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Evaluation of the antiviral activity of *N*-(phosphonoacetyl)-L-aspartate against paramyxoviruses in tissue culture and against respiratory syncytial virus in cotton rats

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Abstract

N-(phosphonoacetyl)-L-aspartate (PALA), a potent inhibitor of L-aspartic acid transcarbamoylase, was evaluated for cytotoxicity and antiviral activity against three different paramyxoviruses in tissue culture, and for antiviral efficacy and toxicity in vivo using a cotton rat-respiratory syncytial virus (RSV) model. Significant in vitro cytotoxicity was observed in proliferating cultures of HEp-2 ($IC_{50} = 250$ $\mu g/ml$) and Vero cells ($IC_{50} = 32$ $\mu g/ml$), but was less evident in cultures containing confluent monolayers (i.e., stationary cells) of these cells, or in cultures of Madin Darby canine kidney (MDCK) cells (these IC_{50} values were all $\geqslant 750$ $\mu g/ml$, with 1000 $\mu g/ml$ being the maximum concentration tested). Mean selective indices (ratio of the median cytotoxic dose : median efficacious dose) of 1, 72 and 146 were obtained against parainfluenza virus type 3, RSV and measles virus, respectively, when PALA was tested against these viruses using confluent HEp-2 and Vero cell monolayers. In cotton rats, significant reductions in pulmonary titers (0.8–1.4 $\log_{10}/g$ lung) compared to pulmonary viral titers in placebo-treated control animals, were consistently seen in cotton rats given $\geqslant 10$ mg of PALA/kg/day (b.i.d.) intraperitoneally on days 1–3 postinfection with either subtype A or B RSV. No toxic effects were noted even in animals given 100 mg of PALA/kg/day for 7 consecutive days.

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1. Introduction

Despite intensive study and efforts to reduce their impact, several viruses belonging to the Paramyxoviridae virus family continue to be medically important. In the United States, respiratory syncytial (RSV) and parainfluenza type 3 (PIV-3) viruses are among the leading causes of serious lower respiratory tract infection in infants and children under 2 years of age, and therefore are of the most concern (Parrot et al., 1973; Glezen et al., 1982; Channock and McIntosh, 1990). Although far less common in the United States, measles virus (MV) is the most lethal paramyxovirus worldwide, causing over 1 million deaths a year (ASM News, 1990). It remains a serious pediatric problem in the U.S. and other developed areas (Englund and Matson, 1989; Centers for Disease Control, 1991).

No vaccines are currently available for the prevention of RSV or PIV-3 infections, and despite the availability of efficacious vaccines against measles, millions of measles cases still occur each year (ASM News, 1990; Englund and Matson, 1989). No antivirals are presently approved for the treatment or prophylaxis of PIV-3 or MV infections and ribavirin is the only available antiviral agent to treat RSV infections. Unfortunately this compound is licensed for use only when given by continuous small particle aerosol (Committee on Infectious Diseases, 1993). Thus for all practical purposes, ribavirin is generally employed only to treat severe RSV infections. Moreover, because ribavirin is a potential teratogen, the utilization of this compound has become somewhat controversial (Bradley et al., 1990). It is clear that there is a need to identify new and safer compounds that can be used to treat medically important paramyxovirus infections.

N-(phosphonoacetyl)-L-aspartate (PALA) has been shown to be a potent inhibitor of L-aspartic acid transcarbamoylase (ATCase) (see Collins and Stark (1971) and Roberts et al. (1976)), the enzyme which catalyzes the condensation of L-aspartate and carbamoyl phosphate to form *N*-carbamoyl L-aspartate. Inhibition of ATCase can control and subsequently decrease the production and concentration of orotic acid, an important intermediate, and uridine, the primary endproduct of the *N*-carbamoyl L-aspartate pathway. In turn, because orotic acid and uridine are required for nucleic acid synthesis, inhibition of ATCase can lead to the inhibition of rapidly proliferating cells (Tsuboi et al., 1977; Leyva et al., 1981; Swyryd et al., 1979, reviewed in Grem et al., 1988).

Inhibitors of ATCase activity also have the potential to inhibit virus since nucleic acid synthesis is also often required for replication of these pathogens. Indeed, we have previously presented preliminary data indicating that PALA has significant antiviral activity against a broad spectrum of viruses in vitro and in vivo (Blough et al., 1993). The purpose of the present studies was to focus on the selective antiviral efficacy of PALA in vitro against three paramyxoviruses (RSV, PIV-3 and MV), and in vivo, in a cotton rat model, against RSV. Evidence is presented that PALA may have utility against RSV and MV infections.

2. Materials and methods

2.1. Animals

All of the cotton rats (*Sigmoden hispidus*) used in these studies were descendants of two pairs of animals obtained in 1984 from the Small Animal Section of the Veterinary Research Branch, Division of Research Services, National Institutes of Health. Test animals were generally between 60 and 110 g at the start of each experiment, and of either sex. However, younger (4- to 6-week-old; 40–60 g) cotton rats were used in the in vivo toxicity experiments described below. All animals were maintained in cages with barrier filters, and fed water and food ad libitum.

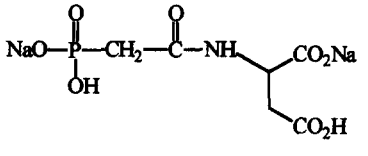
2.2. Tissue culture

Each of the tissue culture cell lines used in these studies was obtained from the American Type Culture Collection (ATCC) Bethesda, MD. HEp-2 (human epithelial carcinoma; ATCC CCL23) tissue culture cells were routinely used to prepare pools of RSV and PIV-3, and to assay for these viruses. Vero (African green monkey kidney; ATCC CCL81) cells were utilized to grow and survey for the presence of MV. Both of these cells lines, as well as Madin Darby canine kidney (MDCK; ATCC NBL-2) cells, were also used in assays designed to determine the mean inhibitory concentration (i.e., the IC_{50} or cytotoxic dose) of PALA.

These cell lines were all grown in monolayer cultures and serially passaged when they became confluent. Eagle's minimal essential medium (MEM; BioWhittaker, Inc., cat. no. 12-611B), supplemented with 10% fetal calf serum (FCS; Sigma Chemical Co., cat. no. F-2138), 100 U/ml penicillin (GIBCO Laboratories, cat. no. 600-5070AG), 100 μg/ml streptomycin sulfate (GIBCO, cat. no. 600-5070AG), 2 mM L-glutamine (GIBCO, cat. no. 320-5030AG), and 0.2% sodium bicarbonate (ICN, cat. no. 16-883-49) was used as a growth medium for these cells. MEM supplemented with 2% FCS was used to maintain HEp-2 cell cultures and in all virus and toxicity assays.

2.3. Compounds

PALA (structure shown in Fig. 1) was obtained as a powder from US Bioscience, West Conshohocken, PA. Prior to each experiment, 500 mg of this material was



N-Phosphonoacetyl-L-aspartate (PALA)

Fig. 1. Structure of *N*-phosphonoacetyl-L-aspartate, disodium salt (PALA).

dissolved in 4.0 ml of sterile distilled water containing 1.0 mg/ml of the disodium salt of ethylenediamine tetraacetic acid (EDTA-Na_2). The pH of the resulting suspension was adjusted to pH 7.2 using 12 N NaOH. The volume of the suspension was then brought to 5.0 ml using the 1 mg/ml EDTA-Na_2 in distilled water (final drug concentration = 100 mg/ml). This stock solution was sterilized by passing the suspension through a 0.2-μm filter (DynaGard, Microghon, Inc., Laguna Hills, CA) and then diluting the material in sterile water (Baxter Healthcare Corp., Deerfield, IL, cat. no. 2F7114) as needed. For in vitro experiments, the stock solution of PALA was diluted in water to a make a 4-mg/ml working concentration. Cotton rats were injected intraperitoneally (i.p.) with graded doses of PALA ranging from 5 to 100 mg/kg/day b.i.d., usually on days 1–3 post virus inoculation.

Ribavirin was used in both in vitro and in vivo experiments as a positive control. This compound was obtained from ViraTek, Covina, CA in powdered form. The compound was dissolved in distilled water to make a 90-mg/ml suspension and sterilized as described above by passage through 0.2-μm filters. This stock suspension of ribavirin was diluted with sterile water to a concentration of 4 mg/ml for in vitro experiments. Cotton rats were intraperitoneally administered 180 mg ribavirin/kg/day b.i.d. on days 1–3 post virus inoculation.

2.4. Viruses

The two RSV A subtypes used in these studies, RSV A2 (ATCC cat. no. VR1302) and RSV Long (ATCC cat. no. VR26), were obtained from the ATCC. RSV 18537, the RSV B subtype used by us, was obtained from Dr. Tony Piedra, Department of Microbiology, Baylor College of Medicine. Stocks of these viruses were prepared by infecting monolayers of HEp-2 cells. When the infected monolayers exhibited approximately 90% syncytia formation, the cells and medium from the monolayers were collected, pooled, sonicated (1 mHz for 5 min) and clarified by centrifugation (450 *g*). The resulting supernatant fluids were passed through a 0.45-μm filter (Acrodisc, cat. no. 4184, Gelman, Ann Arbor, MI), portioned, titered and stored at −70°C.

2.5. Cytotoxicity in vitro

The cytotoxicity of PALA in tissue culture cells was evaluated in sterile 96-well plates (Falcon 3072) as described in detail previously (Wyde et al., 1993), with two exceptions: (1) in some assays confluent cell monolayers (i.e., predominantly stationary cells) were used in place of the approximately 30% confluent monolayers (i.e., predominantly proliferating cells) normally used; and (2) XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide; Sigma Chemical Co., cat. no. X4251) was used instead of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma cat. no. M-2128) to measure mitochondrial respiration (and indirectly cell viability). Regardless of the density of the cell monolayers used, the PALA, ribavirin or placebo (1 mg/ml EDTA-Na_2) were tested in quadruplicate or duplicate in each assay

using serial two-fold dilutions and 2% FCS-MEM as the diluent. When the monolayers in the cell control wells became confluent (assays utilizing proliferating cells) or 48 h after the addition of the test compounds (assays utilizing confluent monolayers), XTT was added to each well. Three hours later the optical density (OD) in each well of each plate was determined using a 96-well plate reader (Molecular Devices UVMax spectrophotometer). The median inhibitory concentration was then determined by calculating the mean concentration (μg/ml) of test compound in the last wells of the replicate rows exhibiting $\leqslant 50\%$ reduction in mean OD compared to the mean OD obtained for the cell control wells.

2.6. Antiviral activity in vitro

Assays to assess the antiviral activity of PALA in tissue culture were also performed in 96-well flat-bottom tissue culture plates (Falcon 3072). In these assays, PALA, ribavirin or 1 mg/ml EDTA-Na_2 was added in quadruplicate or duplicate to the initial wells of the 96-well flat-bottom plates and serially diluted long ways up the plate using 2% FCS-MEM and serial two-fold dilutions (0.05 ml/well). The contents of each well were then transferred to parallel plates containing confluent monolayers of HEp-2 or Vero cells. A 0.05-ml volume of medium containing approximately 100 median tissue culture infectious doses ($TCID_{50}$; multiplicity of infection approximately 0.3) of the appropriate virus was then added to all wells except those set aside as antiviral (wells containing cells and antiviral agent) or tissue (wells containing cells and medium) control wells. All assay plates were incubated at 36°C in a 5% CO_2 incubator until virus control wells exhibited 70–100% cytopathic effects (CPE) including syncytia. All wells were then observed and scored for viral CPE. The mean efficacious concentration (EC_{50}) was calculated after determining the final concentration of antiviral agent in the last wells in each set of quadruplicate rows that completely inhibited CPE. At the conclusion of these assays, the IC_{50} values obtained for PALA and ribavirin were divided by their respective EC_{50} values to obtain the selective index (SI) for each of these compounds.

2.7. Collection of samples

Cotton rats were killed using CO_2 and their lungs removed. These were rinsed in sterile phosphate-buffered saline (PBS; pH 7.2) and weighed. Each set of lungs was then transpleurally lavaged using 3 ml of 2% FCS-MEM media as described previously (Wilson et al., 1980). The resulting lavage suspensions were kept on ice until assayed for RSV.

2.8. Virus quantification

Quantitative viral assays were performed in duplicate in 96-well tissue culture plates (Falcon, cat. no. 3072). In these assays, serial 0.5 $\log_{10}$ dilutions of each test sample

were made in 2% FCS-MEM. Approximately 3×10^3 HEp-2 cells were then added to each well. The plates were placed in a 36°C, CO_2 (5%) incubator for 7 days. Each day, the wells were observed for CPE including syncytia formation. Final readings were made on day 7. The amount of virus present in each suspension was expressed either as mean tissue culture infectious doses ($TCID_{50}$)/ml, or if lung suspensions were involved, geometric mean viral titers (GMT; $\log_{10}$ $TCID_{50}$/g lung). Estimations of $TCID_{50}$ endpoints were determined by the method of Karber (Rhodes and van Rooyen, 1953).

2.9. Antiviral activity in vivo

In vivo experiments were initiated by lightly anesthetizing test cotton rats with Metofane (methoxyfurane, Pitman–Moore, Mundelein, IL). Each animal was then weighed and inoculated i.n. with approximately 100 cotton rat infectious doses ($CRID_{50}$) of RSV in 0.1 ml. On each of the next 3 days (days 1–3), the animals were intraperitoneally (i.p.) injected b.i.d. with placebo (1 mg/ml EDTA-Na_2 in distilled water), ribavirin (180 mg/kg/day) or one of 4 graded doses (5, 10, 20 or 100 mg/kg/day) of PALA. All animals were killed on day 4 after virus inoculation, the day of maximum RSV pulmonary infection in untreated cotton rats. The lungs of each animal were then removed, weighed, and viral titers determined as described above. The minimum efficacious dose of test compound that caused significant reduction of mean pulmonary virus titer in treated cotton rats compared to the mean pulmonary virus titer in control animals given placebo ($CREC_{50}$) was determined at the conclusion of these virus assays.

2.10. Toxicity studies in cotton rats

Cotton rats were anesthetized, weighed and bled from the orbital sinus plexus at the start of each experiment. They were then divided into two groups and injected twice daily i.p. on this and the next 6 days with either 50 mg PALA/kg or placebo (water). Every cotton rat was observed for overt signs of toxicity (i.e., morbidity, death, diarrhea) just prior to each inoculation. On day 8, 16 h after the last inoculation, all of the animals were bled again and reweighed. The blood urea nitrogen (BUN), alanine transferase (ALT), asparagine transferase (AST) and creatine phosphate kinase (CPK) levels in the sera prepared from the blood samples collected from each animal on days 0 and 8 were determined.

2.11. Statistics

The Newman–Keuls multiple comparison test and Kruskal–Wallis analysis of variance (ANOVA) were used to compare geometric mean RSV titers obtained for each cotton rat group. (Lung fluid samples with undetectable virus titers were assigned a value of 1.0 for statistical evaluation.) Student's *t*-test was used to compare mean body weights, BUN and serum enzyme levels on days 0 and 8. These tests and the determination of all descriptive statistics (i.e., GMT $\pm$ S.D.) were all performed using

Table 1
Comparison of the cytotoxicity of ribavirin and PALA in proliferating and stationary monolayers of HEp-2, Vero and MDCK cells

Cell line	Growth condition	Mean inhibitory concentration (IC_{50}; $\mu g/ml$) [a]	
		PALA	Ribavirin
HEp-2	Proliferating [b]	250 ± 75	$> 1000 \pm 0$
HEp-2	Stationary	750 ± 37	$> 1000 \pm 0$
Vero	Proliferating	32 ± 63	$> 1000 \pm 0$
Vero	Stationary	750 ± 50	$> 1000 \pm 0$
MDCK	Proliferating	750 ± 0	$> 1000 \pm 0$
MDCK	Stationary	886 ± 26	$> 1000 \pm 0$

Note: Assays were performed in 96-well tissue culture plates. All wells were observed for cytopathic effects and/or the inhibition of cellular replication using a quantitative colorimetric XTT assay as described in Materials and methods.

[a] Means ± S.D. from two replicate experiments.

[b] In assays using proliferating cells, wells were seeded with approximately 3×10^3 of the appropriate tissue culture cells (i.e., monolayers were about 30% confluent at the start of the assay). In assays utilizing stationary cells, the wells were seeded with approximately 1×10^4 cells (i.e., the monolayers were confluent at the start of the assay).

True Epistat, a statistical program designed by T.L. Gustafson of Epistat Services, Richardson, TX, for IBM compatible computers.

3. Results

3.1. Cytotoxicity of PALA in vitro

A comparison of the IC_{50} values obtained for PALA and ribavirin in proliferating and stationary monolayers of HEp-2, Vero and MDCK cells is shown in Table 1. As indicated by the consistent > 1000 $\mu g/ml$ IC_{50} values displayed for ribavirin in all tests, this compound had no apparent cytotoxicity to any of these tissue culture cells lines, regardless of their replicative state. In contrast, PALA exhibited marked cytotoxicity in proliferating HEp-2 and Vero cells (mean IC_{50} values of 250 and 32, respectively) and some cytotoxic activity in stationary cultures of all three cells lines (the mean IC_{50} values in the stationary cultures ranged from 750 to 886 $\mu g/ml$).

3.2. Antiviral activity of PALA in vitro

A comparison of the antiviral activity of PALA and ribavirin against RSV, PIV-3 and MV in tissue culture-based assays is shown in Table 2. As indicated by the mean EC_{50} values (4–24 $\mu g/ml$) and selective indices (52–292) shown in this table, ribavirin exhibited significant selective antiviral activity against all three of the paramyxoviruses used in these studies. In contrast, PALA exhibited no significant selective antiviral activity against PIV-3 (mean SI = 1), good activity against RSV (mean SI = 72) and

Table 2
Comparison of the antiviral activity of PALA and ribavirin in Hep-2 and Vero tissue culture cells against respiratory syncytial (RSV), parainfluenza type 3 (PIV-3) and measles (MV) viruses

Cell line	Virus	EC_{50} PALA[a,b] ($\mu g/ml$)	SI PALA (IC_{50}/EC_{50})	EC_{50} ribavirin ($\mu g/ml$)	SI ribavirin (IC_{50}/EC_{50})
Hep-2	PIV-3	750 ± 89	1 ± 1	17 ± 5	63 ± 17
Hep-2	RSV	37 ± 22	72 ± 118	4 ± 2	292 ± 83
Vero	MV	8 ± 3	146 ± 42	24 ± 16	52 ± 27

Note: Assays were performed in 96-well tissue culture plates as described in Material and methods using confluent monolayers.
[a] Displayed are means ± S.D. from two replicate experiments.
[b] IC_{50}, median inhibitory (cytotoxic) concentration; EC_{50}, median efficacious concentration that inhibited virus 100%; SI, selective index.

maximal activity against MV (mean SI = 146). Similar selective indices were obtained in assays using RSV A or B subtypes (data not shown).

3.3. Antiviral activity of PALA in cotton rats

Table 3 compares pulmonary RSV titers observed in cotton rats given placebo (1 mg/ml EDTA-Na_2), ribavirin or differing doses of PALA (b.i.d. up to 100 mg/kg/day) following experimental inoculation with this virus. As indicated by the values presented

Table 3
Mean (± S.D.) pulmonary respiratory syncytial virus (RSV) titers in cotton rats given ribavirin or PALA following experimental infection

Expt.	Group	Test compound	Dose (mg/kg/day)	Mean RSV titer [a] (log_{10}/g lung)	Reduction RSV titer (log_{10}/g lung)
1	1	None (placebo)	0	4.2 ± 0.4	–
	2	Ribavirin	180	**2.8 ± 0.3**	**1.4**
	3	PALA	5	3.4 ± 0.4	0.8
	4	PALA	10	**3.0 ± 0.3**	**1.2**
	5	PALA	20	**3.1 ± 0.3**	**1.1**
2	1	None (placebo)	0	3.9 ± 0.3	–
	2	Ribavirin	180	**2.8 ± 0.4**	**1.1**
	3	PALA	5	3.1 ± 0.5	0.8
	4	PALA	10	**2.9 ± 0.4**	**1.0**
	5	PALA	20	**2.5 ± 0.8**	**1.4**
3	1	None (placebo)	0	4.2 ± 0.3	–
	2	PALA	100	**3.4 ± 0.1**	**0.8**

Note: Cotton rats were inoculated with RSV Long or RSV A2 intranasally on day 0 and given placebo, ribavirin or PALA (b.i.d., i.p.) on days 1–3. All animals were killed on day 4, at which time lungs were removed and assessed for virus.
[a] Values in bold indicate means which are significantly different ($P < 0.05$) from the mean pulmonary virus titers in placebo controls using the Kruskal–Wallis non-parametric ANOVA test; number of animals/group in Expts. 1 and 2 $\geqslant 7$; number of animals/group in Expt. 3 equalled 4.

in bold type, significant reductions in pulmonary RSV titers (compared to control animals comparably injected daily with placebo) were observed in both experiment 1 and 2 in cotton rats administered 180 mg ribavirin/kg/day or $\geqslant$ 10 mg PALA/kg/day. However, no significant reductions in mean pulmonary virus titers were observed in either experiment in cotton rats administered 5 mg PALA/kg/day. These results suggested that the minimum efficacious dose of PALA in cotton rats (i.e., the $CREC_{50}$) was between 5 and 10 mg/kg/day. A similar $CREC_{50}$ value was obtained in experiments utilizing a RSV subtype B strain (RSV 18537; data not shown).

In experiment 3, the cotton rats were administered 100 mg PALA/kg/day. Although a significant reduction of virus compared to pulmonary RSV levels in control animals was observed in this experiment, the reduction was only 0.8 $\log_{10}$/g lung. Together the results obtained in the three experiments suggested that PALA did not act in a dose-dependent manner. This statement is based on the fact that similar reductions in pulmonary virus titers were observed in these experiments (0.8–1.4 $\log_{10}$/g lung) despite the testing of a wide range of drug dosage (5–100 mg/kg/day).

3.4. Toxicity testing of PALA in cotton rats

In in vivo toxicity studies, cotton rats were administered 100 mg PALA/kg/day (b.i.d.) for 7 consecutive days. Significant loss in body weight, marked changes in BUN or levels of selected serum enzymes, diarrhea, morbidity and mortality were selected as indicators of drug-induced toxicity. The cotton rats were weighed and sera were collected from the test animals on day 0 (just before the start of the experiment) and day 8 (16 h after the last inoculation of PALA).

Although the cotton rats were inoculated daily with 10 times the protective dose of PALA (i.e., with 100 vs 10 mg PALA/kg/day), no significant differences were observed in BUN or serum enzyme levels in sera collected at this time (data not shown). A significant increase in mean body weight (14 g) was observed in the test animals over the 8-day test period (data not shown). However, such rapid weight gain is typical in cotton rats of this age (4–6 weeks) and weight (40–60 g), and was not significantly different from the 10-g increase in weight observed in untreated cotton rats over the 8-day observation period (data not shown). No diarrhea, morbidity, death or other untoward symptoms were evident in any of the test groups.

4. Discussion

In the present studies, the potential of *N*-(phosphonoacetyl)-L-aspartate (PALA) to be used as an antiviral agent against several paramyxoviruses was evaluated. In tissue culture, PALA exhibited significant cytotoxicity against all three cell lines used in these studies (HEp-2, Vero, and MDCK; Table 1). This cytotoxicity was most pronounced in monolayers of HEp-2 and Vero cells containing proliferating cells (IC_{50} = 32–750 μg/ml), and was less evident in monolayers containing predominantly stationary cells ($IC_{50} \geqslant 750$ μg/ml for all test cell lines). These results were not surprising as PALA

has been reported to inhibit the proliferation of dividing cells (Tsuboi et al., 1977; Leyva et al., 1981; Swyryd et al., 1979; reviewed in Grem et al., 1988), presumably by suppressing ATCase-mediated condensation of L-aspartate and carbamoyl phosphate to form *N*-carbonyl L-aspartate and ultimately the production and concentration of orotic acid and uridine which are essential for nucleic acid synthesis (Collins and Stark, 1971; Roberts et al., 1976). In fact, this same mechanism was most probably responsible for the significant selective indices that were obtained in these studies in vitro against RSV (SI = 72) and MV (SI = 146; Table 2). More puzzling was the apparent failure of PALA to inhibit PIV-3, a virus closely related to both RSV and MV and which has a similar replication strategy as these two viruses. Nevertheless, based on the antiviral activity seen in the in vitro studies, antiviral and toxicity studies were begun in a cotton rat-RSV model.

The in vivo studies confirmed the in vitro antiviral activity seen against RSV (Table 3). In these tests, significant reductions in mean pulmonary RSV titers, compared to virus levels in the lungs of control animals, were observed in cotton rats administered $\geqslant$ 10 mg PALA/kg/day (b.i.d.) for 3 days following experimental inoculation of the animals with RSV. These results indicated that the EC_{50} for PALA in cotton rats against RSV was between 5 and 10 mg/kg/day. However, since the reductions in pulmonary RSV titers were not substantially different in animals given 10, 20 or 100 mg/PALA/kg/day, it was concluded that once inhibitory concentrations were achieved, the activity of PALA against RSV was not dose dependent.

Despite the cytotoxicity observed in tissue culture assays, no evidence of toxicity due to PALA was observed in in vivo toxicity studies, although relatively high doses of this compound (i.e., 100 mg/kg/day or $\geqslant 10 \times$ the minimum $CREC_{50}$) were administered i.p. (b.i.d) for 7 consecutive days to young (5- to 7-week-old), rapidly growing cotton rats. In these studies BUN, ALT and AST levels were used to monitor liver function, CPK was used to follow effects on muscle and loss of weight, morbidity, mortality and diarrhea were used to assess the general well-being of inoculated animals. The only significant change observed in treated animals was a marked increase in mean body weight, and this increase was not significantly different from that observed in untreated, comparably aged cotton rats (14 vs 10 g in untreated animals; $P > 0.05$). In spite of these encouraging results, some caution should be used in interpreting these data. The effects of PALA on rapidly proliferating tissues (e.g., bone marrow or T- and/or B-cells), and the consequences of long-term treatment (such as that required by immunosuppressed patients infected with RSV; i.e., weeks to months) were not determined. This was due primarily to a limited supply of drug and the fact that cotton rat cells do not do well in in vitro assays.

In summary, the data presented in this report confirm preliminary findings presented previously (Blough et al., 1993), indicating that PALA has selective antiviral activity against paramyxoviruses, albeit not all. The data obtained also support earlier findings that PALA may be toxic to proliferating cells, and thus possesses the potential to cause adverse effects in humans. However, the data also suggest that by employing short-term regimens and reduced doses, PALA may be useful in treating both RSV- and MV-induced acute respiratory infections in immunocompetent children without being toxic. Further studies are necessary to determine this.

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