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Short Communication

# In vitro and in vivo enhancement of ddI activity against Rauscher murine leukemia virus by ribavirin

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**Abstract**

Ribavirin has been reported to enhance the activity of ddI against HIV. We explored this enhancement of antiviral activity in Rauscher murine leukemia virus (RMuLV) models in vitro and in vivo. The significant finding in these studies was that combinations of the drugs exhibited virus titer reductions that were greater than would be expected if the drug interactions were simply additive. These effects were designated synergistic by the method of Prichard and Shipman (Prichard, M.N. and Shipman, C., Jr. (1990) A three-dimensional model to analyze drug–drug interactions, Antiviral Res. 14, 181–206). In addition to the antiviral synergy, we also observed some synergistic toxicity in the animal model.

*Keywords:* Rauscher murine leukemia virus; Ribavirin; Dideoxyinosine; Enhancement

Ribavirin enhances the antiviral activity of purine nucleosides (Allen et al., 1982; Baba et al., 1987; Shannon, 1990; Balzarini et al., 1990; Bondoc et al., 1991). Perhaps of greatest significance is the enhanced activity of ribavirin in combination with the 2′,3′-dideoxypurine nucleosides, especially 2′,3′-dideoxyinosine (ddI) against human

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immunodeficiency virus (HIV). Recently, combination chemotherapy evaluations of ddI and ribavirin were initiated to assess the potential for enhancement of anti-HIV activity in man (AIDS Clinical Trials Group protocol 231). Previously, Balzarini et al. (1990) showed that treatment with ribavirin in combination with ddI (200, 100 and 50 mg/kg/day) prolonged the lives of infant mice infected with Moloney murine sarcoma virus (MSV). In that study only one dose of ribavirin (40 mg/kg/day) was combined with the ddI. The studies described here involve 3 doses of ddI and 3 doses of ribavirin, alone and in combination, in a checkerboard design using adult mice challenged with Rauscher murine leukemia virus. The studies also assessed the potential for combined toxicities of the compounds in adult animals as this information might reflect what could happen in humans. The antiviral effects of the drug combinations were evaluated using the Prichard and Shipman (1990) three-dimensional model for analyzing multi-drug interactions.

In vitro antiviral assays were performed by the UV-XC plaque assay developed by Rowe et al. (1970) and modified by Shannon et al. (1974) and Hollingshead et al. (1992). SC-1 cells were grown in 6-well tissue culture plates. After overnight incubation, triplicate wells were inoculated with 0.5 ml of virus and 2.0 ml of the drug solution (1.25 ×). On day 3 post virus inoculation, the cultures were irradiated with ultraviolet light and XC cells were added. On day 3 post UV irradiation, the cultures were fixed with 10% buffered formalin and stained with 0.1% crystal violet. The plaques were counted with the aid of a dissection microscope. For this combination experiment, 4 concentrations (4, 2, 1 and 0.5 $\mu$g/ml) of ddI were assayed alone and in combination with 4 concentrations (6, 3, 1.5 and 0.75 $\mu$g/ml) of ribavirin. Activity was measured as the percent reduction in plaques in the drug-treated samples compared to the virus controls. In addition, the supernatants were collected from the wells at day 3 and titrated for the amount of virus present to assess reductions in the virus titer. For virus titration, serial 1-$\log_{10}$ dilutions of supernatant were prepared in minimal essential medium (MEM) +5% fetal bovine serum. SC-1 cells were grown overnight and treated with 25 $\mu$g/ml of DEAE-dextran for 20 min, followed by a wash with PBS. The sample dilutions (0.5 ml) were placed on duplicate monolayer cultures of SC-1 cells in 6-well tissue culture plates and the virus was allowed to adsorb for 1 h. The samples were then removed and washed once with PBS to remove any residual drug that might have been in the supernatants. The plates were then incubated and processed as indicated above.

The antiviral effects of the drug combinations versus the antiviral effects of each drug alone were evaluated mathematically by the three-dimensional technique of Prichard and Shipman (1990). This approach identifies regions of greater than or less than expected antiviral activity as determined by the Bliss independence equation. Purely additive interactions would appear as a flat plane in three-dimensional space. Synergistic interactions are depicted as a peak over the region where synergy occurs with the height representing the intensity of the synergy at each point.

In the in vitro assay, ddI and ribavirin were evaluated alone and in combination. Ribavirin reduced plaque counts by percentages ranging from 45 down to $< 1\%$ and ddI reduced plaque counts by 42% down to 2%. The numeric data from the 95% confidence level synergy plot are presented in Table 1. Analysis of the results using the Prichard and Shipman system indicated a volume under the curve of 325 $\mu g^2\%$. In the Prichard

Table 1
The synergy plot: effect of ribavirin on the in vitro anti-Rauscher MuLV activity of ddI

| Ribavirin concentration ($\mu$g/ml) | ddI concentration ($\mu$g/ml) | | | | |
|---|---|---|---|---|---|
| | 0 | 0.5 | 1 | 2 | 4 |
| | Percent inhibition above expected | | | | |
| 6 | 0 | 17 | 36 | 27 | 13 |
| 3 | 0 | 16 | 8 | 23 | 22 |
| 1.5 | 0 | 14 | 11 | 29 | 47 |
| 0.75 | 0 | 6 | 0 | 25 | 31 |
| 0 | 0 | 0 | 0 | 0 | 0 |

and Shipman system, values greater than 100 $\mu g^2\%$ are indicative of strong synergy. Using percent plaque reduction as the parameter evaluated, the peak increase (47%) in plaque reduction over that expected for additivity occurred when 4 $\mu$g/ml of ddI was combined with 1.5 $\mu$g/ml of ribavirin. When virus titer reductions were evaluated, the peak reduction (2.3 $\log_{10}$) occurred at the maximum combined concentrations of 4 $\mu$g/ml ddI plus 6 $\mu$g/ml ribavirin. The points at which the peak synergy occurred are different for the plaque assay than for the titer reduction assay. This is due to the fact that the high concentration of each drug reduced plaque reduction by $> 40\%$ and consequently it was not possible to show appreciable synergy in the plaque assay at high concentrations. In contrast, in the titer reduction assay, neither drug reduced titers alone at the concentrations tested and the peak of synergy occurred at the high concentration of the two drugs together.

In the in vitro assay, neither drug alone was cytotoxic and combinations did not show increased cytotoxicity.

The RMuLV used in the in vivo study was prepared as a 10% suspension from pooled spleens of RMuLV inoculated NIH Swiss mice. The spleens were homogenized and the cell debris was removed from the supernatant by low speed centrifugation. The virus suspension was titrated in SC-1 cells by the UV-XC plaque assay method.

For the animal study, male NIH Swiss mice (16–18 g) were obtained from the NCI Animal Program. Groups of 5 uninfected mice were used to observe toxicity and groups of 10 mice were used in the antiviral evaluations. All animal studies were conducted in accordance with the USPHS guidelines. The virus was administered intravenously in a 0.1-ml volume, producing a challenge dose of 870 plaque forming units (PFU) per mouse. The ddI was administered subcutaneously (s.c.) 3 times per day at total doses of 600, 400, 200 mg/kg/day alone and in combination with 3 doses of ribavirin. The total doses of ribavirin (120, 90, 60 mg/kg/day) were also given s.c., alone as well as in combination, with the 3 doses of ddI. The compounds were administered s.c. daily starting 4 h post i.v. inoculation of virus on a 08.00, 16.00 and 24.00 h schedule for 21 days (0–20 days).

Parameters of infection which were compared included: spleen weight (weighed to the nearest 10 mg) and titers of RMuLV in serum samples collected on day 21. Serial 1-$\log_{10}$ dilutions of serum were prepared in MEM and assayed by the UV-XC plaque assay described above.

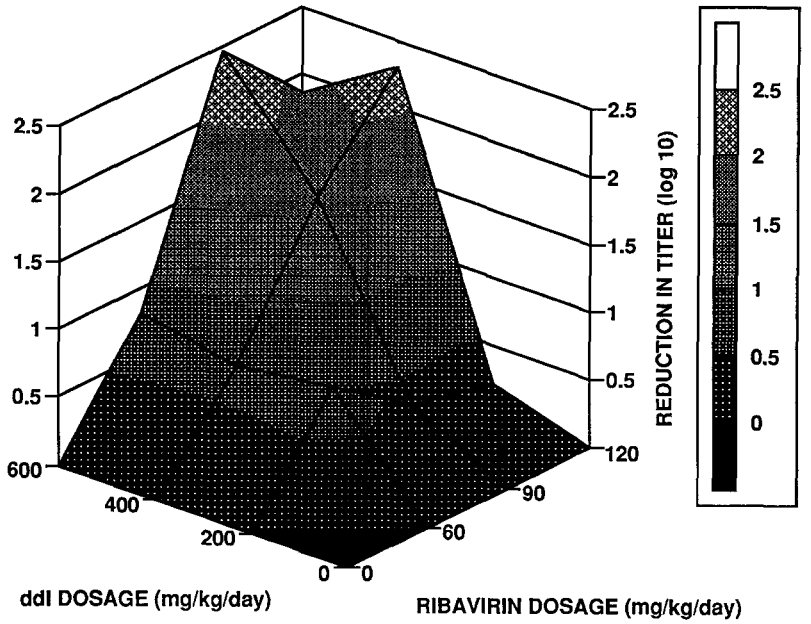
Table 2
Effect of ddI and ribavirin alone and in combination on serum virus titers of mice infected with Rauscher murine leukemia virus [a]

| Group | Titer ($log_{10}$ /ml) | Probability [b] |
|---|---|---|
| Diluent | 4.64 | |
| ddI (mg/kg) | | |
| 600 | 4.69 | > 0.05 |
| 400 | 5.08 | > 0.05 |
| 200 | 4.96 | > 0.05 |
| Ribavirin (mg/kg) | | |
| 120 | 4.51 | > 0.05 |
| 90 | 4.76 | > 0.05 |
| 60 | 4.46 | > 0.05 |
| ddI + ribavirin (mg/kg) | | |
| 600 + 120 | 2.66 | < 0.0005 |
| 600 + 90 | 2.31 | < 0.00005 |
| 600 + 60 | 3.66 | 0.043 |
| 400 + 120 | 2.74 | 0.015 |
| 400 + 90 | 3.56 | 0.089 |
| 400 + 60 | 4.11 | 0.160 |
| 200 + 120 | 4.61 | > 0.05 |
| 200 + 90 | 4.72 | > 0.05 |
| 200 + 60 | 4.27 | > 0.05 |

[a] The animals were inoculated i.v. with 870 PFU of RMuLV on day 0.
[b] Probability by Mann–Whitney *U*-test.

In analyzing the spleen weight reduction data, ddI alone marginally reduced spleen weights by 28, 7.3 and 10.5% at dosages of 600, 400 and 200 mg/kg/day. Ribavirin reduced spleen weights by 67, 56 and 48% at dosages of 120, 90 and 60 mg/kg/day. The various combination groups showed increased spleen weight reduction above the expected additive effects, but it was not appreciable and was not considered significant by the Prichard and Shipman (1990) technique.

When serum virus titrations were analyzed, ddI alone did not reduce titers. Ribavirin alone reduced titers only 0.1–0.2 $log_{10}$ units which is within the expected variation associated with the model. Therefore, the results indicated that neither drug alone was effective in reducing serum virus titers. In contrast, several of the drug combinations produced a 1.6–2.4 $log_{10}$ reduction in virus titer (Table 2). The Prichard and Shipman synergy plot is shown in Fig. 1 with its corresponding table. The figure is uncorrected for experimental variation, however statistical analysis showed 4 out of 9 points were greater than 1 standard deviation above the expected additive interactions. As shown in Table 2, the reductions seen with 600 mg ddI per kg/day and all 3 dosages of ribavirin were statistically significant using the Mann–Whitney *U*-test to compare the titers. In addition, the reduction seen with the 400-mg/kg/day dose of ddI with the high dose of ribavirin (120 mg/kg/day) was also statistically significant ($P < 0.05$).




| ddI Dosage (mg/kg/day) | Ribavirin Dosage (mg/kg/day) | | | |
|---|---|---|---|---|
| | 0 | 60 | 90 | 120 |
| | Reduction in Serum Titer ($Log_{10}$) Above Expected | | | |
| 600 | 0 | 0.82 | 2.47 | 1.86 |
| 400 | 0 | 0.68 | 1.64 | 2.30 |
| 200 | 0 | 0.78 | 0.36 | 0.21 |
| 0 | 0 | 0 | 0 | 0 |

Fig. 1. The antiviral synergy plot. Effect of ribavirin on the anti-Rauscher MuLV activity of ddI measured by reduction in serum virus titers.

All drug-treated groups gained weight throughout the treatment period although not quite to the same extent as the diluent control animals. In general, the animals appeared clinically normal, except the group receiving the high doses of ddI (600 mg/kg/day) and ribavirin (120 mg/kg/day) which became icteric by day 8. These animals did not appear ill or show any other signs of toxicity. Upon necropsy, all animals receiving ddI at 600 mg/kg/day in combination with all doses of ribavirin showed livers with altered color (brownish). In addition, the mice which received the 400-mg/kg/day dose of ddI and the 120-mg/kg/day dose of ribavirin also had discolored livers. These findings suggest that the synergistic antiviral activity occurred in the presence of synergistic toxicity.

Detection of synergy is dependent upon having drug combinations at proper ratios and concentrations. Furthermore, it is necessary that the drugs be distributed to the cells

targeted by the infecting virus and it is also necessary for the drugs to be metabolized by the target cells. In planning for the in vitro experiments, the high doses of each drug that were used were approximately the 50% inhibitory doses. In contrast, in the in vivo experiments, we selected doses based on information concerning both toxicity and activity. Doses exceeding the highest dose level that we used should have had greater antiviral activity, but limitations existed both in drug availability and drug tolerance by the animals. So in this experiment, we selected doses that we knew might not be active alone, but we also knew that those doses would not be toxic alone. If both drugs were metabolized in the infected cells, then synergy might occur. The problems we had selecting doses for the in vivo study would also be faced by clinical investigators planning human trials. Selection of the wrong doses or wrong ratios could result in lack of synergy. For example, if we had selected doses lower than 400 mg/kg/day of ddI, we would not have seen synergy.

As previously published by Streeter et al. (1973), Balzarini et al. (1991) and Bondoc et al. (1991), ribavirin inhibits inosine monophosphate (IMP) dehydrogenase and guanylate biosynthesis. This IMP dehydrogenase inhibition results in an increase in IMP pools and a decrease in guanosine triphosphate (GTP) pools. IMP is presumed to be the phosphate donor for the conversion of ddI to ddIMP by 5′-nucleotidase. Therefore, increasing IMP increases the amount of IMP available to serve as phosphate donor for this conversion. After the conversion of ddI to ddIMP, the ddIMP is converted to ddadenosine monophosphate (ddAMP) and the phosphorylation is continued to ddadenosine triphosphate (ddATP) which is the active molecule that inhibits the HIV reverse transcriptase. Presumably these interactions occurred in vivo in the above-mentioned adult mouse model, since neither ddI nor ribavirin was active alone, but combinations were significantly effective in lowering serum virus titers. As mentioned previously, this synergistic antiviral activity was accompanied by some liver toxicity. Nevertheless, it is possible that ribavirin in humans may enhance the ddI activity and reduce the amount of ddI that is necessary for anti-HIV activity.

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