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Antiviral and immunomodulating inhibitors of experimentally-induced Punta Toro virus infections ☆

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Abstract

A major component of a US Army Medical Research and Development Command-supported program to discover and develop new drugs for the treatment of Rift Valley fever, sandfly fever, and Crimean-Congo hemorrhagic fever has been to study candidate test materials against hepatotropic infections of C57BL/6 mice induced by the related but less biohazardous Punta Toro virus (PTV). The effects of 75 compounds, some of which were considered immunomodulators in their primary mechanism of activity, were studied in the PTV infection model. Of these, ribavirin, ribamidine, ribavirin 2′,3′,5′-triacetate, tiazofurin, tiazofurin-5′-monophosphate, tiazofurin-2′,3′,5′-triacetate, selenazofurin, pyrazofurin, 3-deazaguanine, and 3-deazaguanosine were considered significantly inhibitory, acting against the infection by a direct antiviral (non-immunomodulatory) fashion. These compounds had therapeutic indices (TI) ranging from ≥ 5 to 65, using increased survivors as the evaluation parameter. Immunomodulators considered significantly inhibitory to this infection were poly (ICLC), ampligen, human recombinant interferon-α-A/D, MVE-1, MVE-2, AM-3, AM-5, mannozym, bropirimine, CL246,738, phenyleneamine, and 7-thia-8-oxoguanosine. Utilizing increased survivor numbers as measure of activity, these inhibitors had TI ranging from ≥ 16 to 1000. Other antiviral effects exerted by the active compounds included reduction of hepatic icterus, lowered serum glutamic oxaloacetic and pyruvic acid transaminases,

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and inhibition of recoverable serum and liver virus titers. The active immunomodulators were significantly effective when therapy was initiated as late as 48 h after virus inoculation, at a time when clinical signs of the PTV disease were being manifested in the animal.

Keywords: Punta Toro virus; Antiviral; Ribavirin analog; Immunomodulator

1. Introduction

Several viruses in the Bunyaviridae family are significant threats to man. These include the phleboviruses Rift Valley fever (RVFV), sandfly fever (SFV), and Crimean-Congo hemorrhagic fever (CCHFV). Severe epidemics of RVFV have been reported since 1930 throughout much of the African area. An outbreak occurred in Sudan in 1976 with the disease spreading to Egypt in 1977–78 which resulted in an estimated 200 000 human cases and at least 600 deaths (Meegan, 1979; Meegan et al., 1981). The disease continues to occur in Egypt (Anon, 1993). In the last 10 years, there have been outbreaks in the sub-Saharan Africa, the most recent being an ongoing epidemic in Mauritania (Walsh, 1988). The disease induced by SFV is widespread in the Middle East. Approximately 19 000 members of the Allied Armed Forces stationed in that area in World War II were sufficiently afflicted with SFV infections to require hospitalization (Hertig and Sabin, 1964; Sabin, 1948). Oldfield et al. (1991), in a recent review, emphasized the potential for SFV infections as a significant factor in a prolonged Gulf War conflict. The infection induced by CCHFV is becoming recognized as an important zoonotic disease of humans in the Middle East, Eastern Europe and Asia (for a review, see Oldfield et al., 1991). The disease caused by this virus may be fatal in up to 50% of infected patients (Pak et al., 1975).

Extensive studies have been undertaken through the support of the US Army Medical Research and Development Command to develop drugs which would have potential for treating these infections. As a first phase of this development, test substances were evaluated in vitro for efficacy against the Punta Toro virus (PTV), which is a virus closely related to the above disease agents but which is considerably less pathogenic for man. Compounds found to have in vitro efficacy, as well as selected immunomodulators, were then evaluated in mice infected with the hepatropic strain of PTV. Compounds considered to have suitable inhibitory activity against the PTV infection in the mouse model were then evaluated under stringent biosafety conditions against experimentally-induced infections of RVFV, SFV, and CCHFV at the US Army Medical Research Institute for Infectious Diseases (USAMRIID).

The hepatotropic PTV infection model in mice has been described previously (Pifat and Smith, 1987; Sidwell et al., 1988a).

Individual reports have previously been published describing the anti-PTV effects of the majority of the active compounds discovered in this project (Huffman et al., 1989; Mead et al., 1991; Sidwell et al., 1988a, 1988b, 1990, 1992; Smee et al., 1990, 1991) although several considered highly active (tiazofurin-5′-monophosphate, pyrazofurin, 3-deazaguanosine, human recombinant interferon-α-A/D, AM-5, CL246,738 and phenyleneamine) have never been reported previously; many of the less active materials have not been described previously. The primary purpose of this report is to bring together all of the results of 6 years' of in vivo PTV inhibition studies.

2. Materials and methods

2.1. Virus

The Adames strain of PTV was provided by Dr. Dominique Pifat of USAMRIID. The virus was first isolated from the serum of an individual infected with the virus in Panama in 1972. It was passaged twice in Vero cells, then twice in LLC-MK_2 cells. Plaques were isolated each time from these cells, and a large pool made from these cells following confirmation of virus identity by serum neutralization.

2.2. Animals

Three week-old (10–12 g) C57BL/c mice were obtained from Simonsen Laboratories (Gilroy, CA). All were quarantined 24–48 h prior to use, and maintained on standard mouse chow and tap water.

2.3. Compounds

All compounds were provided through USAMRIID. Unless designated as labile, each was usually prepared 1 day prior to being used in an experiment, utilizing the vehicle considered most appropriate. Water-insoluble compounds were subjected to 15–30 min treatment in a sonifying water bath, warmed to 45°C, vortexed, and used as a suspension if a full solution was not achieved. Each was distributed to sterile injection bottles, sealed and stored at 4°C until used. 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) was included in each series of experiments as a known positive control. The anti-PTV activity of ribavirin was described previously (Stephens et al., 1980; Huggins et al., 1984; Sidwell et al., 1988a).

2.4. Experiment design

Initial experiments were run with each test substance utilizing as parameters numbers of survivors and mean day to death. In these experiments, mice were infected with 10^5 plaque-forming units of virus (80% lethal dose) and usually treated with an approximate maximum tolerated dose (MTD), the MTD/2, and the MTD/4 of each compound (as determined by preliminary range-finding toxicity evaluations). Treatments were s.c., bid $\times$ 5 beginning 4 h pre-virus inoculation unless other treatment regimens were recommended. Materials considered to be immunomodulatory were usually administered intraperitoneally (i.p.) every other day beginning 24 h pre-virus inoculation. A total of 10 infected mice were used in each dosage, with 20 infected animals treated with placebo (drug vehicle) as virus controls. Five sham-infected mice received each drug dosage as toxicity controls, and 5 additional animals served as normal controls. The toxicity and normal controls were held in a room separate from the infected area. The animals were examined daily for death through to day 21. Treated and untreated mice were weighed on day 0 and again 18 h after final drug treatment to ascertain weight loss

or failure to gain weight. A single dose of ribavirin was run in parallel as a positive control.

Follow-up studies were run to confirm and extend the initial findings of antiviral activity. In these experiments, the parameters assessed included, in addition to increased survivors and mean day to death, reduction of hepatic icterus (liver score readings of 0 [normal] to 4 [maximum discoloration]), serum glutamic oxaloacetic and pyruvic acid transaminases (SGOT, SGPT), and recoverable virus from liver and serum of infected animals 3 or 4 days post-virus inoculation. In these experiments, 20 infected mice received each drug dosage, with 40 animals serving as virus controls. One-half of each group was killed on days 3 or 4 for measurement of the above parameters. Toxicity, normal, and positive controls were as described above.

Titration of SGOT and SGPT was done using colorimetric kits (Sigma Chemical Co., St. Louis, MO), with spectrophotometric readings being performed in duplicate with a microplate autoreader (EL309, BioTek Instruments, Inc., Winooski, VT). Livers were homogenized to a 10% (w/v) suspension prepared in minimum essential medium (MEM). Liver homogenates and serum were assayed for PTV titer by diluting each 10-fold to a final concentration of 10^{-5}; 0.2 ml of each dilution were added to triplicate wells of LLC-MK_2 cell monolayers in 96-well microplates, with viral cytopathic effect determined 5 days after incubation at 37°C and 50% endpoints determined as previously described (Sidwell et al., 1988a).

The treatment regimens in these experiments were often changed according to the substance being evaluated. Where sufficient material was available for compounds appearing to have anti-PTV activity, per os (p.o.) treatments were also studied.

2.5. *Statistical evaluations*

Increases in number of survivors were analyzed using chi-square analysis with Yates' correction. Increases in mean day to death (MDD) of animals dying on or before day 21 and reductions in SGOT and SGPT and virus levels in liver and serum were evaluated using the Student's t test. Ranked sum analysis (Wilcoxon test) was used to compare inhibition of mean liver scores. Therapeutic indices (TI) were determined by dividing the 50% lethally toxic dose (LD50) of each compound by the minimum effective dose, which was the lowest dose which caused a statistically significant ($P < 0.05$) improvement in disease parameter.

3. Results

A total of 946 experiments were run over a 6-year period, with 150 compounds evaluated. A number of these compounds were identified by code only, so the activity of only 75 total compounds will be described in this report. None of these coded compounds were considered markedly active against the PTV infection. An example of the anti-PTV activity of a compound, ribavirin-2′,3′,5′-triacetate (RTA) (considered to be active in these experiments) is seen in Table 1. This experiment also illustrated the

Table 1
Effect of oral gavage treatment [a] with ribavirin 2′,3′,5′-triacetate on Punta Toro virus infections in mice

Dosage (mg/kg/day)	Toxicity Controls		Infected, Treated							
	Surv./Total	Host wt. Change [b] (g)	Surv./Total	Mean day to death [c] (days)	Mean liver Score [d]	Mean SGOT [d,e]	Mean SGPT [d,e]	Mean liver Virus titer [d] (log_{10})	Mean serum Virus titer [d] (log_{10})	Total WBC [d] ($\cdot 10^6$)
4000	0/5	−3.5	0/10	7.4 **	1.3 **	1264 **	273 **	0.0 **	0.0 **	2.4 **
1280	5/5	0.8	5/10 **	8.8 **	0.1 **	587 **	70 **	0.0 **	0.0 **	3.3 **
400	5/5	1.8	8/10 **	9.5	0.0 **	554 **	452 **	0.0 **	1.1 **	4.2 **
128	5/5	3.1	10/10 **	> 21.0 **	0.2 **	196 **	165 **	0.0 **	0.3 **	5.6 **
40	5/5	2.1	8/10 **	6.0	2.7 *	803 **	473 **	2.2 **	3.0 **	4.0 **
12.8	5/5	2.2	2/10	4.8	3.6	1522	984	5.2	4.9	1.1
0 (H_2O)	–	–	0/20	4.1	3.8	1858	1061	5.3	6.2	0.6

[a] bid×5 beginning 24 h post-virus inoculation.
[b] Difference between weight at start of treatment and 18 h after final treatment. Normal control weight gain: 2.7 g.
[c] Animals dying on or before day 21.
[d] Data from 10 animals/dose killed on day 5.
[e] Sigma-Fraenkel units/ml.
* $P < 0.05$, ** $P < 0.01$.

type of experiments run throughout this study. Therapy with RTA, administered p.o. bid for 5 days beginning 24 h post-virus inoculation, resulted in significant disease inhibition using all infection parameters. Antiviral activity was seen at doses as low as 40 mg/kg/day. In this experiment, total numbers of white blood cells (WBC) were also determined, since these declined markedly during PTV infection; therapy significantly prevented this decline. The total WBC count in normal mice run in parallel was $6.2 \cdot 10^6$. The LD50 of RTA was estimated by regression analysis plot to be 2250 mg/kg/day; the TI of the compound utilizing every disease parameter except mean day to death was thus $2250 \div 40$, or 56.

Compounds considered to be non-immunomodulators in their mechanism of antiviral activity, which were significantly inhibitory to the PTV infection, are summarized in Table 2. Since multiple parameters were used in the evaluation of these compounds, the TI of the compound as measured by each parameter is shown. In addition to ribavirin, all were considered chemical analogs of ribavirin. All of these significantly inhibitory compounds that were evaluated by the p.o. route of administration were also active against the PTV infection when given by that treatment route.

The immunomodulating compounds considered to be significantly inhibitory to the PTV disease are summarized in Table 3. The maximum TI's using each infection parameter are again shown for each material. One cytokine, human recombinant interferon-α-A/D (rIFN-α-A/D) was included in this group of PTV inhibitors. Table 4 illustrates the activity of rIFN-α-A/D when administered i.p. qd for 9 days beginning 4 h post-virus inoculation. Treatments with this cytokine administered beginning 24 h or later after virus exposure was less efficacious, with only the maximal dose used being effective (data not shown). More detailed data have been described on the activity of the majority of the other immunomodulators as indicated by the footnotes in Table 4. It is important to point out that many were effective therapeutically, i.e., when treatment began as late as 48 h after virus inoculation.

Compounds considered to be non-immunomodulating as their principle biological activity, which had a moderate inhibitory effect on the PTV infection, are seen in Table 5. These materials were usually not significantly active in inhibiting all disease parameters; shown in the table is the maximum TI of each compound and the parameter(s) with which antiviral activity was seen. Also included is an indication of whether the antiviral activity was dose-responsive, i.e., maximum efficacy seen at the highest nontoxic dose used. These moderately inhibitory compounds were widely divergent in their chemical structures.

Immunomodulators considered to have moderate inhibitory effects on the PTV disease are seen in Table 6. These included several derivatives of the highly active immunomodulator, bropirimine, and two cytokines, human recombinant interleukin-2 (rIL-2) and granulocyte-macrophage colony stimulating factor (gmCSF).

Compounds which were not considered active against the PTV infection are summarized in Table 7. A number of these materials, due to an inadequate quantity available, were evaluated only at doses which were well tolerated by the animals; these are indicated by the comments in Table 7. It is possible that if higher dosages had been used, a significant PTV inhibitory effect may have occurred.

A number of compounds identified only by Army number or by very incomplete

Table 2
Non-immunomodulating compounds considered significantly inhibitory to hepatotropic Punta Toro virus infections in mice [a]

Compound	No. expts. run	Maximum therapeutic indices [b]					Orally active
		Survivor increase	Liver score reduction	SGOT/SGPT reduction	Liver virus inhibition	Serum virus inhibition	
Ribavirin [e]	25	65	65	200	200	200	+
Ribamidine [f,g]	24	65	65	200	65	200	+
Ribavirin 2′,3′,5′ triacetate [g]	20	56	65	200	65	65	+
Tiazofurin [h]	10	16	65	65	8	8	+
Tiazofurin-5′-monophosphate	2	≥ 5. [c]	≥ 5	≥ 5	≥ 5	≥ 5	? [d]
Tiazofurin 2′,3′,5′ triacetate [g]	3	≥ 9	≥ 3	≥ 36	≥ 18	≥ 18	?
Selenazofurin [g,h]	7	6	12	6	3	3	+
Pyrazofurin	6	≥ 8	≥ 8	≥ 4	≥ 8	≥ 8	+
3-Deazaguanine [g]	7	16	27	27	8	8	+
3-Deazaguanosine	6	8	16	16	16	16	?

[a] Initial treatment regimen, in which TI's were maximal for all compounds, was s.c. bid×5 beginning 4 h pre-virus inoculation; later, follow up experiments utilized other treatment routes (i.p. and p.o.), delayed initiation of therapy and single treatments.
[b] LD50 dose ÷ minimum effective dose.
[c] ≥ = LD50 dose not achieved.
[d] ? = No oral test run.
[e] Stephens et al. (1980), Huggins et al. (1984), Sidwell et al. (1988a).
[f] Sidwell et al. (1988b).
[g] Huffman et al. (1989).
[h] Smee et al. (1990).

Table 3
Immunomodulating compounds considered significantly inhibitory to hepatotropic Punta Toro virus infections in mice

Compound	No. expts. run	Maximum Therapeutic Indices [a]					Orally active
		Survivor increase	Liver score reduction	SGOT/SGPT reduction	Liver virus inhibition	Serum virus inhibition	
Poly (ICLC) (polyriboinosinic-polyribocytidylic acid stabilized with poly-2-lysine and carboxymethylcellulose) [f]	14	1000	100	1000	3125	3125	–
Ampligen (poly I●poly C_{12},U) [f]	22	1000	100	100	100	100	–
hu rIFN-α-A/D (human recombinant interferon-α-A/D)	5	≥ 100 [c]	≥ 32	≥ 320	≥ 320	≥ 320	? [d]
MVE-1 (low molecular weight maleic anhydride divinyl ether copolymer) [f]	12	≥ 275	≥ 138	≥ 138	≥ 275	≥ 138	–
MVE-2 (1:2 divinyl ether-maleic anhydride cyclic polymer) [f]	12	≥ 16	≥ 16	≥ 16	≥ 8	≥ 16	–
AM-3 (inmunoferon, a glucomannon polysaccharide from *Candida utilis*) [e,f]	17	125	1	≥ 40	≥ 40	≥ 40	–
AM-5 (a glucomannon polysaccharide from *Candida utilis*) [d]	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	≥ 10	+

Mannozym (glucomannan polysaccharide from *Sacchromyces cerevisiae*) [f]	18	≥ 129	≥ 65	≥ 129	≥ 1	≥ 1	−
Bropirimine (5-bromo-2,3-dihydro-2-imino-6-phenyl-4(1H)pyrimidinone) [f,g]	22	20	20	40	40	20	+
CL246,738 (3,6-bis[2-p-peridinoethoxy]acridine trihydrochloride	3	≥ 32	≥ 4	≥ 8	≥ 8	≥ 8	+
Phenyleneamine	8	≥ 16	≥ 8	≥ 8	≥ 8	≥ 8	+
7-thia-8-oxoguanosine [f,h]	6	26	26	52	26	13	?

[a] Initial treatment regimen, in which TI's were maximal for all compounds, was i.p. every other day beginning 24 h pre-virus inoculation; later, followup experiments for selected compounds, utilized other treatment routes (s.c. and p.o.), delayed initiation of therapy, varied frequency of treatments, and single treatments.

[b] LD50 dose ÷ minimum effective dose.

[c] ≥ = LD50 dose not achieved.

[d] ? = No oral test run.

[e] AM-3, 5 vary in molecular weight.

[f] Sidwell et al. (1992).

[g] Sidwell et al. (1990).

[h] Smee et al. (1991).

Table 4
Effect of intraperitoneal treatment[a] with human recombinant interferon-α-A/D on Punta Toro virus infections in mice

Dosage (units/ mouse/day)	Toxicity controls		Infected, treated						
	Surv./ total	Host wt. Change [b] (g)	Surv./ total	Mean day to Death [c] (days)	Mean liver score [d]	Mean SGOT [d,e]	Mean SGPT [d,e]	Mean liver Virus titer [d] (log_{10})	Mean serum Virus titer [d] (log_{10})
$10^{5.0}$	5/5	6.6	10/10 **	> 21.0 **	1.5 **	1204 **	933 **	1.1 **	1.1 **
$10^{4.5}$	5/5	7.4	8/10 **	4.0	1.8 **	3469 **	2848 **	2.2 **	2.8 **
$10^{4.0}$	5/5	7.3	5/10 **	5.2	3.3	5650 **	4645 **	4.7 *	5.3 **
$10^{3.5}$	5/5	7.7	1/10	5.9	3.2	3921 **	3057 **	4.5 *	4.9 **
0 (saline)	–	–	0/20	4.4	3.9	9100	7600	5.9	6.5

[a] Treatment i.p. once daily for 9 days beginning 4 h post-virus inoculation.
[b] Difference between weight at start of treatment and 18 h after final treatment. Normal control weight gain: 6.9 g.
[c] Animals dying on or before day 21.
[d] Data from 10 animals/dose killed on day 5
[e] Sigma-Fraenkel units/ml.
* $P < 0.05$, ** $P < 0.01$.

Table 5
Non-immunodulating compounds considered moderately inhibitory to Punta Toro virus infections in mice

Compound	Maximum TI—Any parameter	Active orally?	Dose-responsive
Thioformycin B [d]	≥ 24 (MDD) [a]	+	yes
Formycin B [d]	16 (survivors) [a]	? [b]	yes
9-β-D-ribofuranosylpurine-6-thiocarboxamide [d]	24 (MDD)	+	yes
1-β-d-ribofuranosyl-1,2,4-triazole-3-carboxamide acetic acid	≥ 4 (survivors, liver score, SGOT, SGPT)	?	yes
Actidione	≥ 4 (survivors, liver score, virus)	?	yes
Phyllanthoside	3 (survivors, SGOT)	?	yes
Uridine 2′,3′-dialdehyde	8 (MDD, SGOT, SGPT)	?	no
Thymidine riboside 2′,3′-dialdehyde	6 (MDD only) [c]	?	yes
6-Ethylthioriboside	12 (survivors, SGOT, SGPT)	+	no
6-Azauridine	4 (survivors)	?	yes
Narciclasine	~ 8 (survivors, liver score, SGOT, SGPT, virus)	?	yes
3-T-Butyl-1-adamantylthiourea	~ 32 (liver score, virus)	?	no
8-Bromoguanosine	5 (survivors) [c]	−	no
1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide acetic acid	~ 10 (MDD)	?	yes

[a] Required treatment 3 or 4 times daily for efficacy to be seen.
[b] ? = Not tested.
[c] Required single i.p. treatments pre-virus exposure for efficacy to be seen.
[d] Huffman et al. (1989).

names were omitted from the data presented. None were considered highly active against this infection.

4. Discussion

These experiments, carried out over a 6-year period, have identified a number of compounds which are strong inhibitors of *Phlebovirus* infection. Ribavirin, described previously as being highly active against the PTV infection (Stephen et al., 1980; Huggins et al., 1984; Sidwell et al., 1988a), was utilized as a positive standard. This compound was run in parallel with every test compound, and consistently exhibited the antiviral activity expected. Detailed experiments have been previously reported using the ribavirin analogs ribamidine (Sidwell et al., 1988b; Huffman et al., 1989), tiazofurin and selenazofurin (Huffman et al., 1989; Smee et al., 1990). A brief report on the in vitro and in vivo anti-PTV activity of the majority of the ribavirin analogs was published by Huffman et al. (1989).

The mechanism of action of ribavirin and its analogs against PTV is as yet unclear, but is assumed to be somewhat multifaceted. It is known that the 5′-monophosphate metabolite of ribavirin is inhibitory to IMP dehydrogenase, leading to inhibition of viral

Table 6
Immunodulating compounds considered moderately inhibitory to Punta Toro virus infections in mice [a]

Compound	Maximum TI—Any Parameter	Active orally?	Comment
rIL-2 (human recombinant interleukin-2) [d,e]	~ 16 (liver score, SGOT, SGPT, virus titers) ~ 8 (survivors)	? [b]	No toxic dose achieved
gmCSF (granulocyte-macrophage colony stimulating factor)	≥ 8 (MDD)	?	No toxic dose achieved
CL259,763 (N-[4-[(4-fluorophenyl)sulfonyl]-phenyl]acetamide) [e]	100 (liver score, SGOT, SGPT)	+	
AIPP (5-iodo-2,3-dihydro-2-imino-6-phenyl-4)1H)-pyrimidinone) [f]	4 (liver score, virus titers)	+	Higher doses prevented death
ABMP (5-bromo-2,3-dihydro-2-imino-6-methyl-4)1H)-pyrimidinone) [f]	8 (liver score, SGOT, SGPT, virus titers)	+	Erratic dose response
Oxamisole (2,3,5,6,7,8-hexahydro-2--phenyl-8,8-dimethoxyimidazo[1,2-a]pyridine)	8 (MDD)	+	Higher doses prevented death
ACPP (5-chloro-2,3-dihydro-2--imino-6-phenyl-4(1H)-pyrimidinone) [f]	15 (SGOT, SGPT)	+	Higher doses prevented death
AM-6 (glucomannon polysaccharide from *Candida utilis*) [c]	≥ 16 (MDD)	?	Higher doses prevented death

AM-7 (glucomannon polysaccharide from *Candida utilis*) [c]	≥ 16 (MDD)	?	Higher doses prevented death
Compound	Maximum TI—Any Parameter	Active	Comment
AM-8 (glucomannon polysaccharide from *Candida utilis*) [c]	4 (survivors)	?	
AComF$_2$PP (5-chloro-2,3-dihydro-2-imino-6-(2,3-difluorophenyl)-4(1H)-pyrimidinone) [f]	2 (liver score, SGOT, SGPT, virus titers)	+	
ABmFPP (5-bromo-2,3-dihydro-2-imino-6-(3-fluoro-phenyl)-4(1H)-pyrimidinone) [f]	8 (survivors, MDD)	+	
LY253,963 (1,3,4-thiadiazol-2-ylcyanamide) [d]	52 (MDD)	+	Erratic dose response
GE132 (Germanium)	~16 (survivors, liver score, SGOT, SGPT, virus titers)	+	Erratic dose response

[a] Initial treatment regimen, in which TI's were maximal for all compounds, was i.p. every other day beginning 24 h pre-virus inoculation; later, followup experiments for selected compounds, utilized other treatment routes (s.c. and p.o.), delayed initiation of therapy, varied frequency of treatments, and single treatments.

[b] ? = Not tested.

[c] AM-6, 7, 8 vary in molecular weight.

[d] Mead et al. (1991).

[e] Sidwell et al. (1992).

[f] Sidwell et al. (1990).

Table 7
Compounds considered inactive against Punta Toro virus infections in mice

Compound	Treatment route	Treatment schedule	Comment
Enviroxime	s.c., p.o.	qd, bid, tid×5, single	
Glycerrhetic acid	s.c., i.p.	bid, tid×5	
Suramin	s.c., p.o.	single, bid, tid×5	
3-Bromo-4-chloropyrazolo-[3,4-d]pyrimidine [a]	s.c., i.p.	single, qd, bid, tid×5	
Formycin	s.c.	single, bid×5	
7-Deoxynarciclasin	s.c.	bid×5	
Pancratistatin	s.c.	qd×7	
Isoprinosine	p.o.	bix×5	
Streptonigrin	s.c., i.p.	single, qd, bid, tid×5	
Thymine riboside 2′,3′-dialdehyde	i.p.	single, bid×5	
Bryostatin 2	i.p.	single, bid×5	
Ribavirin tetrahydropyrimidine	s.c.	bid×5	Nontoxic at all doses used
Ribavirin-5-OH-tetrahydropyrimidine	s.c.	bid×5	Nontoxic at all doses used
Neurotropin	i.p., p.o.	single, qd×3, every other day×3, every 3 days×2	
1-(4-methoxybenzyloxy)adenosine perchloric acid salt	s.c., i.p.	bid×5	Nontoxic at all doses used
Pseudolycorine HCl	s.c.	qd×5	Nontoxic at all doses used
3-Acetamido-7-amino-6-methyl-7H-5-triazolo[5,1-C]-S-triazole	s.c., i.p.	bix×5	Nontoxic at all doses used
2,3-Dihydro-5-iodothiophene-1,1-dioxide	s.c., i.p.	single, bid×5	
Trans-3-chloro-2-iodotetrahydrothiophene-1,1-dioxide	s.c., i.p.	single, bid×5	
1-Aminoadenosinium mesitylenesulfonate	s.c., i.p.	bid×5	Nontoxic at all doses used
Noxymethylpennicillinic acid	s.c.	single, qd, bid×5	
5′-N,N-diethylthiocarbamate-5′-deoxy-5′-thioadenosine	s.c., i.p.	qd, bid×5	Nontoxic at all doses used
Imexon (4-imino-1,3-diazobicyclo[3.1.0]-hexan-2-one)	i.p.	qd×5	
2-Thia-6-azauridine	i.p.	bid×5	Nontoxic at all doses used
2-Thia-6-azauridine-2′,3′,5′-triacetate	s.c.	bid×5	Nontoxic at all doses used

[a] Huffman et al. (1989).

nucleic acid synthesis (for a review, see Sidwell et al., 1985), and it is probable the analogs of this compound act in a similar fashion. Ribavirin also interferes with mRNA translation, possibly by being incorporated into the mRNA cap (Goswami et al., 1979). Ribamidine, while also an inhibitor of IMP dehydrogenase presumably as its monophosphate metabolite, is also a strong competitive inhibitor of purine nucleoside phosphorylase (Willis et al., 1980). Tiazofurin and selenazofurin and their analogs are also inhibitors of IMP dehydrogenase in their nucleotide forms (Streeter et al., 1973), but had a lesser degree of anti-PTV activity than ribavirin or ribamidine, possibly due to a limited bioavailability or rapid clearance from the animal (Smee et al., 1990). RTA, the triacetyl form of ribavirin which exhibited anti-PTV activity at a level essentially equal to that of ribavirin, has been reported to have very significant activity against the unrelated type A influenza virus (Stephen et al., 1977). The compound may be considered a pro-drug of ribavirin, and probably requires enzymatic removal of the acetyl groups for antiviral activity to be rendered. Pyrazofurin, a long-recognized nucleoside with a broad-spectrum of antiviral activity (for a review, see Cadman, 1983), had a TI that was considerably lower than that of ribavirin. This compound was quite toxic to mice by the treatment regimens used, with an LD50 of approximately 8 mg/kg/day. 3-Deazaguanine and 3-deazaguanosine, first reported by Cook et al. (1975), are inhibitors of IMP dehydrogenase and hypoxanthine guanine phosphoribosyltranferase (Streeter and Koyama, 1976). They have previously been shown to have activity against influenza, herpes, and Friend leukemia viruses in vivo (Allen et al., 1977).

A variety of immunomodulators exhibited striking inhibition of the PTV disease; this activity of both the active immunomodulators and the more directly antiviral compounds was often seen with delayed treatment initiation (Sidwell et al., 1992), at a time when the disease was manifested as major hematologic changes, high virus titers in liver and blood, liver discoloration and marked increases in SGOT and SGPT (Pifat and Smith, 1987; Sidwell et al., 1992).

A common mechanistic action for many of these immunomodulators appeared via the induction of interferon (IFN) with or without a secondary IFN effect, such as stimulation of natural killer cells. However, other mechanisms may be involved in protection of the mice. Support of the IFN induction premise comes from studies showing that mice resistant to lethal effects of PTV are rendered sensitive to the virus infection when treated with anti-IFN antibody (Pifat and Smith, 1987). Smee et al. (1991) have also demonstrated that the activity of 7-thia-8-oxoguanosine is eliminated by treatment of the infected mice with anti-IFN-α/β antibodies during therapy with this compound. The majority of the PTV disease-inhibitory immunomodulators are recognized IFN inducers. Poly (ICLC) and ampligen are potent IFN inducers, exerting their antiviral activity by IFN-mediated activation of nonspecific and specific immune responses (Carter et al., 1985; Levin, 1983). Bropirimine and the related pyrimidinones are also known to be rapid IFN inducers, although they also have a spectrum of other immunomodulatory properties including macrophage activation, augmentation of natural killer (NK) cell activity, induction of polyclonal B-cell response, induction of interleukin-1 and 2, enhancement of antigen-mediated antibody formation, and stimulation of bone marrow proliferation (for a review, see Wierenga, 1985). The pyran copolymer MVE-2, which is

a recognized activator of macrophages (Carrano et al., 1984), was found by us to also induce IFN in low levels (Sidwell et al., 1992). The related, lower molecular weight pyran copolymer, MVE-1, which has similar immunological properties to MVE-2 (Carrano et al., 1984), has not been studied for IFN induction. As noted above, 7-thia-8-oxoguanosine has also been shown to be a rapid inducer of IFN (Smee et al., 1990).

Arguments for additional immunological effects other than IFN playing a role in protecting mice from lethal PTV infections are based on studies of several immunomodulators considered highly active which are not apparent IFN inducers. The fungal-origin polysaccharide, AM-3, was previously shown by us not to induce IFN at varying times after injection of mice (Sidwell et al., 1992); AM-3 is reported to enhance macrophage function, induce IL-2 production, and stimulate NK cell activity (Moya et al., 1987; Roja et al., 1986). It is understood that AM-3, 5, 6, 7, and 8 are all related compounds differing only in an increasing molecular weight as the number increases. Mannozym was similarly not found to induce IFN in mice (Sidwell et al., 1992), but is a strong activator of macrophages (Bartocci et al., 1982).

The compound CL246,738 (3,6-bis[2-p-peridinoethoxy]acridine trihydrochloride) has previously been shown to protect mice from infections induced by arboviruses, murine cytomegalovirus, and type 2 herpesvirus (Sarzott et al., 1989; Morahan et al., 1991; Kende, 1992; Kunder et al., 1993). This antiviral activity was found to be mediated by IFN when administration of anti-IFN-α/β antiserum abrogated the compound's antiviral effectiveness (Kunder et al., 1993). Wang et al. (1986) showed that CL246,738 stimulates macrophages to release IFN-α, which subsequently activates NK cells.

Three cytokines were evaluated in this virus infection model; human recombinant IFN-α-A/D was considered markedly active, whereas human recombinant interleukin-2 (rIL-2) and granulocyte-macrophage colony stimulating factor (gmCSF) had a moderate degree of efficacy. Since PTV is being used as a chemotherapy model for this virus, it is significant that IFN-α has also been found effective in treating RVFV infections in monkeys (Morrill et al., 1989). The PTV-inhibitory effects of IL-2 have been previously described by us (Mead et al., 1991). gmCSF has only recently come under study as an antiviral agent as recombinant forms have become available; to date, mixed antiviral results in which both inhibition and enhancement of viral replication have been reported, primarily in studies with the human immunodeficiency virus (for a review, see Warren and Sidwell, 1993). This cytokine has a spectrum of immunologic effects, including stimulation and differentiation of precursor cells into granulocytes and monocytes, enhancement of antibody-dependent cellular cytotoxicity, and augmentation of neutrophil oxidative metabolism, chemotaxis and phagocytosis (Groopman et al., 1987).

Evidence to date indicates that this PTV infection model is a good predictor of antiviral efficacy against the more biohazardous related viruses, RVFV, CCHV, and Hantaan viruses. Ribavirin and RTA have been found equally effective against all these viruses (Stephen et al., 1980; Huggins et al., 1984). The immunomodulators poly (ICLC), ampligen, CL246,738, IFN-α, bropirimine, and AIPP have been shown to have similar levels of antiviral efficacy against RVFV infections of mice (Kende, 1992). Thus the results obtained in these antiviral studies with the PTV animal model would appear to have potential relevance for treating these serious infections of man.

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