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Comparison of the anti-respiratory syncytial virus activity and toxicity of papaverine hydrochloride and pyrazofurin in vitro and in vivo

Philip R. Wyde, Brian E. Gilbert and Mark W. Ambrose

Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas, U.S.A.

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Summary

Based on reports describing their broad antiviral activity, the toxicity and antiviral efficacy of papaverine hydrochloride and pyrazofurin against respiratory syncytial virus (RSV) infection were tested in vitro in tissue culture cells and in vivo in cotton rats. Papaverine inhibited RSV replication in vitro; however, the median minimal toxic dose–median minimal inhibitory concentration ratios (MTD_{50}:MIC_{50}) in vitro and in vivo for papaverine were <4. Further work with this compound was discontinued. In contrast, pyrazofurin inhibited RSV replication in vitro (a mean MIC_{50} of 0.04 μg/ml was obtained) and in vivo (RSV pulmonary titers were significantly reduced consistently in cotton rats given daily 10 mg/kg doses compared to untreated control animals). However, some toxic effects were observed in both the in vitro and in vivo tests of this compound. The remaining potential of pyrazofurin as an anti-RSV compound is discussed.

Pyrazofurin; Papaverine; Respiratory syncytial virus

Introduction

Respiratory syncytial virus (RSV) remains a major cause of acute respiratory disease in children under two years of age. No vaccines are currently licensed for use in preventing RSV infection. The only antiviral approved for use against RSV is ribavirin. However, this compound is only licensed for use when delivered by

Correspondence to: P.R. Wyde, Department of Microbiology and Immunology, Baylor College of Medicine, Houston, TX 77030, U.S.A.

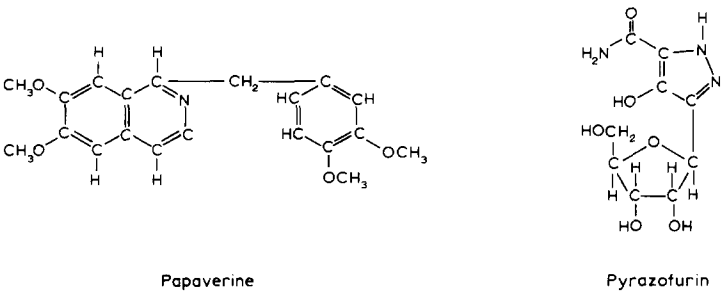

Fig. 1. Chemical structure of papaverine and pyrazofurin.

small particle aerosol, making treatment with ribavirin both expensive and practical only for treatment of severe disease. Identification and development of other compounds with anti-RSV activity would be highly desirable.

Papaverine (6,7-dimethoxy-1-veratrylisoquinoline) (Fig. 1) has been reported to inhibit measles virus in human neural and non-neural cells (Yoshikawa and Yamanouchi, 1984), and to inhibit cytomegalovirus (CMV) in human skin-muscle cells (Albrecht et al., 1987). Pyrazofurin (Fig. 1) also has been reported to inhibit a broad range of viruses in vitro including vesicular stomatitis, measles, polio, coxsackie, vaccinia and respiratory syncytial viruses (Descamps and De Clercq, 1978; Kawana et al., 1985, 1987). To our knowledge the antiviral activity of papaverine or pyrazofurin in vivo against RSV has not been reported. In this report we describe studies testing and comparing the toxicity and antiviral activities of these two compounds in tissue culture and in cotton rats. Although both compounds exhibited anti-RSV activity in vitro, only pyrazofurin was active in vivo.

Materials and Methods

Animals

All cotton rats (*Sigmoden hispidus*) used in these studies were bred from 2 pair obtained from the Small Animal Section, Veterinary Research Branch, Division of Research Services, National Institutes of Health. Test animals were 21 to 49 days old at the start of each experiment and of either sex. All animals were maintained in cages with barrier filters and fed water and food ad libitum.

Virus

Seed RSV A2 virus was obtained from the American Type Culture Collection (Rockville, MD). A stock of this virus was prepared by infecting flasks of HeLa tissue culture cells. When the monolayers in these flasks exhibited approximately 90% syncytia formation, the medium from the flasks was collected, pooled and clarified by centrifugation (450 × *g*). The clarified supernatant fluid was passed

through a 0.45 μM filter (Acrodisc, Cat. No. 4184, Gelman, Ann Arbor, MI), portioned and stored at −70°C. The titer of this pool was 1×10^7 plaque forming units/ml.

Tissue culture

Starting cultures of HeLa (ATCC CCL2), L929 (ATCC CCL1), HEp2 (ATCC CCL23) and Madin Darby canine kidney (MDCK; ATCC NBL-2) cells were obtained from the American Type Culture Collection (ATCC). These cultures were serially passaged whenever they became confluent, using Eagle's minimum essential medium (MEM) supplemented with fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 μg/ml), sodium bicarbonate (0.2%), and L-glutamine (2 mM/ml).

Compounds

Pyrazofurin, 3-β-D-ribofuranosyl-4-hydroxypyrazole-5-carboxamide, was obtained from Calbiochem (Cat. No. 5741094, San Diego, CA). Papaverine hydrochloride was obtained from Sigma Chemical Co. (Cat. No. P3510; St. Louis, MO).

Virus quantification

Virus in pools or in lung lavage suspensions were titered in 12-well plates containing 50–70% confluent monolayers of HeLa tissue culture cells. Serial half $\log_{10}$ dilutions of each test sample were made in 5% FCS-MEM. Medium was then removed from each well and 0.1 ml of each dilution was added to the appropriate well. After a 90 min adsorption period with periodic rotation of the plates, MEM containing 5% FCS was added to each well. At the end of seven days of incubation at 37°C in a 5% CO_2 incubator, each well was observed for syncytia formation. Wells exhibiting syncytia were considered to be infected with RSV. Mean virus titers ($\log_{10}$) were then determined by calculating the means of the last dilutions in replicate rows that contained virus.

Cytotoxicity measurement

Cytotoxicity assays were performed in 96-well flat bottom tissue culture plates (Falcon 3072). In these assays, each test compound was serially diluted in 5% FCS MEM using serial 2-fold dilutions, starting at a concentration of 1 mg/ml. Approximately 5×10^3 HeLa, L929 or MDCK (canine-derived) tissue cells was then added to appropriate wells; each compound was tested in quadruplicate in each tissue line. At this cell concentration, the monolayers that formed following settlement and attachment of cells were about 30–50% confluent. Control wells containing cells and media, but no antiviral, were included in each assay. These control wells were observed for cell growth. When they were confluent, all wells were observed for cytopathic effects (CPE) and for cell growth. Toxicity was considered

to have occurred if ≥20% of the cells in a monolayer exhibited CPE (i.e., were rounded or exhibited other marked morphologic changes compared to cells in media control wells), or if monolayers remained ≤50% confluent. The median minimal toxic dose (MTD_{50}) was calculated (in μg/ml) for each tissue by determining the concentration of test compound in the last wells of the quadruplicate rows exhibiting ≥20% CPE or ≤50% confluency.

Antiviral measurements in vitro

Each compound was tested for antiviral activity in 96-well flat bottom tissue culture plates (Falcon 3072). In these assays each compound was serially diluted in quadruplicate in 5% FCS-MEM using serial two-fold dilutions (0.05 ml/well). A 0.05 ml volume of RSV A2, usually containing about 100 median tissue culture infectious doses ($TCID_{50}$), was then added to all but antiviral and tissue control wells. Next, 0.1 ml of HeLa cells (approximately 5×10^3 cells) was added to each well. Control wells containing antiviral and no virus (antiviral control), containing virus but no antiviral (virus control) and containing medium without virus or antiviral (tissue control), were included in each test. A back titration of the challenge virus was also run with each assay. All assay plates were incubated at 37°C for 5 to 7 days in a 5% CO_2 incubator. When virus control wells exhibited 70–100% CPE including syncytia, all wells were observed. The median minimal inhibitory concentration (MIC_{50}) was calculated after determining the concentration of antiviral in the last wells in the quadruplicate rows exhibiting ≤50% CPE compared to the CPE in virus control wells.

Collection of lungs and nose washes

Cotton rats were killed and the lungs of each animal collected. Each lung was transpleurally lavaged using 3 ml of 5% FCS-MEM as described previously (Wilson et al., 1984). The animals were then decapitated and the lower jaw was removed from each head. Nose washes were collected by pushing 1 ml of 5% FCS MEM through each nostril and capturing effluent from the posterior opening. Lung fluids and nose washes were assessed for virus levels as described above. Lungs required for histologic examination were similarly removed, but they were placed into buffered formalin and processed as described next.

Histological methods and evaluations

After fixation in buffered formalin for a minimum of 24 h, all lung tissues were embedded in low-melting point paraffin, sectioned at 5 μm thickness, and stained with hematoxylin and eosin. The stained sections were coded by number and observed in a blinded fashion for histopathologic evidence.

Antiviral activity in vivo

Generally, the following protocol was used to test each compound for antiviral activity in cotton rats: on day 0 animals were weighed, anesthetized lightly with ether and inoculated intranasally (i.n.) with approximately 1×10^6 plaque forming units of RSV A2 in 0.1 ml. On days +1, +2 and +3, animals were inoculated intraperitoneally (i.p.) with 0.1 ml of either sterile water, or the appropriate concentration of papaverine or pyrazofurin. On day +4 (the day of maximum pulmonary infection in untreated cotton rats) all animals were killed and reweighed. The lungs of each animal were then removed and weighed. Each nose wash and lung fluid was assayed for RSV levels as described above.

Toxicity studies in cotton rats

Extra animals which received placebo or antiviral, but not virus, were included in each test group. Lungs from these extra animals were removed, fixed in formalin, processed and studied for histologic evidence of toxicity.

Other toxicity studies were carried out in which uninfected cotton rats were given daily i.p. injections of test antiviral for up to eight consecutive days. At the end of eight days each animal was weighed, killed and its lungs removed for histologic studies.

Pyrazofurin metabolism

To ascertain if pyrazofurin was metabolized in blood, pyrazofurin was mixed with freshly drawn, heparinized whole cotton rat blood. At fixed intervals aliquots were removed from the mixture and processed for high performance liquid chromatography (HPLC) in a manner similar to that previously done for ribavirin (Gilbert and Wyde, 1988). Briefly, pyrazofurin (40 μg/ml, 154 μM) was added and 1.0 ml samples were taken at 0, 0.25, 0.5, 1.0, 2.0 and 2.7 h. Incubations were at 37°C with occasional mixing. At each interval samples were collected and centrifuged at 13000 rpm for 10 min. The plasma fraction (hematocrit = 45%) was removed and an equal volume (ca. 0.45 ml) of water was added to the red blood cell (RBC) fraction. This fraction was frozen and thawed once. Both plasma and RBC fractions were then mixed with $HClO_4$ (2.5%, final concentration), allowed to sit on ice for 15 min and centrifuged at 13000 rpm for 10 min. Supernatant fractions were removed, neutralized with 10 N KOH and subjected to reverse-phase HPLC. Quantitation of pyrazofurin by HPLC was performed using a Microsorb C18 stainless steel column and 1% methanol in 20 mM NH_4PO_4 buffer (pH 5.1) as the mobile phase. Monitoring was performed at 200 nm. This system is capable of detecting pyrazofurin at a level of 0.4 μM (0.1 μg/ml).

Detection of a possible phosphorylated derivative of pyrazofurin in plasma and/or RBC fractions was also performed by HPLC. This was done utilizing a Particil SAX-10 ion exchange resin and 0.45 M potassium phosphate buffer (pH 4.1) as the mobile phase. Monitoring was performed at 200 nm. This latter method has

proven capable of separating ribavirin triphosphate from UTP, CTP, dTTP, ATP, GTP and dGTP. A pyrazofurin triphosphate standard was not available for comparison.

Statistics

Means, standard errors of the means, standard deviations and Student's *t*-tests were calculated using Stats Plus, a statistical program designed by Human Systems Dynamic Corp., Northridge, CA, for the Apple IIe computer.

Results

Cytotoxicity in tissue culture

A comparison of the cytotoxicity of papaverine and pyrazofurin in HeLa, L929 and MDCK tissue culture cells is shown in Table 1. In these studies papaverine caused test cells to become markedly distorted and elongated at doses intermediate between concentrations of this compound that caused death ($\geq$250 μg/ml) and doses that had no apparent effects on cells (21–78 μg/ml). Such marked morphologic changes were considered abnormal and for this reason were judged to be a drug induced cytopathic effect. The MIC_{50} values for papaverine presented in Table 1 reflect this judgement. No similar morphologic distortions were seen in monolayers of cells incubated with pyrazofurin, and in general, induction of CPE and significant inhibition of tissue culture cell growth occurred at equivalent concentrations of this drug.

As indicated by the data presented in Table 1, papaverine and pyrazofurin had comparable MTD_{50} values in HeLa or L929 cells. In contrast, pyrazofurin was markedly more cytotoxic in MDCK cells than papaverine (MTD_{50} for papaverine = 78 ± 16 μg/ml compared to 0.2 ± 0.1 μg/ml for pyrazofurin, $P<0.001$ using Student's *t*-test). The MTD_{50} value of 0.2 ± 0.1 μg/ml for pyrazofurin in MDCK cells was the lowest MTD_{50} value obtained in these studies.

TABLE 1

Comparison of the median minimal toxic dose (MTD_{50}) of papaverine hydrochloride and pyrazofurin in human-derived HeLa, mouse-derived L929 fibroblast and Madin Darby canine kidney cells[a]

Drug	Minimal toxic dose (μg/ml) in[b]		
	HeLa	L929	MDCK
Papaverine	47 ± 17	21 ± 5	78 ± 16
Pyrazofurin	24 ± 6	57 ± 26	0.2 ± 0.1

[a]Tissue controls were observed until they were confluent (usually 3 to 4 days). At that time all wells were observed for cytopathic effects and/or the inhibition of cellular replication.
[b]Mean value ± SEM for $\geq$3 experiments.

TABLE 2

Comparison of the antiviral efficacy (MIC_{50}) of papaverine hydrochloride and pyrazofurin against respiratory syncytial virus A2 in HeLa tissue culture cells

Compound	Minimal inhibitory conc. (μg/ml) in experiment[a]			Mean ± SD[b]
	1	2	3	
Papaverine	6.3 (33)[c]	25.0 (333)	7.1 (333)	12.8 ± 10.6
Pyrazofurin	0.02 (333)	0.01 (10)	0.1 (100)	0.04 ± 0.05

[a]Minimal inhibitory concentration = mean concentration of papaverine or pyrazofurin that inhibited virus CPE ≥50% as compared to control wells not containing antiviral but challenged with virus. Number of replicates/test=four.

[b]Mean of the three MIC_{50} values obtained in experiments 1, 2 and 3.

[c]The titer of challenge virus (in $TCID_{50}$) used in each experiment is indicated in parenthesis.

Antiviral activity in tissue culture

A comparison of the antiviral activity of papaverine and pyrazofurin against RSV in HeLa cells is shown in Table 2. As indicated by the MIC_{50} values shown in this table, pyrazofurin was significantly more inhibitory to RSV than papaverine, irrespective of the challenge dose used. (The challenge dose in median tissue culture infectious doses is indicated by the numbers in parentheses.) The mean MIC_{50} value for papaverine in the three experiments was 12.8 ± 10.6 μg/ml compared to a mean MIC_{50} value of 0.04 ± 0.05 for pyrazofurin (a 320-fold difference; $P<0.01$ using Student's *t*-test with two tails).

Therapeutic index

In vitro therapeutic indices (i.e., the ratio of the MTD_{50}:MIC_{50}) were calculated for papaverine and pyrazofurin using the MTD_{50} values obtained in HeLa cells and presented in Table 1, and the mean MIC_{50} values obtained in HeLa cells and shown in Table 2. The in vitro therapeutic index (T.I.) for papaverine was 3.6 (47/12.8) and for pyrazofurin 600 (24/0.04).

Toxicity in cotton rats

Single doses of papaverine ≥50 mg/kg, administered intraperitoneally, were toxic to cotton rats; animals given such doses became lethargic, comatose or died. Single doses of 40 mg/kg or less had no overt effects on animals.

No similar effects were seen in cotton rats given comparable single doses of pyrazofurin. However, weight losses (10–25%) were observed in uninfected animals given daily 10 mg/kg doses of this compound for 8 successive days (data not shown). No such weight losses were observed in sham inoculated control animals. Compared to comparably stained sections prepared from untreated control animals, no significant histopathologic changes were seen in hematoxylin and eosin stained sections of lung, spleen, liver and kidneys from these animals.

TABLE 3

Respiratory syncytial virus titers in nasal washes and lungs of cotton rats treated with papaverine hydrochloride[a]

Papaverine concentration (mg/kg/day)	Geometric mean RSV titer (log_{10}) ± SD			
	Nasal wash		Lung (/g)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
0	4.3 ± 2.0[b]	3.6 ± 1.7	4.9 ± 2.3	3.4 ± 1.7
20	4.3 ± 2.1	3.0 ± 1.5	4.8 ± 2.2	3.8 ± 1.3
40	4.0 ± 1.9	3.2 ± 1.2	5.6 ± 2.8	3.5 ± 1.7

[a]Cotton rats were inoculated with virus on day 0, treated with papaverine once daily (i.p.) on days +1, +2 and +3 and killed on day +4. At that time lungs and nasal washes were collected and assessed for virus levels.
[b]No means obtained in this study were significantly different from untreated controls (all P values > 0.05).

Antiviral activity in cotton rats

Table 3 presents the geometric mean pulmonary (log_{10}/g lung) and nasal wash titers (log_{10}/wash) of RSV in untreated control cotton rats and cotton rats treated daily with 20 or 40 mg/kg of papaverine. No significant reductions ($P>0.05$ using Student's t-test) in RSV levels in lungs or nasal washes were observed in these experiments in animals given papaverine compared to levels of virus in lungs and nose washes of animals given placebo.

In contrast, as indicated by the values presented in Table 4, cotton rats given 10 mg/kg doses of pyrazofurin on day +1 through +3 after experimental challenge with RSV had significant reductions in RSV titers in their noses and lungs compared to levels of this virus in noses and lungs of sham inoculated control animals ($P<0.05$). Reductions of virus in nose washes from treated animals in experiments 1 and 2 were 2.0 and 0.6 log_{10}, respectively, and 1.3 and 1.2 log_{10}/g of lung. Var-

TABLE 4

Antiviral efficacy of pyrazofurin against respiratory syncytial virus infection in cotton rats[a]

Pyrazofurin concentration (mg/kg/day)	Geometric mean RSV titer (log_{10}) ± SD			
	Nasal wash		Lung (/g)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
None (PBS)	4.1 ± 0.5	3.7 ± 0.3	4.3 ± 0.5	4.4 ± 0.3
1	3.6 ± 0.5	3.5 ± 2.5	3.8 ± 0.7	4.3 ± 0.2
3	3.1 ± 0.8[b]	3.7 ± 0.3	3.4 ± 0.5	3.7 ± 0.9
10	2.1 ± 1.5	3.1 ± 0.3	3.0 ± 0.5	3.2 ± 0.8

[a]Animals were inoculated intranasally with approximately 1×10^6 PFU RSV A2 on day 0. On days +1, +2 and +3 each animal was inoculated intraperitoneally with phosphate buffered saline (PBS) or pyrazofurin. Animals were killed and lungs and nasal washes tested for virus on day +4.
[b]Underlined values indicate statistical significance ($P \leq 0.05$) compared to controls (PBS) using Student's t test; number of animals/group = 4 or 5.

iable reductions in virus were seen in animals administered 3 mg/kg of pyrazofurin per day. With the exception in experiment 1 of a 1 $\log_{10}$ decrease in virus in nose washes of treated animals, none of the decreases (which ranged from 0 to 0.9 $\log_{10}$) were statistically significant compared to virus levels in untreated controls. No significant reduction in RSV levels in lung fluids or nose washes were observed in animals given 1 mg/kg/day of pyrazofurin on days +1 to +3 after experimental challenge with RSV; reductions in RSV levels ranged only from 0.1 to 0.5 $\log_{10}$ in this group compared to untreated controls.

Metabolism of pyrazofurin

When pyrazofurin (154 μM) was added to heparinized cotton rat blood and allowed to incubate at 37°C for 2.7 h, the pyrazofurin was rapidly metabolized in the plasma fraction (Fig. 2) with a half-life of approximately 15 min. Pyrazofurin could not be detected in the RBC fraction (data not shown). Using ion exchange resins to detect possible phosphorylated derivatives of pyrazofurin, a new peak was observed in the plasma fraction at about 24.5 min; the mobility pattern observed resembled that of other nucleoside triphosphates. Since pyrazofurin-5′-triphosphate (PFTP) was not available as a standard, identification and quantification of the new peak as PFTP was not possible. Nevertheless accumulation occurred during the first hour, then declined, and by the end of the experiment no peak was detectable. This pattern suggested that the new product was further metabolized; however, no additional new peaks were detected. No derivatives were detected in the RBC fraction (data not shown).

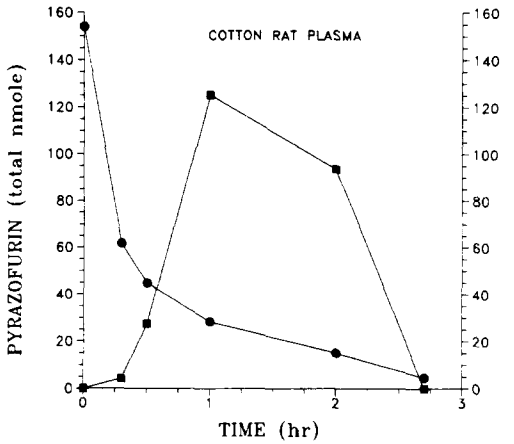

Fig. 2. Metabolism of pyrazofurin in heparinized cotton rat blood. To follow the in vitro metabolism in the blood of cotton rats, 154 μM (40 μg/ml) pyrazofurin was added to heparinized blood, incubated at 37°C and assayed at various times for pyrazofurin by HPLC. Pyrazofurin (●) was metabolized in the plasma fraction. A new peak (■), detected by ion exchange HPLC, was observed, suggesting the formation of a phosphorylated derivative. No pyrazofurin or metabolic product(s) was detected in the RBC fraction.

Discussion

These studies were concerned primarily with the toxicity and anti-RSV activity of papaverine hydrochloride and pyrazofurin in tissue culture and in cotton rats. Impetus to do the studies came from the dearth of prophylactic or therapeutic agents available for use against serious RSV infection, and from reports that indicated that both papaverine and pyrazofurin could inhibit replication of DNA and RNA viruses in different tissue culture cells (Albrecht et al., 1987; Descamps and De Clercq, 1978; Kawana et al., 1987; Wyde et al., 1978), including paramyxoviruses (Descamps and De Clercq, 1978; Kawana et al., 1987; Yoshikawa and Yamanouchi, 1984).

Inhibition of measles virus in vitro by papaverine is thought to occur via the drug's inhibition cyclic nucleotide phosphodiesterase (Lugnier and Stoclet, 1974; Shannon, 1977; Triner et al., 1970), leading to a blockade of essential virus protein phosphorylation (Yoshikawa and Yamanouchi, 1984). Other factors may be involved. In this compounds inhibition of DNA viruses, Albrecht et al. (1987) found that the anti-CMV activity of papaverine was most pronounced when papaverine was given before or within six hours of virus infection. Regardless, papaverine did inhibit replication of RSV in vitro in these studies. The MIC_{50} obtained against RSV A2 in HeLa cells was 12.8 μg/ml (Table 2). This value compared to reported MIC_{50} values for papaverine against CMV and measles of 0.38 (Albrecht et al., 1987) and 1.52 μg/ml (Yoshikawa and Yamanouchi, 1984), respectively.

As discussed in the Results section, our evaluation of the toxicity of papaverine was somewhat subjective. (We considered marked morphologic changes and distortion of tissue cells in ≥20% of the cells of a monolayer to be a toxic manifestation even though these cells were not dead.) Since the concentration of papaverine that induced this marked morphologic alteration was nearly six-fold less than the concentration that caused cell death, our calculated MTD_{50} was six-fold lower than one based on ≥20% cell death. (The MTD_{50}:MIC_{50} ratio using our criteria was 3.6.) Regardless of this subjectivity, no significant anti-RSV activity was observed in cotton rats even at the maximum daily dose given (40 mg/kg on days +1 through +3; Table 3). Higher doses could not be given as single doses of papaverine ≥50 mg/kg given i.p. caused cotton rats to go into a catatonic state, and in many instances to die. These doses are well in excess of those recommended for humans (4.3 mg/kg for a 70 kg person; Gutowski et al., 1978) when papaverine is used as a smooth muscle relaxant. Because of its apparent toxicity and lack of anti-RSV activity in cotton rats, we have stopped all further testing of papaverine.

In contrast to papaverine, pyrazofurin inhibited RSV replication both in vitro and in vivo. As depicted in Table 2, the mean MIC_{50} values obtained in three experiments was 0.04 μg/ml. In cotton rats, daily doses of 10 mg/kg/day i.p. significantly reduced pulmonary and nasal titers of RSV compared to control animals (Table 4). Reductions in RSV titers were also observed at lower daily doses, but these reductions were inconsistent, and on the whole, not statistically different from levels of virus in nasal washes and in lung fluids from control animals.

Unfortunately, pyrazofurin exhibited some toxicity in both the in vitro and in

vivo tests. In in vitro tests pyrazofurin had comparable cytotoxicity in HeLa and L929 cells as papaverine (Table 1), but was markedly more toxic in MDCK cells. MDCK cells differ from most cultured cells in that they maintain some of their specialized in vivo (kidney) functions in vitro (Cereijido et al., 1978; Leighton et al., 1970; Lever, 1979; Misfeld et al., 1976). It is not known if the toxicity seen in these cells is predictive of potential kidney toxicity, if and when the drug is used in vivo. We did not note any histopathologic evidence of toxicity in sections of kidney prepared from cotton rats treated with pyrazofurin for eight consecutive days. However, no kidney function tests were done, as we lack this capability.

Pyrazofurin did not induce diarrhea, overt stress or death when it was given i.p. at 10 mg/kg/dose for eight continuous days. We did, however, observe a 10 to 25% weight loss in treated animals that was not observed in sham inoculated control animals. Such significant weight losses are suggestive of untowards drug effects.

It has been reported that the maximum doses of pyrazofurin that can be tolerated by mice for five consecutive days is 5 mg/kg, and that patients given >5 mg/kg intravenously once weekly regularly produced intolerable mucositis (Gutowski et al., 1978). Thus the minimum consistent effective dose of pyrazofurin obtained in these studies in cotton rats against RSV is higher than the reported toxic dose of this compound for humans. This finding would normally discourage further testing of pyrazofurin. However, the high therapeutic index obtained in these studies (T.I.= 600), the anti-RSV activity seen in cotton rats, and the general non-availability of safe compounds active against RSV makes us hesitant to dismiss outright further testing of pyrazofurin.

It may be possible to reduce the toxicity of pyrazofurin and maintain its antiviral efficacy. It has been reported that the major product of pyrazofurin metabolism, orotic acid, accumulates and peaks in the blood of patients 2 to 3 days after administration of pyrazofurin, suggesting that this compound is not rapidly cleared from the body (Gutowski et al., 1978). If true, accumulation of pyrazofurin (or one of its by-products) following daily injections may be responsible for the toxicity associated with this compound. Results from our in vitro mixing studies using cotton rat blood (shown in Fig. 1) indicate that pyrazofurin is rapidly degraded (half life = 15 min). An apparent by-product, presumably orotic acid, was also rapidly degraded (T 1/2 = 15 min). However, we were unable to identify the metabolic products formed in our tests, as control reagents are not available. Identity of the by-product is important as it is possible that cotton rats metabolize pyrazofurin differently than humans. Regardless, if accumulation of pyrazofurin or one of its by-products does occur in humans, it may be possible to reduce toxicity associated with this compound by using intermittent treatment schedules. It is also possible that intermittent administration, combination therapy or delivery by continuous small particle aerosol would reduce toxic manifestation and not affect efficacy. Precedence for this exists; small particle aerosols of ribavirin have proven to be more efficacious and less toxic for treating RSV-induced disease than parenterally administered ribavirin (Gilbert and Wyde, 1988; Wilson et al., 1984). More practical perhaps, a derivative of this compound that has equivalent antiviral efficacy to pyrazofurin but less toxicity should be sought. Previous and apparently

intensive efforts to do this have failed (Petrie et al., 1986). It is hoped that the high selective index seen in previous (Descamps and De Clercq, 1978; Kawana et al., 1987; Shannon, 1977) and the present study will encourage new efforts in this direction.

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