Biological response modifiers in combination with antivirals against experimentally-induced virus infections

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RW SIDWELL, JH HUFFMAN, DF SMEE, et al. Biological response modifiers in combination with antivirals against experimentally-induced virus infections. Can J Infect Dis 1992;3(Suppl B):49B-54B. Biological response modifiers (BRMs) have particular promise when used in combination with more standard antiviral agents for treatment of viral diseases. Reported here are a series of studies which have used two BRMs in combination with the antiviral drug, ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) in treatment of experimentally-induced phlebovirus (Punta Toro virus) infections in mice. The positive BRMs studied include bropirimine (2-amino-5-bromo-6-phenyl-4[3H]pyrimidinone) given orally at dosages of 25, 50 and 100 mg/kg/day beginning 24 h after virus inoculation, and 7-thia-8-oxoguanosine administered intraperitoneally at dosages of 6.3, 12.5 and 25 mg/kg/day given 24 and 31 h after virus inoculation. In each experiment, multiple dosages of both BRM and ribavirin were selected to range from ineffective levels to, in certain cases with ribavirin, lethally toxic levels. Ribavirin was always administered orally twice daily for three days starting 24 h after virus inoculation. Both drug combinations were considered synergistic, increasing the therapeutic index compared to either drug used alone, and significantly reducing the evidence of ribavirin toxicity. Efficacy was seen as increased survivors, decreased virus recovery from tissues and blood, and lowered glutamic oxalic and pyruvic transaminase levels in the serum.

Key Words: *7-thia-8-oxoguanosine, Antiviral, Bropirimine, Immunomodulator, Phlebovirus, Punta Toro virus, Ribavirin*

Modificateurs de la réponse biologique en association avec des antiviraux contre des infections virales induites expérimentalement

Les modificateurs de la réponse biologique (MRB) sont particulièrement prometteurs lorsqu'utilisés en association avec des agents antiviraux classiques dans le traitement des maladies virales. Sont présentées ici des études au cours desquelles deux MRB ont été utilisé en association avec l'antiviral Ribavirine (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) dans le traitement d'infections à phlebovirus (virus Punta Toro) induites expérimentalentent chez la souris. Parmi les MRB positifs étudiés, notons la bropirimine (2-amino-5-broino-6-phényl-4[3H]pyrimidinone) administrée par voie orale à raison de 25, 50 et 100 mg/kg/jour débutant 24 heures après l'inoculation du vi,rus, et de la 7-thia-8-oxoguanosine administrée par voie intrapéritonéaley à raison de 6,3, 12,5 et 25 mg/kg/jour, 24 et 31 heures après l'inoculation du virus. Dans chaque expérience, des posologies multiples des deux MRB et de la Ribavirine furent sélectionnées. Elles variaient d'inefficaces à toxiques. Elles étaient même potentiellement fatales dans certains cas avec la Ribavirine. La ribavirine a été administrée par voie orale, deux fois par jour, durant 3 jours, 24 heures après l'inoculation du virus. Les deux associations médicamenteuses furent synergiques. L'indice thérapeutique était augmenté par comparaison avec l'un ou l'autre des médiciments utilisé seul. L'on nota également une réduction significative des signes da toxicité associés à Ribavirine. Le nombre plus élevé de survivants, la quantité moindre de virus prélevée dans les tissus et le sang et des taux abaissés de transaminases glutamiques oxaliques et pyruviques sériques suggerent que le traitement était efficace.

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THE NUCLEOSIDE ANALOGUE 1-β-D-RIBOFURANOSYL-1,2,4-triazole-3-carboxamide (ribavirin) is significantly inhibitory to a broad spectrum of virus infections (1,2). However, ribavirin is known to be associated with various adverse effects. Toxicity, primarily manifested as anemia, is seen at doses usually only slightly exceeding those needed for therapeutic effects (3), which thus reduces the compound's therapeutic index and potential usefulness.

One approach to enhance the antiviral efficacy of ribavirin and possibly to reduce the toxicity of the drug is to use it in combination with other substances. Biological response modifiers (BRMs), by virtue of their immunomodulatory properties, appear ideal candidates for such combination chemotherapy studies. Experimental in vivo antiviral studies with combinations of other nucleoside analogues and BRMs have shown definite synergy. These positive interactions include inhibition of type 2 herpesvirus infections in mice by combinations of vidarabine and vidarabine 5′-monophosphate plus alpha-interferon (IFNα) (4) and vidarabine plus acyclovir plus poly (ICLC) (5), and inhibition of murine retrovirus infections by 3′-azido-3′-deoxythymidine plus IFNα (6). Of particular pertinence to the present manuscript is the report by Kende et al (7) which showed enhanced therapeutic efficacy against Rift Valley fever virus infections in mice using the combination of ribavirin and poly (ICLC).

This report describes the efficacy of combinations of ribavirin and two distinctly different BRMs: bropirimine (2-amino-5-bromo-6-phenyl-4[3H]pyrimidinone) and 7-thia-8-oxoguanosine (TOGuo), against experimentally induced phlebovirus (Punta Toro virus [PTV]) infections of mice. Ribavirin has previously been shown to inhibit markedly the virus infection (8), and both bropirimine and TOGuo have also exerted strong activity against this virus infection when used alone (9-11).

MATERIALS AND METHODS

Virus: The Adames strain of PTV was used, which has been described previously (8). The virus was originally isolated from a patient in Panama. It was twice plaque purified, pools made, and the virus titrated in cells and in mice.

Animals: Specific pathogen-free female C57BL/6 mice weighing 10 to 12 g were obtained from Simonsen Laboratories (California). They were quarantined 24 h prior to use, housed five or 10 to a cage, and fed Wayne Laboratory Chow and tap water ad libitum.

Test compounds: Ribavirin, bropirimine and TOGuo were provided by the United States Army Medical Research Institute for Infectious Diseases. Ribavirin was prepared in sterile physiological saline. Bropirimine was suspended in 0.4% carboxymethylcellulose (CMC). TOGuo was dissolved in 2% sodium bicarbonate in water at pH 8.6 to 8.9.

Virus titrations: Liver homogenates (10% weight per volume) and serum were assayed for PTV titre by adding 0.1 mL of varying 10-fold dilutions to triplicate cups of Rhesus monkey kidney (LLC-MK2) cell monolayers in 96-well microplates. Viral cytopathic effect was determined after six days of incubation at 37°C, and tissue culture 50% infective dose endpoints were determined (12).

TABLE 1
Treatment regimens for drug combination studies in the Punta Toro virus animal model

Number	Compounds tested	Treatment route	Beginning of treatment	Schedule
1	Ribivarin +	Oral	+ 24 h	bid x3
	bropirimine	Oral	+ 24 h	qid x3
2	Ribivirin +	Oral	+ 24 h	bid x3
	TOGuo	IP	+ 24 h, + 31 h	bid x1

IP Intraperitoneal

Serum transaminase determinations: Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were assayed using colorimetric kits from Sigma Chemical Co (Missouri). Spectrophotometric readings for these assays were performed in duplicate using an automated microplate reader (EL309; Bio-Tek Instruments, Inc, Vermont).

Experiment design: Two objectives were sought in these studies. The first was to determine if BRMs would reduce the usual lethal or near-lethal toxicity of high doses of ribavirin. The second was to ascertain if BRMs would enhance the anti-PTV effect of inactive or marginally active doses of ribavirin. Thus, one or two usually lethal doses of ribavirin, as well as several low doses of the drug were used in these studies. Two drug combinations were studied: Ribavirin plus bropirimine, and ribavirin plus TOGuo (Table 1). Five to six experiments were run in parallel, according to the following general scheme.

Ribavirin at four or five dosages: a lethally toxic dose and three or four marginally PTV-active or inactive dosages, ie, dosages that have previously been reported (8) to have moderate to no inhibitory effects on the PTV infection. Since a variety of disease parameters were used, these dosages usually did not prevent death but may reduce AST, ALT or tissue virus titres.

- The immunomodulators bropirimine or TOGuo at three or four doses ranging from active to inactive against the PTV infection.
- Ribavirin at all doses used in the first experiment plus one of the immunomodulators used at the highest dose only.
- Ribavirin at all doses used in the first experiment plus one of the immunomodulators used at the mid-dose only.
- Ribavirin at all doses used in the first experiment plus one of the immunomodulators used at the low dose only.

TABLE 2
Effects of combination therapy with ribavirin and bropirimine on Punta Toro virus infections in mice (combination 1)

Ribavirin (mg/kg/day)	Bropirimine (mg/kg/day) 0	25	50	100	0	25	50	100
	Survivors/Total				Mean liver score†			
0	0/20	2/10	9/10**	10/10**	3.7	3.4	0.4**	0.2**
3.1	0/10	**7/10****	8/9**	10/10**	3.0	**2.6**	**0.1****	0.5**
6.3	0/10	0/10	**10/10****	10/10**	3.7	**2.6**	**0.1****	0.4**
12.5	2/10	1/10	**10/10****	10/10**	3.8	**1.2****	**0.3****	0.4**
25	9/10**	**10/10****	**10/10****	10/10**	1.1**	1.4**	**0.0****	0.3**
50	10/10**	9/9**	10/10**	10/10**	0.1**	0.1**	**0.0****	**0.0****
1200	7/10**	**10/10****	**10/10****	10/10**	0.3**	**0.1****	**0.0****	0.4**
	Mean liver virus titre‡				Mean serum virus titre‡			
0	6.2	5.4	0.5**	0.4**	6.4	5.7	0.5**	0.7**
3.1	6.2	**3.7****	2.1**	0.4**	5.0	**4.3****	0.7**	**0.4****
6.3	6.4	**4.9**	**0.0****	1.1**	6.5	**4.5****	**0.0****	1.6**
12.5	5.9	**2.7****	1.8**	0.9**	6.3	**2.6****	**1.6****	**0.3****
25	2.6**	4.9**	**0.0****	1.0**	2.8**	5.2**	**0.0****	**0.0****
50	0.0**	0.3**	0.8**	0.0**	0.3**	**0.0****	**0.2****	**0.0****
1200	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**	0.2**	0.0**
	Mean AST activity§				Mean ALT activity§			
0	11,794	3790**	189**	94**	10,960	3290**	205**	66**
3.1	12,902	**247****	236**	120**	11,281	**719****	**142****	**64****
6.3	14,383	**206****	**93****	529**	12,072	**747****	**51****	547**
12.5	12,570	**52****	**158****	**90****	10,610	**174****	**155****	**26****
25	2179**	**204****	**115****	**81****	2033**	**812****	**36****	**21****
50	262**	**21****	**74****	**75****	170**	**24****	**27****	**14****
1200	205**	**118****	**61****	148**	30**	37**	**15****	36**

*Bold-faced data indicate an improvement over either drug used alone at the dosages indicated; †Scores assigned to livers removed on day 3 post virus inoculation; ‡Geometric means (log_{10} $CCID_{50}$/mL) determined on liver serum samples taken on day 3 post virus inoculation; §Sigma-Fraenkel unilt/mL of serum samples taken on day 3 post virus inoculation; **P<0.01*

In each experiment, 20 mice infected subcutaneously with 10^5 plaque forming units (an approximately 95% mouse lethal dose) of PTV were treated with each drug dose or one combination of each drug, and 40 infected mice were treated with the appropriate vehicle. Five sham-infected mice at each drug dosage served as toxicity controls, and 10 additional animals were used as normal controls. The uninfected, treated animals and normal controls were weighed immediately prior to initial treatment and again 18 h following final treatment. On infection day 3, mice from one cage (10 animals) of each treatment group, one cage (five mice) of normal controls, and two cages of virus controls (20 mice) were anesthetized, bled, and their livers removed. Hepatic icterus, characterized by liver discoloration, was assigned a score of 0 (normal) to 4 (maximal discoloration). The livers were frozen at –80°C, and later thawed and assayed for infectious virus. The serum was frozen at –80°C and later thawed and assayed for AST and ALT levels and for virus titre. Animals not killed on day 3 were observed for death through 21 days post virus inoculation.

Statistical evaluations: Survivor increases were evaluated using χ^2 analysis with Yates' correction. Increases in mean survival time of animals dying before day 21 and reductions in AST, ALT, and virus levels in liver and serum were analyzed using the *t* test. Liver score inhibition was compared with ranked sum analysis.

Drug combination effects were evaluated using fractional inhibitory concentration (FIC) indices as described by Berenbaum (13) to determine if the drug interactions were synergistic, additive, or antagonistic. The FIC was determined by the formula:

$$FIC = \frac{MIC \text{ of drug A in combination}}{MIC \text{ of drug A alone}} + \frac{MIC \text{ of drug B in combination}}{MIC \text{ of drug B alone}}$$

The interpretation of the FIC values was: FIC less than 0.5 equalled synergism; FIC 0.5 to 0.9 equalled suggestive synergism; FIC approximately 1 equalled additive; FIC 1.1 to 1.9 equalled partial antagonism; FIC 2 or more equalled antagonism.

RESULTS

Ribavirin plus bropirimine: Treatment combination of ribavirin and bropirimine is summarized in Table 2. Ribavirin was essentially inactive against the PTV infection when used at dosages of 12.5 mg/kg/day or less. Bropirimine was significantly effective at the 50 and 100 mg/kg/day dosages. At the lowest dose, 25 mg/kg/day, only AST and ALT levels were significantly affected. Treatment with the drug combination was considered strongly synergistic using all parameters (Table 3).

TABLE 3
FIC indices for drug combinations evaluated against Punta Toro virus infections in mice

Combination	Evaluation parameter	FIC index	Mean FIC	Interpretation
1	Survivors	≤0.6	≤0.6	Synergistic
Ribavirin +	Liver score	≤0.6		
bropirimine	Liver virus	≤0.6		
	Serum virus	≤0.6		
	AST	≤0.6		
	ALT	≤0.6		
2	Survivors	1.7	0.6	Synergistic
Ribavirin +	Liver score	0.6		
TOGuo	Liver virus	0.2		
	Serum virus	0.4		
	AST	0.6		
	ALT	0.4		

ALT Alanine aminotransferase; AST Aspartate aminotransferase; FIC Fractional inhibitory concentration

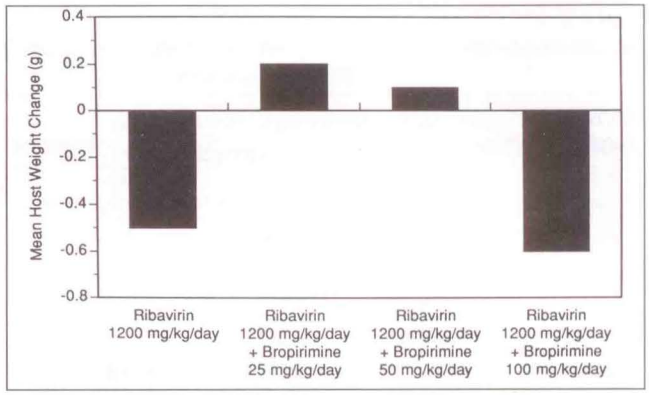

Figure 1) *Effects of bropirimine treatment on ribavirin-indiced weight loss in uninfected mice*

Ribavirin's toxicity at 1200 mg/kg/day was manifested in this experiment as significant weight loss (Figure 1). When toxicity control mice were treated with both ribavirin and bropirimine, this host weight loss was not observed except at the highest dosage of bropirimine (Figure 1). In this experiment, normal controls gained 1.4 g during the treatment period, and toxicity control mice receiving bropirimine also gained weight as follows: 1.6 g at the 25 mg/kg/day dose, 0.5 g at the 50 mg/kg/day dose, and 2.2 g at the 100 mg/kg/day dose.

Ribavirin plus TOGuo: The second combination study, which used ribavirin plus TOGuo, is summarized in Table 4. In this study, viral challenge was inadvertently less than in the previous combination experiment. This resulted in a 25% survival occurring in the placebo-treated infected mice, and the other disease parameters were somewhat reduced compared with the other studies. Antiviral effects of both ribavirin and TOGuo were readily demonstrated; with the ineffective doses of ribavirin being similar to the other experiments. TOGuo was effective using all disease parameters at the 25 mg/kg/day dosage only; the 12.5 mg/kg/day dose significantly prevented death but was not considered significantly inhibitory to the disease using the other disease parameters. The 6.3 mg/kg/day dose of TOGuo was ineffective when used alone. Treatment with the drug combination resulted in increased disease reduction using all parameters. A calculation of FIC indices suggested the drug combination was synergistic (Table 3).

TABLE 4
Effects of combination therapy with ribavirin and TOGuo on Punta Toro virus infections in mice (combination 2)

Ribavirin (mg/kg/day)	TOGuo (mg/kg/day) 0	6.3	12.5	25	0	6.3	12.5	25
	Survivors/Total				Mean liver score†			
0	5/20	2/10	8/10**	8/10**	2.4	2.9	2.0	0.0**
6.3	1/10	0/10	**9/10****	**9/9****	2.8	**2.3**	**0.7**	0.7**
12.5	0/10	**5/10**	7/9**	**10/10****	3.2	**2.5**	2.7	0.1**
25	9/10**	**10/10****	**10/10****	**10/10****	1.4**	**0.5****	**0.5****	0.2**
1250	2/10	**10/10****	**9/9****	**10/10****	0.1**	0.2**	**0.0****	0.2**
	Mean liver virus titre‡				Mean serum virus titre‡			
0	3.8	3.4	2.9	0.0**	4.1	4.1	3.1	0.0**
6.3	5.2	**2.9**	**1.7***	0.3**	5.9	**3.4**	**2.4****	0.4**
12.5	5.3	4.3	**2.7**	1.2**	6.1	5.6	3.2	0.0**
25	2.4	**0.8****	**0.3****	0.0**	2.0**	**0.2****	**0.0****	0.0**
1250	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**
	Mean AST activity§				Mean ALT activity§			
0	753	1196	742	114**	863	1373	825	73**
6.3	4887	**1135**	**488**	201**	5810	**1149**	**417***	136**
12.5	4872	3270	**708**	199**	5531	3925	**609**	153**
25	741	**334****	**231****	**112****	734	**274****	**159****	**51****
1250	140**	215**	260**	169**	25**	51**	51**	29**

*Bold-faced data indicate an improvement over either drug used alone at the dosages indicated; †Scores assigned to livers removed on day 3 post virus inoculation; ‡Geometric means (log_{10} $CCID_{50}$/mL) determined on liver serum samples taken on day 3 post virus inoculation; §Sigma-Fraenkel unit/mL of serum samples taken on day 3 post virus inoculation; *P<0.05; **P<0.01*

The combination treatment also appeared to lower ribavirin's lethal toxicity, when the highest dose of TOGuo was used with the 1250 mg/kg/day dose of ribavirin (Table 5). The mice used in this experiment were approximately two days younger than those used in the bropirimine study, which may account for the apparent slight increase in ribavirin's toxic effects in this experiment.

TABLE 5
Reduction of ribivarin-induced murine toxicity by TOGuo therapy

Drug	Dose (mg/kg/day)	Survivors (%)
Ribavirin	1250	0
	6.3 to 25	100
TOGuo	6.3 to 25	100
Ribavirin + TOGuo	1250 + 6.3	0
	1250 + 12.5	0
	1250 + 25	80**
	6.3 to 25 + 6.3 to 25	100

***P<0.01 versus ribavirin alone*

DISCUSSION

Ribavirin is known to be immunosuppressive at high dosage levels. This has included suppression of the primary immune response in mice to sheep red blood cells (14), reduction of serum antibodies to various virus challenges (15), moderate inhibition of cellular immune response to EL-4 tumour cells (16), reduced guinea pig contact hypersensitivity to dinitrochlorobenzene (16), and inhibition of adjuvant-induced arthritis in rats (17). In contrast, the immune modulators used in this study have significant immunostimulatory properties which may be reversing ribavirin's high dosage toxicity by neutralizing the immunosuppression induced by ribavirin. It has been observed, however, in separate unpublished studies that mice dying from high dose ribavirin therapy exhibit significant bleeding into the small and large intestine. Such observations suggest an adverse effect on capillary walls which may not be related directly to the drug's immunosuppressive effects. it is not clear what role the immunomodulators used in the present study would have in reversing this capillary wall breakdown.

Bropirimine is a recognized IFN inducer (18) which has been shown previously to be active used alone against in vivo PTV infections (10). This material also activates macrophages, augments natural killer cell cytotoxicity, induces polyclonal B cell response, interleukin-1 and interleukin-2, enhances antigen-mediated antibody formation, and stimulates bone marrow proliferation (19). Pifat and Smith (20) have shown PTV to be highly sensitive to IFN induction, so it is probable that bropirimine's anti-PTV effects are primarily a result of the material's rapid IFN induction.

The anti-PTV activity of TOGuo has been described (11). This compound stimulates B cell blastogenesis, activates natural killer cells and macrophages, enhances antibody-dependent cell cytotoxicity, induces tumour necrosis factor, and induces IFN (21). Smee et al (11) have shown that the anti-PTV effects of TOGuo are completely eliminated by concomitant treatment of PTV-infected mice with anti-IFN antibody, indicating that the PTV disease inhibitory effects of this compound are also closely related to its IFN induction.

Ribavirin is currently being used clinically in the United States for treatment of severe respiratory syncytial virus infections in young children. The compound is administered in a small particle aerosol to enhance its antiviral efficacy and to lessen any potential toxic effects. The drug has been reported to be a strong inhibitor of phlebovirus infections such as those induced by PTV, Rift Valley fever virus and sandfly fever virus (8,22,23). These infections are primarily hepatotropic, which probably require parenteral application of the drug, hence increasing the possibility of toxicological effects being manifested.

Both Rift Valley fever and sandfly fever are serious diseases of Africa and the Middle East. Severe epidemics of Rift Valley fever have been known since 1930 through much of Africa. An outbreak occurred in Sudan in 1976, with the disease apparently spreading to Egypt in the next two years with an estimated 200,000 human cases and over 600 deaths (24). During World War II, sandfly fever was a serious affliction of troops in the Mediterranean area, with 3 to 10% of all troops infected at the same time and attack rates of over 50% reported in some units (25). Both diseases are insect transmitted. PTV was used in the present studies since it is closely related to both Rift Valley fever and sandfly fever viruses, is also transmitted by biting insects, and induces a disease in mice very similar to that induced by Rift Valley fever; importantly, it causes a much less severe disease in humans (20). In addition, PTV is not readily transmitted in the laboratory. Similar sensitivities to antiviral compounds have been seen against all three viruses (8,22,23,26).

These data indicate that use of immunomodulators in combination with the moderately selective antiviral drug ribavirin appear to be useful both in increasing the antiviral efficacy of all compounds employed and in lessening the toxicity of ribavirin.

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