral effects of RBV could be ascribed to its mutagenic role, inducing error-prone replication. The mechanism of action of RBV is controversial (reviewed in references 14, 24, and 37). But since RBV has little if any direct activity against HCV in vivo (4, 10, 11), none of the hypotheses concerning the mechanism of action of the drug

adequately explains the known clinical synergy of action between the two drugs observed in HCV-infected patients (16). In order to ascertain the potential for direct synergy of antiviral effects between IFN- α and RBV, we performed a drug-drug combination analysis using related members of the family *Flaviviridae*.

The family *Flaviviridae* comprises three genera: *Hepacivirus*, *Pestivirus*, and *Flavivirus* (25). The structural organization of the genomes of representative members of the *Flaviviridae* is shown in Fig. 1. The genus *Hepacivirus* includes only HCV. Since there are no robust methods by which cultured cells can be infected with and replicate HCV virions, other methods are often employed in the study of anti-HCV agents. HCV RNA replicons (26; reviewed in reference 2) are robust systems that have shown utility in the evaluation of antiviral agents (6, 12, 15, 22). However, since replicons do not faithfully reproduce all steps of the HCV replication cycle, surrogate viruses are still widely utilized for the identification and characterization of anti-HCV agents. The type member of the genus *Pestivirus*, bovine viral diarrhea virus (BVDV), is often utilized for this purpose (1, 36, 41). The pestiviruses BVDV and HCV have similar genomic RNA structures, and both utilize an internal ribosome entry site (IRES) for translation initiation. Yellow fever virus (YFV) is the type member of the genus *Flavivirus*. This virus has also been employed as a model of HCV replication for the evaluation of antiviral agents (29). However, since its genomic structure is further removed phylogenetically from that of HCV than is that of BVDV, and because it uses a cap-dependent rather than an IRES-mediated mechanism of translation initiation, it is seldom employed individually as an in vitro model of HCV. Rather, YFV is often used in the evaluation of antiviral compounds in range-of-action studies. These studies are typically employed to see if compounds act specifically against HCV or BVDV or nonspecifically against multiple viruses, in order to ascertain whether the drug in question has a broad spectrum of activity against all *Flaviviri-*

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FIG. 1. Structural organization of the RNA genomes of representative members of the *Flaviviridae*. The diagram is based on the sequences of HCV-1 (GenBank accession number AF009606), BVDV NADL (AJ133738), and YFV 17D (X03700).

dae viruses. This is the case with both of the broadly acting antiviral compounds IFN- α and RBV, which have activity against both BVDV and YFV. It is probable that the mechanisms of action of these drugs against these *Flaviviridae* viruses are similar.

The antiviral effects of IFN- α (IFN α -2b; PBL Biolabs, New Brunswick, N.J.) and RBV (ICN, Costa Mesa, Calif.) were assessed in inhibition of cytopathic effect assays, (CPE) performed as previously described with human immunodeficiency virus (39) with minor changes. The assay involves the killing of cells by the cytopathic *Flaviviridae* members BVDV and YFV and the inhibition of cell killing by active antiviral compounds. We used BVDV (strain NADL; American Type Culture Collection [ATCC]) grown in Madin-Darby bovine kidney (MDBK) cells (ATCC), or YFV (strain 17D; ATCC) grown in Vero cells (ATCC). The MDBK cells were propagated in Dulbecco's modified essential medium (DMEM) containing phenol red with 10% horse serum, 1% glutamine (Glu) and 1% penicillin-streptomycin (PEN-STR), while the CPE inhibition assays were performed in DMEM without phenol red with 2% horse serum, 1% Glu, and 1% PEN-STR. The Vero cells were propagated in DMEM containing 10% fetal bovine serum, 1% Glu, and 1% PEN-STR, and the CPE inhibition assays were performed in DMEM without phenol red with 2% FBS, 1% Glu, and 1% PEN-STR. All medium components were from Invitrogen Life Technologies (Carlsbad, Calif.). The cells were grown at 37°C in a 5% CO₂ incubator. On the day preceding the assay, cells were trypsinized (1% trypsin-EDTA [Invitrogen Life Technologies]), pelleted (1,200 $\times$ g, 10 min), counted in a hemocytometer, and plated out at 10⁴/well in 96-well flat-bottom BioCoat plates (Fisher Scientific, Pittsburgh, Pa.) in a volume of 100 μ l per well using the appropriate growth medium. The next morning the medium was removed, and a pretitrated aliquot of virus was removed from the freezer (−80°C) and thawed immediately prior to use. The amount of virus used was the maximum dilution in the appropriate assay medium that would yield complete cell killing (>80% destruction of the cell monolayer) at the time of maximal CPE development. For BVDV, this was at day 7 postinfection, while maximal CPE development was seen in YFV at day 13 to 14. Cell viability was determined with CellTiter 96 reagent (Pro-

TABLE 1. Antiviral activity and cytotoxicity of IFN- α and RBV against BVDV and YFV

Virus	Treatment (n)	Mean $\pm$ SD		
		IC ₅₀	TC ₅₀	TI
BVDV	IFN- α (8)	16 $\pm$ 11 IU/ml	>5,000 IU/ml ^a	>310 $\pm$ 210 ^a
	Ribavirin (12)	9.8 $\pm$ 6.2 μ M	35 $\pm$ 13 μ M	4.7 $\pm$ 2.5
YFV	IFN- α (6)	110 $\pm$ 55 IU/ml	>5,000 IU/ml ^a	>45 $\pm$ 23 ^a
	Ribavirin (7)	210 $\pm$ 86 μ M	>410 μ M ^c	>2.0 $\pm$ 0.80 ^a

^a TC₅₀ was not reached.

mega, Madison, Wis.) according to the manufacturer's protocol, using a Vmax plate reader (Molecular Devices, Sunnyvale, Calif.). Each 96-well plate contained 12 wells with complete medium alone (medium control blanks), six cell control wells (cells only), and six virus control wells (cells plus virus). Drugs were tested at six concentrations each, diluted in assay medium in a half-log series. The highest concentration of IFN- α used was 5,000 IU/ml, while RBV was used at 82 μ M (20 μ g/ml) for the BVDV assays and at 410 μ M (100 μ g/ml) in the YFV assays. At each concentration there were one drug colorimetric control well (medium and drug), duplicate toxicity control wells (cells plus drug), and triplicate antiviral efficacy wells (cells and virus and drug). The average background- and drug color-corrected data for percent CPE reduction and percent cell viability at each drug concentration were determined relative to the cell controls and displayed graphically. An in-house computer program calculated the inhibitory drug concentration which reduced the CPE by 50% (IC₅₀) and the toxic drug concentration which caused the reduction of viable cell numbers by 50% (TC₅₀) automatically by interpolation from the plots. A therapeutic index (TI) for each active compound was determined by dividing the TC₅₀ by the IC₅₀.

A summary of the antiviral evaluation experiments is shown in Table 1. IFN- α showed good activity against BVDV (IC₅₀ = 16 $\pm$ 11 IU/ml). This value is somewhat higher than the IC₅₀ of 3 IU/ml that was reported previously when a plaque assay endpoint was utilized with BVDV (30). IFN- α was also active against YFV, but at higher concentrations (IC₅₀ = 110 $\pm$ 55 IU/ml). IFN- α was nontoxic to both MDBK and Vero cells at all concentrations tested, so a TC₅₀ was not reached. RBV had an IC₅₀ of 9.8 $\pm$ 6.2 μ M (2.4 $\pm$ 1.5 μ g/ml). This was lower than the IC₅₀ of 44.6 μ M reported by others for BVDV when a plaque assay endpoint was used (28) but higher than the IC₉₀ of 3.5 μ M for RBV that was reported when a reverse transcriptase PCR endpoint was used (36). We found RBV to have a TC₅₀ of 35 $\pm$ 13 μ M (8.6 $\pm$ 3.3 μ g/ml) in MDBK cells. One previously published study found no RBV toxicity at >500 μ M in confluent BT cells during a BVDV antiviral evaluation (28). However, Stuyver et al. recently found that RBV had a TC₅₀ of 5 μ M in exponentially growing MDBK cells in a BVDV antiviral assay and a TC₅₀ of >75 μ M when confluent cells were utilized (36). Thus, it is possible that differences in the state of confluence of the cells being assayed by different groups may explain these differences in RBV cytotoxicity. We determined that RBV had a narrow TI of 4.7 $\pm$ 2.5 in the BVDV assay, with only a slight margin between the concentration required for antiviral effects and that causing cytotoxicity. RBV was

FIG. 2. Effects of IFN- α and RBV combination on BVDV in MDBK cells. The calculated additive interactions were subtracted from the experimentally determined values based on average (mean) background- and drug color-corrected data to reveal the regions and corresponding drug concentrations at which synergistic or antagonistic interactions affecting antiviral activity (A and B) and drug cytotoxicity (C and D) 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- α 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 experiment was repeated five times with essentially identical results.

active against YFV, with an IC_{50} of $210 \pm 86 \mu M$ ($51 \pm 21 \mu g/ml$), but a TC_{50} was not reached at the concentrations tested ($TC_{50} > 410 \mu M$ [$>100 \mu g/ml$]). The RBV IC_{50} we observed with YFV is comparable to the previously published values of $46 \mu g/ml$ (20) and $28 \mu g/ml$ (29) also determined with inhibition-of-CPE assays, yet it was higher than the $8\text{-}\mu g/ml$ IC_{50} determined with a plaque reduction assay endpoint (18) or the IC_{50} of $>500 \mu M$ found in another study utilizing inhibition of CPE as an endpoint (28). RBV also failed to reach a TC_{50} in most published YFV antiviral evaluation studies (20,

28, 29), while one study found a TC_{50} for RBV of $63 \mu g/ml$ (18). It is possible that differences in the methodologies employed by these investigators may be responsible for these variations.

Drug-drug combination analysis for IFN- α and RBV was subsequently performed with average background- and color-corrected data by using the inhibition-of-CPE assays performed as above, with the MacSynergy II program used to perform the required calculations (31, 32; reviewed in reference 33). The experimental design utilized a checkerboard

FIG. 3. Effects of IFN- α and RBV combination on YFV in Vero cells. The calculated additive interactions were subtracted from the experimentally determined values based on average (mean) background- and drug color-corrected data to reveal the regions and corresponding drug concentrations at which synergistic or antagonistic interactions affecting antiviral activity (A and B) and drug cytotoxicity (C and D) 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- α 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 experiment was repeated four times with essentially identical results.

dilution matrix of the permutations of twofold serial dilutions of each of the drugs, including the drugs used individually. In the BVDV drug-drug combination analysis, the highest concentration of IFN- α used was 50 IU/ml, with RBV used at 82 μ M (20 μ g/ml). In the YFV combination analysis, we used 300 IU of IFN- α per ml as the highest concentration and 1,020 μ M (250 μ g/ml) RBV. The MacSynergy II program calculated the theoretical additive interactions of the drugs based on the Bliss Independence mathematical definition of expected effects for

drug-drug interactions. The Bliss Independence model is based on statistical probability and assumes that the drugs act independently to affect virus replication. The calculated theoretical additive interactions were determined from the dose-response curves of the individual drugs. The calculated additive surface, which represents the predicted additive interaction, was then subtracted from the observed surface to reveal regions of statistically significant greater-than-expected (synergy) or less-than-expected (antagonism) interactions. If the interactions

were additive, the resulting surface appeared as a horizontal plane at 0% above the calculated additive surface in the resulting difference plots. Peaks above this plane in the difference plots were indicative of synergy, while depressions below the horizontal plane indicated antagonism. The 95% confidence intervals for the experimental dose-response surfaces were used to statistically evaluate the data. For each point in the difference plot (itself based on replicate data as described above), if the lower 95% confidence interval limit of the experimental data was found to be greater than the calculated additive surface, the synergy at that concentration of IFN- α and RBV was considered statistically significant. Likewise, if the upper 95% confidence limit of the experimental data was less than the calculated additive surface, the antagonism at that concentration of IFN- α and RBV was considered statistically significant. A region of synergy or antagonism was indicated by a peak or depression composed of a cluster of adjacent cells significantly above or below zero in the difference plot. The volume of the peaks observed in the difference plots (in units of concentration times concentration times percent) was calculated by the program. This peak volume is the three-dimensional counterpart of the area under a dose response curve and is a quantitative measure of synergy or antagonism. Our assignment of the potential importance of the observed volumes of synergy or antagonism observed in the difference plots was based on the guidelines provided in the MacSynergy II user's manual (31) concerning the interpretation of the significance of peak volumes analyzed with 95% confidence intervals. The reproducibility of the peak volumes was assessed using the mean and sample standard deviation based on the results of repeat experiments. Contour plots (two-dimensional representations of the data) were also created from the data to allow for easier identification of the concentration ranges where statistically significant synergistic or antagonistic effects occurred.

Figure 2 shows a representative result of this drug-drug combination analysis obtained with BVDV. As can be seen in the 95% confidence interval difference plot (Fig. 2A) and contour plot (Fig. 2B), there was a synergy of antiviral effects between the two drugs. According to the guidelines found in the MacSynergy II user's manual (31), the size of the synergy volume [86 IU(μ g)/ml²%], derived from 95% confidence interval differential plots as described herein, indicates that this effect may be important in vivo. From an analysis of five independent experiments, we determined that this synergy volume was 66 ± 25 IU(μ g)/ml²% (mean $\pm$ standard deviation). A clear antagonism of antiviral effects [antagonism volume = -690 IU(μ g)/ml²%] between the drugs was also observed; however, this effect was seen only when high concentrations of RBV were employed. This antagonism volume averaged -940 ± 220 IU(μ g)/ml²% in five repeat experiments. Pharmacokinetic data have demonstrated that these concentrations of RBV are not reached in vivo in HCV-infected patients (13, 23, 34, 38). In the analysis of the effects of IFN- α on RBV cytotoxicity in MDBK cells (Fig. 2C and D), we found no significant effects. These experiments demonstrate a clear synergy between the activities of IFN- α and RBV against BVDV at physiologically relevant concentrations of the drugs.

The drug-drug combination analysis was also performed with YFV. Figure 3 shows a representative experiment. A nearly identical pattern was observed in the difference plot of

YFV as that found with BVDV. Figure 3A and B indicate a synergy of antiviral activity between the drugs [synergy volume = 79 IU(μ g)/ml²%] as well as a strong antagonism [antagonism volume = -480 IU(μ g)/ml²%] when the highest concentrations of both drugs were used. Four independent experiments were used to calculate an average synergy volume of 210 ± 120 IU(μ g)/ml²% and an average antagonism volume of -290 ± 150 IU(μ g)/ml²%. Again, based on the guidelines found in the MacSynergy II user's manual (31) concerning the analysis and interpretation of such results, the extent of the synergy volume indicates that this effect is probably important in vivo. While the large antagonism volume might indicate a similar importance for the antagonistic effect on antiviral activity, it again occurs only at the highest, physiologically irrelevant concentrations of the drugs (13, 23, 34, 38). As with BVDV, we found no significant synergy of drug cytotoxic effects (Fig. 3C and D). However, in contrast to the results observed with BVDV, we also found a reproducible antagonism of the cytotoxic effects of RBV against Vero cells by IFN- α [antagonism volume = -85 IU(μ g)/ml²%]. This antagonism was determined to be -330 ± 210 IU(μ g)/ml²% from four independent experiments. According to the MacSynergy II user's manual guidelines, the extent of this antagonism volume indicates that this effect may be important in vivo (31). But the relevance of this drug-drug interaction is uncertain, since this effect was apparent only at very high concentrations of RBV, which may not be relevant in vivo (13, 23, 34, 38).

In conclusion, we have found significant synergistic drug-drug interactions between IFN- α and RBV in terms of effects against both BVDV and YFV at physiologically relevant concentrations. These favorable drug-drug interactions observed with BVDV and YFV as surrogate models of HCV replication suggest that the known clinical synergy between IFN- α and RBV might be accounted for by the direct effects of the drugs on cells rather than through indirect immune-mediated mechanisms. This hypothesis is supported by the recent finding that IFN- α and RBV display synergistic antiviral effects against HCV RNA replicons in vitro (A. K. Yamaga, J. Choi, and J.-H. Ou, Abstr. 9th Int. Meet. HCV Relat. Viruses, abstr. 106, 2002; Y. Tanabe, N. Sakamoto, S. Maekawa, M. Kurosaki, T. Yamashiro, and N. Enomoto, Abstr. 9th Int. Meet. HCV Relat. Viruses, abstr. 95, 2002).

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ADDENDUM IN PROOF

The combination of IFN- α and RBV shows a similar pattern of synergy of antiviral effects, an antagonism of antiviral effects at high concentrations of RBV, and no effects on cytotoxicity in vitro with the flavivirus West Nile virus (J. B. Wells, S. Sloane, M. C. Maland, L. J. Chuvala, P. Fellows, A. Nalca, and T. G. Voss, Abstr. 103rd Gen. Meet. Am. Soc. Microbiol., abstr. T-015, 2003).

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