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ANTIVIRAL EFFICACY OF PYRAZOFURIN AGAINST SELECTED RNA VIRUSES *

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The antibiotic pyrazofurin, 3-(β-D-ribofuranosyl)-4-hydroxypyrazole-5-carboxamide, markedly inhibited the in vitro replication of a number of RNA viruses including Rift Valley fever (RVF), Venezuelan equine encephalomyelitis (VEE), Sandfly, Pichinde, Lassa and LCM virus. Plaque formation was reduced by 80% or more with 2–10 μg/ml of pyrazofurin while 2 μg/ml reduced by 1000-fold the yield of Lassa and LCM virus in a yield reduction assay. In vivo, pyrazofurin failed to protect mice and guinea pigs against a lethal challenge with VEE and Pichinde virus, respectively. On the other hand, pyrazofurin caused a slight increase in the mean time to death of mice infected with RVF virus.

INTRODUCTION

Pyrazofurin is a carbon-linked nucleoside reported to exhibit broad-spectrum antiviral activity against pox-, herpes-, rhabdo-, paramyxo-, toga-, and picornaviruses [2]. Antiviral activity of this compound against a number of other viruses of medical and veterinary importance has not previously been reported. We tested the efficacy of pyrazofurin against Rift Valley fever (RVF), Sandfly fever (SF), Lassa, Pichinde (PIC), Venezuelan equine encephalomyelitis (VEE) and Yellow Fever (YF), viruses routinely included in our antiviral drug screen.

One objective of this research is to identify classes of compounds with antiviral activity and assess structure–activity relationships to obtain insight into design of synthetic

* In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.
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analogues having higher potential antiviral efficacy and reduced toxicity. This report summarizes preliminary comparisons of pyrazofurin with another antiviral drug, ribavirin, which has proven antiviral efficacy but also a moderate degree of toxicity [1].

MATERIALS AND METHODS

Facilities

The highly infectious nature of a number of the viruses used in this study requires special biological containment facilities for studying infectious disease. Special laboratory suites meeting safety design considerations for level 3 and 4 biological containment [5,8] were used in these studies.

Tissue culture media and antiviral compounds

Tissue culture cells were grown in Eagle's minimum essential medium (EMEM) with Earle's salts and 10% fetal calf serum (FCS). Overlay solutions consisted of Eagle's basal medium with Earle's salts; 2% host inactivated FCS and 0.2% agarose. Pyrazofurin as the fermentation product was generously provided by D.C. DeLong (Lilly Research Laboratory, Eli Lilly & Co., Indianapolis, IN). Ribavirin was a gift of R. Smith (Virateck, Covina, CA). Compounds were obtained as powders and reconstituted with Hank's balanced salt solution (HBSS) buffered to pH 7.2 with Hepes (10 mM) for tissue culture experiments or phosphate-buffered (pH 7.4) sodium chloride (PBS, 0.15 M) for in vivo studies.

In vitro experiments

African green monkey kidney cells (Vero) were used in the plaque-reduction assays for RVF (Zagazig 501, 900040), VEE (Trinidad), PIC (3830) and SF (Brownell, Sicilian) while YF (Asibi) was assayed in rhesus monkey kidney cells (LLC-MK_2). Drug solutions (0.3 ml) of appropriate concentrations were added to confluent cell monolayers in 24-well tissue culture trays (Falcon), and immediately followed by 0.1 ml of virus (50–200 pfu) or media in the case of tissue culture toxicity controls. After incubation for 60 min, 0.5 ml of overlay media containing gentamicin (5 μg/ml) was added to each well; cells were incubated further in accordance with the time required to yield optimal plaque formation for each virus. Tissue culture plates were then stained, plaque-forming units (pfu) counted, and tissue culture toxicity assessed visually on the basis of cytopathic effects.

For yield-reduction assays, arenaviruses were adsorbed to Vero cell monolayers at low multiplicity (1 pfu/1000 cells). Following adsorption for 1 h at 37°C, virus inocula were removed, monolayers washed in PBS, and medium (EMEM, 2% FCS) containing pyrazofurin was added to give final concentrations ranging from 0 to 10 μg/ml. Inoculated cells were incubated for 3 days at 37°C, 5% CO_2. Following incubation, supernatant fluids were harvested and assayed for infectious virus by counting pfu on Vero cells [4].

Animal studies

Female Swiss Webster mice, 3–4 weeks of age were challenged subcutaneously (s.c.) with approx. 250 pfu/0.1 ml of RVF or VEE virus on day 0. Drugs, dissolved in phosphate-buffered saline were administered s.c. to mice (approx. 0.1 ml) using treatment schedules described in the tables. Inbred male, strain 13 guinea pigs were inoculated s.c. with $10^{4.1}$ pfu of PIC adapted by sequential passage to kill guinea pigs [4]. On selected days blood was collected in heparinized tubes and assayed for PIC virus infection.

RESULTS

Both pyrazofurin (Fig. 1) and ribavirin (Fig. 2) markedly inhibited replication of all viruses tested. 50% reductions in plaque counts were obtained with 10 μg/ml or less of pyrazofurin. In these assays, SF, RVF, and VEE were most sensitive requiring 2–10 μg/ml of drug to reduce plaque forming by 80% or more. Higher concentrations were required to achieve similar levels of plaque-reduction for YF and PIC. Ribavirin, the other antiviral drug tested, was also effective in reducing plaque formation of all five viruses, although in comparison to pyrazofurin, 10-fold and higher concentrations of ribavirin were generally required to obtain the same levels of inhibition. At the concentrations used neither drug produced visible cytopathic effect, suggesting a reasonable safety margin for both ribavirin and pyrazofurin. Pyrazofurin was highly efficacious in inhibiting the growth of Lassa, LCM and PIC in Vero cells maintained under fluid overlay (Fig. 3). The growth of Lassa and LCM was inhibited nearly 1000-fold at concentrations of pyrazofurin as low as 2 μg/ml. On the other hand, PIC was less sensitive, requiring nearly five times as much pyrazofurin to reach a similar degree of inhibition.

Since in vitro efficacy studies appeared promising, pyrazofurin was next tested in animal models. In mice inoculated with RVF, low doses of pyrazofurin (0.25 mg/kg) given for 5 days with treatment initiated one day before infection resulted in a survival rate of 20% and generally prolonged life (Table 1). At 2.5 mg/kg per day, the drug was

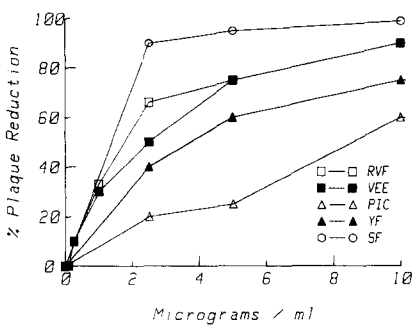

Fig. 1. Inhibition of viral plaque formation by pyrazofurin in vitro.

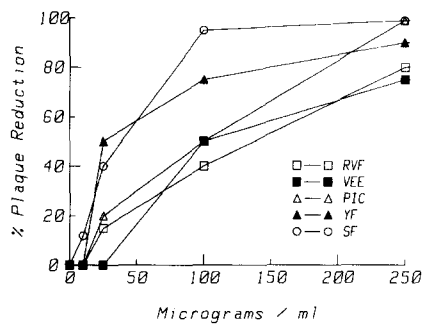

Fig. 2. Inhibition of viral plaque formation by ribavirin in vitro.

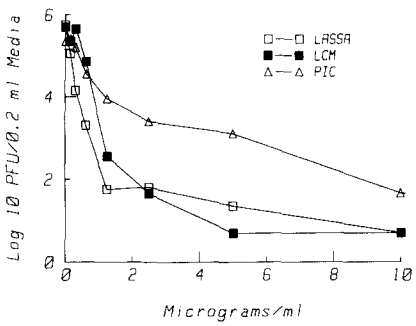

Fig. 3. In vitro growth inhibition by pyrazofurin of Lassa, LCM and Pichinde virus.

uniformly ineffective at protecting mice from death but did increase the time to death. A dose of 1 or 10 mg/kg given for 5 days on alternating days starting on day -1 was without benefit (Table 1). The higher concentration was toxic to mice and resulted in 80% mortality of the drug control group. Ribavirin treatment for 5 days at doses of 25 and 100 mg/kg resulted in the survival of 20 and 60% of RVF-infected mice, respectively (Table 2). Neither pyrazofurin nor ribavirin demonstrated any efficacy in protecting or prolonging the life of VEE-infected mice. In guinea pigs inoculated with PIC, pyrazofurin treatment did not alter the lethal course of infection (Table 3) and viremia (Fig. 4). On

TABLE 1

Survival of Rift Valley fever virus-infected mice treated with pyrazofurin

Virus inoculated	Treatment regimen		No. 21-day survivors[b]	Days of death of mice that died (mean ± S.D.)	P^c
	Schedule[a]	Dose (mg/kg)			
Inoc.	–	0	0	4.4 ± 0.5	–
Inoc.	Daily	0.25	2	6.8 ± 0.5	NS
		1.0	2	5.3 ± 1.3	NS
		2.5	0	7.8 ± 2.3	<0.01
Not. inoc.	–	2.5	10	–	–
Inoc.	–	0	0	4.2 ± 1.0	–
Inoc.	Alt.	1.0	0	4.2 ± 1.0	NS
		5.0	0	4.2 ± 1.0	NS
		10.0	0	4.8 ± 0.6	NS
Not inoc.	–	10.0	2	5.5 ± 0.9	<0.05

[a] Pyrazofurin was given beginning on day -1 of inoculation for either 5 consecutive (daily) or alternative (Alt.) days.

[b] Ten mice per group were inoculated s.c. with 350–400 pfu of RVF virus.

[c] χ^2 test.

TABLE 2

Survival of Rift Valley fever virus-infected mice treated with ribavirin

Virus inoculated	Dose (mg/kg)[a]	No. 21-day survivors[b]	Days of death of mice that died (mean ± S.D.)	P^c
Inoc.	0	0	5.0 ± 0	–
Inoc.	25	1	7.0 ± 4.2	NS
Inoc.	100	6	12.5 ± 1.9	<0.01
Not inoc.	100	10	–	–

[a] Ribavirin was given beginning on day -1 of inoculation for 5 consecutive days.
[b] Ten mice per group were inoculated s.c. with 300 pfu of RVF virus.
[c] χ^2 test.

TABLE 3

Survival of Pichinde virus-infected guinea pigs treated with pyrazofurin

Virus inoculated	Dose (mg/kg)[a]	No. 21-day survivors[b]	Days of death of mice that died (mean ± S.D.)	P^c
Inoc.	0	0	15.3 ± 5.3	–
	1	0	15.1 ± 1.8	NS
	5	0	14.6 ± 2.2	NS
	10	0	9.1 ± 4.3	<0.01
Not. inoc.	10	10	–	–

[a] Pyrazofurin was given 5 times at 3-day intervals beginning on day +3.
[b] Five guinea pigs per group were inoculated s.c. with 4.1 $\log_{10}$ pfu of Pichinde virus.
[c] χ^2 test.

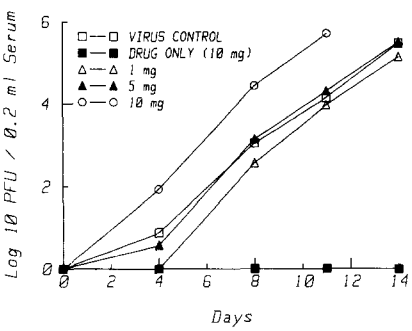

Fig. 4. Viremias in Pichinde virus-infected guinea pigs treated with pyrazofurin; five treatments at 3-day intervals beginning on day + 3.

the contrary, animals in the high dose group died earlier and had viremias 10-fold greater than those of the untreated infected controls or guinea pigs receiving lower doses of drug.

DISCUSSION

The families of Arenaviridae and Bunyaviridae contain highly virulent human pathogens for which no effective therapeutic or prophylactic treatment exists. The high morbidity and mortality associated with such virus infections as RVF and Lassa underscore the need for effective antiviral agents. In vitro and animal models have clearly shown that interferon is apparently ineffective for the treatment of arenavirus infections such as Lassa fever, the efficacy of immune serum treatment for this disease has not been convincingly demonstrated in human populations and may not be practical even if the concept proves to be valid. Of the antiviral drugs tested to date, only ribavirin has been shown to exert a therapeutic effect against arenaviruses in tissue culture as well as animal model systems [4,6]. The overall effectiveness of ribavirin therapy on arenavirus or bunyavirus infections, however, depends on the early initiation of treatment. Although effective in preventing systemic disease, ribavirin treatment often fails to prevent late CNS disease [6] perhaps due to the drug's inability to reach sufficient concentrations in the CNS. Active screening for other effective drugs led to the observation that pyrazofurin was highly effective in vitro against representative arena-, bunya- and togavirus groups at concentrations 10–100 times lower than those used with ribavirin.

The remarkable in vitro antiviral activity of pyrazofurin is attributed to the inhibition of de novo synthesis of nucleotides [7,9]. The addition of uridine reverses the drug's antiviral activity; hence, the inhibition of orotic acid monophosphate decarboxylase by pyrazofurin monophosphate, a metabolite of pyrazofurin is thought to be the basis of the drug's mechanism of action [3,4]. In spite of its effects on such a fundamental cellular metabolic pathway, pyrazofurin exhibits a rather broad safety margin in vitro. Nonetheless, the potent in vitro efficacy and broad margin of safety are not reflected in animal models. In the RVF mouse model, pyrazofurin shows marginal efficacy with doses at 0.25 mg/kg per day. The drug is even less effective in higher doses. Results from the guinea pig PIC model are similar. At the highest dosage level used guinea pigs died earlier than infected controls. The higher viremias in guinea pigs treated with the highest dose suggest that pyrazofurin may alter host defense systems so as to enhance growth or suppress clearance of virus. Both RVF and PIC may have suppressive effects on the reticuloendothelial functions [4,5]. Pyrazofurin may act synergistically to impair reticuloendothelial function resulting in diminished killing or clearance of virus. Descamps and De Clercq [2] suggested that the toxicity of pyrazofurin is probably not related to the structural features of the molecule responsible for antiviral activity. Analogues of pyrazofurin, therefore, may be synthesized which retain the antiviral efficacy but have reduced host toxicity. A study of the biochemical similarities and differences of the antiviral activity of pyrazofurin, ribavirin, and structurally related derivatives could result in compounds with greater potency than ribavirin and considerably less toxicity than pyrazofurin.

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