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Inhibitory Effects of Ribavirin Alone or Combined With Human Alpha Interferon on Feline Infectious Peritonitis Virus Replication In Vitro*

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ABSTRACT

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The antiviral activities of ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; virazole), either alone or in combination with recombinant human leukocyte (alpha) interferon (rHuIFN-α), were evaluated against feline infectious peritonitis virus (FIPV) in feline kidney-cell cultures. The 50% inhibitory dose (ID50) of ribavirin for uninfected, rapidly dividing cells was ~17 μg ml⁻¹ whereas the ID50 for FIPV was 2.5 μg ml⁻¹. The therapeutic index (TI) of ribavirin (i.e. the ratio of the minimum cell-toxic dose to minimum virus-inhibitory dose) was 6.8. Although a dose-dependent inhibition of viral infectivity occurred at non-toxic doses, maximum antiviral effects (>4 log₁₀ reduction in FIPV) occurred at cytotoxic doses.

When low or moderate doses of ribavirin were combined with either 10 or 100 U of rHuIFN-α ml⁻¹, the resulting antiviral effects were significantly greater than the sum of the observed effects from either ribavirin or rHuIFN-α alone. Significant synergistic interactions with rHuIFN-α occurred at ribavirin doses of 1, 5, 12.5 and 25 μg ml⁻¹. Synergistic combinations of rHuIFN-α and ribavirin produced up to an 80-fold or a 200-fold relative increase in FIPV antiviral activities compared with that produced by equivalent doses, respectively, of ribavirin or rHuIFN-α alone.

In cell growth studies, the addition of either 10 or 100 U of rHuIFN-α ml⁻¹ to test doses of ribavirin did not increase the antinecellular effect observed with ribavirin alone; seemingly, the potentiation of ribavirin antiviral activity by rHuIFN-α was independent of any additive cytoxic effects. Potentially, synergistic combinations of the two antiviral agents in vivo may decrease the therapeutic dose of ribavirin required for inhibition of FIPV and thus reduce drug toxicity.

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INTRODUCTION

There are presently no antiviral drugs that are known to be clinically effective against feline infectious peritonitis (FIP), a fatal immunopathologic disease of cats induced by a coronavirus, FIPV (Pedersen, 1986; Scott, 1986). Studies on the in vitro sensitivity of FIPV to antiviral agents are limited so far to evaluation of feline or human interferon (IFN) (Weiss and Toivio-Kinnucan, 1988). As recommended for other viruses (White and Fenner, 1986), a rational approach toward development of antiviral agents for clinical use in cats with FIP would be to screen potential compounds first in vitro (preferably in host species cells). Those agents which show a relatively high therapeutic index (TI; ratio of minimum cell-toxic dose to minimum virus-inhibitory dose) would then be selected for further testing, including toxicology and clinical efficacy studies.

In our preliminary in vitro studies, one of the more promising synthetic antivirals against FIPV was ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; virazole). Ribavirin, a nucleoside analogue related to guanosine, has demonstrated broad-spectrum virostatic activities against both DNA and RNA viruses, including herpes, orthomyxo, paramyxvo, arena and bunya viruses (Nicholson, 1984; Reines and Gross, 1988; Bryson, 1988). Due to potential toxicity, the clinical application of ribavirin in humans has been limited mostly to aerosol therapy of infants with serious respiratory syncytial virus infection or to intravenous or oral administration in adults with influenza A or acute hemorrhagic fever (e.g. Lassa, Junin, Machupo) virus infections (Reines and Gross, 1988; Bryson, 1988). Besides its broad-spectrum activity, a prominent attribute of ribavirin is the lack of development of viral resistance (Nicholson, 1984).

Although ribavirin showed strong activity against FIPV in our pilot studies, its cytotoxicity in feline cells and modest TI suggested toxicity when administered therapeutically to cats. Combinations of different antiviral drugs, notably human IFN and acyclovir, have produced additive or synergistic antiviral effects in vitro against human herpesviruses (Levin and Leary, 1981; Stanwick et al., 1981; Spector et al., 1982). It has been suggested that similar interactions in vivo might allow smaller doses of drugs to be used to decrease the potential adverse clinical effects and enhance efficacy (Bryson, 1988). We anticipated that combinations of ribavirin and human IFN-α, a drug which inhibits FIPV in vitro (Weiss and Toivio-Kinnucan, 1988), might show additive or synergistic inhibitory effects on FIPV and be useful clinically. The objectives of the present study were to document the antiviral activities of ribavirin against FIPV in vitro and to evaluate the potential additive or synergistic effects of ribavirin combined with human IFN-α on replication of FIPV in feline cell cultures.
MATERIALS AND METHODS

Cell culture

Crandell feline kidney (CrFK) cells (Crandell and Despeaux, 1959) were maintained in a growth medium (GM) consisting of minimum essential medium (Gibco). The GM was supplemented with 10% heat-inactivated fetal bovine serum, 100 U of penicillin ml⁻¹, 100 μg of streptomycin ml⁻¹, and 2.5 μg of amphotericin ml⁻¹ along with 2 mM L-glutamine and 1% non-essential amino acids. The cultures were incubated in a humidified chamber at 37°C in an atmosphere of air with 5% CO₂.

Virus

The virulent FIPV strain, FIPV-79-1146 (Pedersen et al., 1984) was obtained from Dr. N.C. Pedersen (University of California, Davis, CA) and was passaged seven times in CrFK in our laboratory. Viral suspensions were prepared from the supernatant fluids of thrice frozen-thawed cells harvested at 80% cytopathic effect (CPE) and were frozen and stored in 1-ml aliquots at −80°C until used. The infectivity titer of the FIPV stock was ~10⁻¹⁸ tissue-culture infectious doses (TCID₅₀) ml⁻¹.

Antiviral agents

The ribavirin (Virazole; Viratek Co; lot No. A030007) was initially stored lyophilized (1 g) at 4°C until diluted for use. A 5-mg ml⁻¹ stock solution of ribavirin was prepared in GM, sterile-filtered through a 0.2-μM cellulose acetate membrane (Nalgene), frozen, and then stored in 1-ml aliquots at −80°C for 1-4 weeks before use.

The recombinant DNA-derived (Bgl-II restriction endonuclease specified) human leukocyte (alpha) hybrid (Subtypes A and D) IFN (rHuIFN-α; Hoffmann-LaRoche, lot No. RO 23-1740) was a gift from Dr. R. Cordts (Roche Laboratories, Nutley, NJ) and was stored lyophilized [at a concentration of 50×10⁶ International Units (U) per vial] at −80°C until diluted in GM and sterile-filtered for use. Antiviral activity (U ml⁻¹) of the rHuIFN-α was determined in the manufacturer's laboratory, using an international reference standard for human leukocyte IFN.

Cell growth studies

The inhibitory effects of ribavirin, rHuIFN-α, or combinations on normal, rapidly dividing CrFK cells were evaluated, using a cell-proliferation assay described previously (Levin and Leary, 1981). Briefly, 25-cm² cell-culture flasks
(Corning) were seeded with ~5×10⁵ fresh trypsinized CrFK cells and the flasks were incubated at 37°C. At 24 h after seeding, when the cell monolayers were about 40–50% confluent, the medium (7 ml) was removed from each flask and replaced with an equal volume of either GM (cell controls), ribavirin, rHuIFN-α, or drug combinations. The cultures were incubated at 37°C and examined daily by phase-contrast light microscopy for cytopathic effects (CPE). At 72 h after drug exposure, triplicate control or drug-treated flasks at each inhibitor concentration were trypsinized, and the number of viable cells in each flask, determined by trypan blue exclusion, was counted in duplicate using a Neubauer hemocytometer.

Antiviral assays

The inhibitory effects of ribavirin alone or in combination with rHuIFN-α on FIPV replication were measured using a microtitration infectivity (CPE)-inhibition assay described previously for IFN (Weiss and Toivio-Kinnucan, 1988). For the antiviral assays of ribavirin, serial log₁₀ dilutions of FIPV were made in GM and 0.05 ml of each dilution added to triplicate wells of a 96-well microtitration plate (Flow) containing 24-h monolayer cultures of CrFK cells. After 1 h of viral adsorption at 37°C, the inoculum was removed and the wells replenished with 0.2 ml of GM only (virus controls) or GM containing different concentrations of ribavirin. The cultures were then incubated at 37°C and examined at 72 h for viral CPE. The 50% endpoint infectivity titers, expressed as the log₁₀ TCID₅₀ per 0.05 ml of FIPV, were calculated using the method of Reed and Muench (1938). The antiviral activity of ribavirin was determined as the log₁₀ difference in titer of the virus control and ribavirin-treated cultures. For the antiviral assays of rHuIFN-α, or combinations of ribavirin and rHuIFN-α, the identical procedure as described for ribavirin was followed, except that the rHuIFN-α (10 to 100 U ml⁻¹) was added to the cultures (0.2 ml per well) at 24 h before and 1 h after viral adsorption [alone or combined in some of the cultures with an equal (0.1 ml) volume of ribavirin]. Uninfected cultures exposed to doses of ribavirin, rHuIFN-α or combinations were included as drug toxicity controls.

Definition of interaction of drug combinations

In order to evaluate the combined effects of two antiviral drugs in cell culture, we adapted the criteria for drug combinations described previously by Valeriote and Lin (1975). The relative decrease of control titer (Yₐ) for an antiviral drug is defined as the reduction in virus titer produced in the presence of drug relative to the control without drug. Similarly, YₐB is the observed titer of control decrease in the presence of both drugs relative to control without
drugs. The calculated titer of control decrease ($Y_c$) for an additive interaction is the product of $Y_A$ and $Y_B$:

$$Y_c = Y_A \times Y_B$$

therefore, the $\log_{10} Y_c$ is equal to the algebraic sum of $\log_{10} Y_A$ and $\log_{10} Y_B$:

$$\log_{10} Y_c = \log_{10} Y_A + \log_{10} Y_B$$

Thus, for a synergistic interaction, $Y_{AB} > Y_c$. If $Y_{AB} < Y_c$, but greater than the most effective agent alone, the interaction is considered subadditive; if $Y_{AB}$ is less than the most effective agent alone, but greater than the least effective agent, interference; if $Y_{AB}$ is less than the least effective agent alone, antagonism. An interaction in which the inhibition produced is equivalent to the most effective agent alone is considered as indifference.

**Statistical methods**

Analysis of data was performed using a computerized statistical analysis program (Abstat; Anderson-Bell). Correlation between viable cell counts and ribavirin concentration was made by multiple linear regression analysis of $\log_{10}$ corrected mean cell values. Statistical tests of differences in mean FIPV titer reductions between combination drugs ($Y_{AB}$) and ribavirin alone and between $Y_{AB}$ and $Y_c$ (for synergistic interactions) were performed using the Student's $t$-test on paired samples (1-tailed). The Student's $t$-test also was used to compare mean cell numbers of untreated vs. drug-treated or ribavirin-treated vs. combination drug-treated cultures.

**RESULTS**

**Ribavirin toxicity in CrFK cells**

Compared with viable cell numbers in untreated CrFK cells, the numbers of cells in cultures treated with ribavirin at doses ranging from 1 to 100 $\mu$g ml$^{-1}$ were reduced (Fig. 1). The mean $\log_{10}$ cell numbers in ribavirin-treated cultures were related inversely ($r = -0.89; P < 0.001$) to drug concentration. The dose of ribavirin which inhibited 50% of mean control cell numbers in untreated cultures (50% inhibitory dose; ID$_{50}$) was $\sim 17$ $\mu$g ml$^{-1}$. Morphologically, mild toxic changes consisting of villous membrane projections and stranding of cytoplasm initially were observed at a dose of 50 $\mu$g ml$^{-1}$; these changes were moderate to marked at doses $\geq 100$ $\mu$g ml$^{-1}$ and were accompanied by rounding or detachment of cells and small gaps in the cell monolayer.
Fig. 1. The effect of ribavirin on growth of non-infected CrFK cells at 72 h. Results are expressed as the geometric mean ± SD log_{10} total cell counts of three identical 25-cm² cell-culture flasks each counted twice. The multiple linear regression formula is given. The mean log_{10} cell count in untreated CrFK cell cultures (n = 3) was 6.58 ± 0.02.

Fig. 2. The inhibitory effect of ribavirin at various concentrations on replication of FIPV at 72 h. Results are expressed as the geometric mean ± SD log_{10} decrease in mean titer (TCID_{50} per 0.05 ml) of virus control (untreated) cultures on the basis of three separate titrations. The log_{10} titer of virus control cultures was 4.67 ± 0.58 TCID_{50} per 0.05 ml.

Effect of ribavirin on replication of FIPV

Treatment of FIPV-infected cell cultures with ribavirin resulted in a dose-dependent inhibition of infectivity (Fig. 2). The ID_{50} of ribavirin for FIPV was approximately 2.5 µg ml⁻¹. The TI of ribavirin in FIPV-infected CrFK cells was 6.8 (17/2.5). Maximal inhibition of virus (≥ 4 log_{10} TCID_{50}) occurred at ribavirin doses of 50–100 µg ml⁻¹ or more; these inhibitory doses, however, often produced mild to moderate CPE in uninfected CrFK cells. Complete
inhibition of FIPV by ribavirin was not achieved at a multiplicity of infection (m.o.i.) ≥ 0.3.

Effect of ribavirin and rHuIFN-α combinations on FIPV

Since the higher antiviral doses of ribavirin were more potent that rHuIFN-α, the titer of control decrease observed with each combination of drugs (Y_AB) was evaluated statistically against the decrease in titer obtained by the ribavirin alone (Y_ribavirin). All combinations of ribavirin and rHuIFN-α tested were significantly (P < 0.01) more effective in inhibiting FIPV than were equivalent doses of ribavirin alone (Table 1). Except for the very lowest doses (1 μg of ribavirin ml⁻¹ combined with 10 U of rHuIFN-α ml⁻¹), all drug combinations were also more effective than equivalent amounts of rHuIFN-α alone.

For drug interactions, the observed decrease in FIPV titer of control for combinations of rHuIFN-α and ribavirin was significantly greater (P < 0.05) than the calculated decrease in titer for additive interactions:

\[ \log_{10} Y_{AB} > \log_{10} Y_{C} \] (Table 1)

Synergistic drug interactions occurred at doses of 10 U of rHuIFN-α ml⁻¹

| Concentration | FIPV⁻¹ | Log₁₀ Y_AB | (Log₁₀ Y_ribavirin) | Log₁₀ Y_C |
|---------------|--------|------------|---------------------|-----------|
| Ribavirin/rHuIFN-α (μg ml⁻¹) (U ml⁻¹) |        |            |                     |           |
| 1/10          | 0.9 ± 0.1* | (0.1 ± 0.1) | 1.0 ± 0.1           |
| 1/100         | 2.0 ± 0.3* | (0.1 ± 0.1) | 1.3 ± 0.3           |
| 5/10          | 2.2 ± 0.2* | (0.8 ± 0.3) | 1.7 ± 0.2           |
| 5/100         | 2.3 ± 0.2* | (0.8 ± 0.3) | 2.0 ± 0.2           |
| 12.5/10       | 3.2 ± 0.4* | (1.5 ± 0.3) | 2.4 ± 0.3           |
| 12.5/100      | 3.4 ± 0.1* | (1.5 ± 0.3) | 2.7 ± 0.6           |
| 25/10         | 3.8 ± 0.2* | (2.5 ± 0.3) | 3.4 ± 0.2           |
| 25/100        | 3.8 ± 0.2* | (2.5 ± 0.3) | 3.7 ± 0.2           |

\( Y_{AB} = \) relative decrease in FIPV titer (TCID₅₀ per 0.05 ml) observed at 72 h in the presence of both drugs relative to control without drug (mean ± SD of three separate titrations). \( Y_{C} = \) calculated decrease in titer of control for additive interaction \( (Y_{ribavirin} \times Y_{rHuIFN-α}) \), \( \log_{10} Y_{C} = \) the algebraic sum of the observed log₁₀ decrease in titer of control for the individual drugs \( (\log_{10} Y_{ribavirin} + \log_{10} Y_{rHuIFN-α}) \) (the titer of FIPV in the absence of drugs was 10⁴.₃ TCID₅₀ per 0.05 ml; the log₁₀ decrease in titer of control in the presence of either 10 or 100 U of rHuIFN-α ml⁻¹ was 0.9 or 1.2, respectively).

*Significant decrease in titer of control (combination vs. ribavirin alone), P < 0.01.

*Synergistic interaction \( (\log_{10} Y_{AB} > \log_{10} Y_{C}), P < 0.05 \).
TABLE 2

Effect of ribavirin, rHuIFN-α, and combinations on growth of uninfected CrFK cells at 72 h

| Ribavirin concentration (μg ml⁻¹) | Cell No.¹ with given rHuIFN-α dose |  |  |
|----------------------------------|------------------------------------|--|--|
|                                  | None                               | 10 U ml⁻¹ | 100 U ml⁻¹ |
| 0                                | 6.35 ± 0.06                        | 6.31 ± 0.04 | 6.25 ± 0.11 |
| 5                                | 6.25 ± 0.02*                       | 6.21 ± 0.11ᵇ | 6.15 ± 0.15ᵃᵇ |
| 12.5                             | 6.04 ± 0.30ᵃ           | 5.97 ± 0.10ᵃᵇ | 6.03 ± 0.05ᵃᵇ |
| 25                               | 5.99 ± 0.13ᵃ           | 5.96 ± 0.05ᵃᵇ | 5.86 ± 0.06ᵃᵇ |

¹Total cell count per 25 cm² cell-culture flask, expressed as the mean ± SD log₁₀ cell counts of three identical flasks each counted twice.

*Significant decrease in mean cell numbers vs. untreated cells, P < 0.05.

⁺Cell count in presence of both ribavirin and rHuIFN-α does not differ significantly from count with ribavirin alone, P > 0.2.

Combined with 5, 12.5 or 25 μg of ribavirin ml⁻¹ and at doses of 100 U of rHuIFN-α ml⁻¹ combined with 1 or 12.5 μg of ribavirin ml⁻¹.

Compared with equivalent doses of the individual drugs, the combination of ribavirin and rHuIFN-α at doses of 5 μg ml⁻¹ and 10 U ml⁻¹, respectively, produced a 20–25-fold increase in FIPV antiviral activities. Similarly, the combination of 12.5 μg of ribavirin ml⁻¹ and 10 or 100 U of rHuIFN-α ml⁻¹ provided a 50–80-fold increase in antiviral activities compared with the equivalent dose of ribavirin, and produced a 160–200-fold increase in activities compared with equivalent doses of rHuIFN-α. Except for a 10-fold increase in antiviral effect of 100 vs. 10 U of rHuIFN-α ml⁻¹ combined with 1 μg of ribavirin ml⁻¹, increasing the rHuIFN-α concentration from 10 to 100 U ml⁻¹ had minimal effect (0.1–0.2 log₁₀ increase) on the magnitude of viral inhibition produced by synergistic drug combinations.

Cytosuppressive effects of drug combinations

Ribavirin doses of 5, 12 or 25 μg ml⁻¹, either alone or combined with 10 or 100 U of rHuIFN-α ml⁻¹, inhibited the growth of uninfected, rapidly dividing CrFK cells (Table 2); the rHuIFN-α alone was not cytosuppressive. The addition of either 10 or 100 U of rHuIFN-α ml⁻¹ to test doses of ribavirin did not increase the anticellular effect produced by the ribavirin alone (P > 0.2).

DISCUSSION

Ideally, agents screened in vitro for antiviral activities against a particular virus should demonstrate significant inhibition of virus and minimum toxicity for normal host cells. Agents with a TI of at least 10, and preferably much
higher, are considered for further evaluation (White and Fenner, 1986). In the present study, we found that ribavirin produced a dose-dependent inhibition of FIPV and had an antiviral ID$_{50}$ of 2.5 µg ml$^{-1}$. Despite good antiviral activity, the drug was suppressive to normal, rapidly dividing feline cells at relatively low concentrations and had an anticellular ID$_{50}$ of only 17 µg ml$^{-1}$. Consequently, the calculated TI of ribavirin for FIPV (6.8) was somewhat smaller than the minimal value recommended to justify further testing. The reason for the relative sensitivity of feline cells to ribavirin was not clear. Ribavirin has a modest inhibitory effect in normal cells both on cellular DNA synthesis and also on capping of messenger (m)RNA (Sidwell, 1980; White, 1984). The drug is cytotoxic to resting cell lines in other species only at concentrations of 200–1000 µg ml$^{-1}$ (Sidwell, 1980). The relative toxicity of ribavirin in resting (vs. dividing) feline cells, however, was not determined in our study.

The precise mechanism(s) responsible for the antiviral actions of ribavirin are not well-defined and may differ for different groups of viruses (Dolin, 1985). Ribavirin (an analogue of guanosine) may exert its antiviral (and, to a lesser degree, anticellular) effect by reducing intracellular guanosine nucleotide pools, thereby inhibiting DNA and RNA synthesis. Ribavirin may also inhibit the enzyme mRNA guanylyltransferase (which is responsible for capping of viral mRNA), thereby interfering with translation and promoting susceptibility of viral mRNA to endonuclease (White, 1984).

Considering the antiviral activity of ribavirin observed by us against FIPV, we decided to investigate potential additive antiviral (and cytotoxic) effects of ribavirin combined with rHuIFN-α. Our rationale for testing the combination of rHuIFN-α and ribavirin was based on two findings. Firstly, synergistic effects of human IFN combined with antiviral agents such as acyclovir or ganciclovir (DHPG) have been reported against herpesviruses (Stanwick et al., 1981; Levin and Leary, 1981; Eppstein et al., 1985). Secondly, rHuIFN-α has antiviral activities against FIPV (Weiss and Toivio-Kinnucan, 1988). Unlike the antiviral mechanism of ribavirin, IFN produces an antiviral state in uninfected cells primarily by inducing an enzyme, 2,5 A polymerase, which eventually promotes the degradation of viral mRNA by endonuclease (Jacobsen, 1986). Potentially, combinations of drugs each with a different antiviral action (as in the case of ribavirin and rHuIFN-α) could enhance the sum of effects produced by each agent alone (i.e. drug synergism). Ideally, clinical efficacy would be increased and the adverse effects of the drugs reduced.

In the present study, dual combinations of ribavirin (even at doses as low as 1 µg ml$^{-1}$) and rHuIFN-α produced significant synergistic antiviral effects against FIPV in vitro; moreover, the antiviral effects of the combination were 80–200-fold greater than the effects of the drugs alone. The drugs themselves appeared to be compatible; i.e. the ribavirin did not antagonize or interfere with the antiviral state induced previously by rHuIFN-α and, conversely, the rHuIFN-α did not alter the subsequent action of ribavirin in infected cells.
The addition of rHuIFN-α to doses of ribavirin did not increase the anticellular effect of the ribavirin alone suggesting that enhancement of ribavirin activity by rHuIFN-α was independent of any additive cytotoxic effects.

Although antiviral doses of ribavirin and rHuIFN-α were used (in our study) in an in vitro virus inhibition assay, and the IFN was given both pre- and post-infection, it is still appropriate to consider clinical application of this drug combination in cats infected with FIPV. Generally, IFN has been considered to protect cells against viruses; it can still be active, although considerably less so, when given post-infection and may indeed act differently in virus-infected cells (Munoz and Carrasco, 1987). Significant antiviral activities of rHuIFN-α and synergistic interactions with acyclovir have been observed against feline herpesvirus when rHuIFN-α was given post-infection (Weiss, 1989). Also, additive effects of acyclovir and rHuIFN-β given post-infection against human cytomegalovirus have been reported (Spector et al., 1982). The reasons for the antiviral and synergistic or additive activities of IFN post-infection were not clear. Possibly, the IFN produced an antiviral state in uninfected cell populations (particularly in cultures infected at low m.o.i.); or, as described in murine retroviruses, it may have affected post-translational events, such as virus assembly or release (Billiau et al., 1973; Friedman and Chang, 1977). Potentially, the combined antiviral effects of ribavirin and rHuIFN-α could be further enhanced in vivo by certain immunomodulatory actions of IFN believed to be important in virus killing, such as activation of macrophages and increased cytotoxicity of natural killer cells and cytotoxic T-lymphocytes (White, 1984).

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