Antiviral Research, 20 (1993) 145–154

AVR 00607

The antiviral activity of SP-303, a natural polyphenolic polymer, against respiratory syncytial and parainfluenza type 3 viruses in cotton rats

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(Received 28 July 1992; accepted 29 September 1992)

Summary

SP-303, a naturally occurring polyphenolic polymer (average M.W. = 2100 Da), was tested in cotton rats (*Sigmoden hispidus*) for antiviral activity against respiratory syncytial (RSV) and parainfluenza type 3 (PIV3) viruses, and for acute toxicity. Significant reductions in pulmonary RSV titers, compared to pulmonary RSV titers in comparably treated control animals, were seen in cotton rats given 1–10 mg SP-303/kg/day intraperitoneally (i.p.) on days 1 through to 3, after experimental inoculation with RSV. The minimum efficacious dose of SP-303 against PIV3, when given i.p. for 3 days, was 3 mg/kg/day. Higher doses of SP-303 could not be given i.p., as doses $\geqslant 30$ mg/kg/day given once daily by this route for 3 or more consecutive days caused both significant weight loss and death in infected or uninfected animals. Although no toxicity was observed following oral administration of up to 270 mg of SP-303 daily for 3 days, this compound had variable antiviral activity when given by this route.

SP-303; Respiratory syncytial; Parainfluenza virus type 3; Cotton rat

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Introduction

Respiratory syncytial virus (RSV) and parainfluenza virus type 3 (PIV3) are leading causes of serious lower respiratory tract infection in children under 2 years of age (Channock and McIntosh, 1990; Glezen, Loda and Denny, 1982). There are no vaccines currently approved for use against either of these viruses. The solitary antiviral drug available for treatment of RSV, ribavirin, is only licensed for use in infants, and only when delivered by continuous small particle aerosol. No antiviral drugs are currently licensed for use against PIV3.

SP-303, a naturally occurring polyphenolic polymer derived from an *Euphorbiaceae* shrub, has been reported to have antiviral activity in tissue culture against orthomyxo- and *Paramyxoviruses* (Wyde, et al., 1991). This compound has also been reported to reduce RSV titers in monkeys experimentally infected with this virus, when administered intravenously beginning 4 h prior to virus inoculation (Soike, Zang and Meyerson, 1992). In the present studies, the antiviral efficacy of SP-303 given therapeutically, intraperitoneally (i.p.) or orally, to cotton rats experimentally infected with either RSV or PIV3 was evaluated. The results of preliminary experiments testing the acute toxicity of SP-303 in cotton rats are also presented and discussed.

Materials and Methods

Animals

All cotton rats (*Sigmoden hispidus*) used in these studies were descendants of two pairs of animals obtained from the Small Animal Section (Veterinary Research Branch, Division of Research Services, National Institutes of Health) in 1984. Test animals were between 70–110 g 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.

Tissue culture

A continuous human epithelial cell line, HEp2, was used to grow and test for the presence of RSV. These cells were obtained from the American Type Culture Collection (ATCC CCL23). Whenever flasks containing these cells became confluent, they were serially passaged using Eagle's minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 μg/ml), sodium bicarbonate (0.2%) and L-glutamine (2 mM). The medium and all supplements were obtained from Sigma Chemical, St. Louis, MO.

Viruses

RSV A2 was obtained from the ATCC (cat. no. VR1302). Stocks of this virus were prepared by infecting monolayers of HEp2 cells. When these monolayers

SP-303

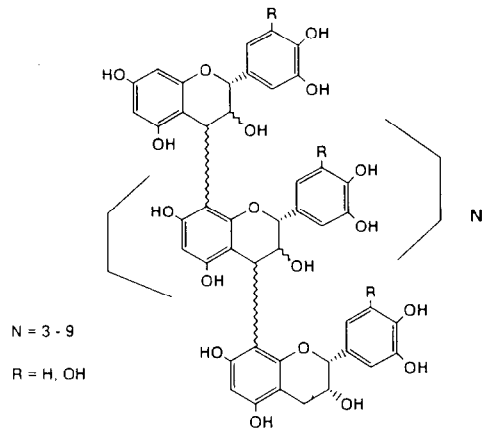

Fig. 1. Purposed structure of SP-303. This compound has been assigned as a polymer consisting of 3–9 monomers containing four related catechin and gallocatechin isomers. The mean number of monomers (N) for SP-303 is thought to be = 7.

exhibited approx. 90% syncytia formation, the medium from the monolayers was collected, pooled and clarified by centrifugation (450 × g). The resulting clarified supernatant fluids were passed through a 0.45 μM filter (Acrodisc, cat. no. 4184, Gelman, Ann Arbor, MI), portioned and stored at −70°C.

Compounds

SP-303 was submitted for antiviral testing by Shaman Pharmaceuticals, San Carlos, CA. This compound was initially extracted by Shaman from a shrub (family *Euphorbiaceae*) found throughout Central and South America and then purified by them using liquid–liquid partitioning and preparative chromatography on three different systems. Primarily, on the basis of nuclear magnetic resonance and analytical chromatography studies, SP-303 has been assigned as a polymer consisting of 3 to 9 monomers containing four related catechin and gallocatechin isomers (see Fig. 1). The material tested had an average length of 7 monomers and an average molecular weight of approx. 2100 Da. The characteristic red-brown color of SP-303 is thought to be due to the presence of small amounts (< 1% by weight) of an anthrocyanin/quinone moiety present in each preparation. Efforts to more completely elucidate the specific arrangement and linkages of the monomeric units are ongoing.

Ribavirin, used as a positive antiviral control in some experiments was obtained rom Viratek, Costa Mesa, CA. Our use of this compound to treat cotton rat experimentally infected with RSV has been previously described (Wyde et al., 1987; Gruber et al., 1987). Both SP-303 and ribavirin were suspended in sterile distilled water (Gibco, cat. no. 670-5230AJ) for injection.

Virus quantification

Assays to detect and quantify virus in different preparations were generally performed in quadruplicate in 96-well tissue culture plates (Falcon 3072, Lincoln Park, NJ). In these assays, serial 3-fold dilutions of each virus sample were made in 2% FCS-MEM. Approx. $3 \cdot 10^3$ HEp2 cells were then added to each well. The plates were placed in a 35°C, 5% CO_2 incubator for 7 days. Wells were observed daily for formation of syncytia. The amount of virus present in each suspension was expressed either as median tissue culture infectious doses ($TCID_{50}$/ml) or mean pulmonary virus titers ($\log_{10}$ $TCID_{50}$/g lung). Calculation of endpoints were made using the method of Karber (Rhodes and van Rooyen, 1953).

Collection of lungs

Lungs were removed, weighed and rinsed in sterile phosphate-buffered saline (PBS; pH 7.2). Each lung was transpleurally lavaged using 3 ml of 2% FCS-MEM as described previously (Wilson et al., 1980). Virus titers obtained using the lavage procedure are comparable to titers obtained using lung homogenates (unpublished data). The advantage of using lavage fluids is that there is little obfuscation of the bottom wells of assay plates assessing reductions of pulmonary virus in animals treated with an antiviral compound or placebo, thus increasing the sensitivity of these assays.

Experimental design for antiviral testing

Cotton rats were lightly anesthetized with ether on day 0, weighed and inoculated intranasally (i.n.) with approx. 100 median cotton rat infectious doses ($CRID_{50}$) of RSV or PIV3 in 0.1 ml. In most experiments, sterile water (placebo), SP-303 or ribavirin were administered i.p. or orally by gavage (0.1 ml), usually on days +1, +2 and +3 after virus inoculation. Animals were killed and weighed on day +4 after virus inoculation, the day of maximum RSV pulmonary infection in untreated cotton rats. At this time, the lungs of these animal were removed, weighed and assayed for virus levels as described above. The minimum efficacious dose of SP-303 was calculated by determining the minimum dose of 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.

To assess if residual drug was present in the lungs of killed animals, in some experiments, uninfected animals were given 30 mg SP-303 daily for 3 days. Sixteen hours after the last injection, the lungs from these animals were processed as described above. 3–10 $TCID_{50}$ of RSV was then added to portions of the resul-ting fluids and to comparable volumes of 2% FCS-MEM control fluids. Virus titers in these suspensions were determined as described above and compared.

Drug toxicity

Weight loss, morbidity and mortality in test animals were used as indicators

of acute drug toxicity, and were looked for in each experiment. In addition, lungs from selected animals were removed and processed for histologic examination as described below.

Histologic studies

Lungs designated for histologic studies were fixed in buffered formalin for at least 24 h, embedded in low-melting point paraffin and sectioned at 5 μM thickness. Sections were then stained with hematoxylin and eosin, and observed blinded by an observer.

Statistics

Student's t test was used to compare mean percent weight changes. Geometric mean pulmonary virus titers (GMPT) were compared using the Mann-Whitney U rank sum test. These tests and the determinations of GMPT, means and standard deviations were all performed using True Epistat, a statistical program designed by T.L. Gustafson of Epistat Services, Richardson, Texas, for IBM compatible computers.

TABLE 1

Comparison of the in vivo antiviral efficacy of SP-303 and ribavirin administered intraperitoneally to cotton rats experimentally infected wit RSV[a]

Daily treatment (mg/kg)	Pulmonary RSV titers (GMT ± S.D.) $\log_{10}$/gm lung	% Reduction virus titer	Significance[b] (Mann–Whitney) (P-value)
Experiment 1			
Placebo	4.7 (0.3)	–	–
SP-303 (1.0)	4.1 (0.3)	75	n.s. (0.06)
SP-303 (3.0)	3.3 (0.4)	96	$P=0.03$
SP-303 (10.0)	3.1 (0.3)	97	$P=0.03$
Ribavirin (90)	2.8 (0.4)	99	$P=0.03$
Experiment 2			
Placebo	4.4 (0.3)	–	–
SP-303 (1.0)	2.9 (0.5)	97	$P=0.03$
SP-303 (3.0)	2.8 (0.4)	97	$P=0.03$
SP-303 (10.0)	2.8 (0.6)	97	$P=0.03$
Ribavirin (90)	2.9 (0.3)	97	$P=0.03$
Experiment 3			
Placebo	4.9 (0.5)	–	–
SP-303 (1.0)	4.2 (0.3)	80	n.s. (0.06)
SP-303 (3.0)	4.1 (0.3)	84	n.s. (0.06)
SP-303 (10.0)	3.3 (0.4)	97	$P=0.03$
Ribavirin (90)	2.8 (0.4)	99	$P=0.03$

[a]Cotton rats were inoculated intranasally with approx. 100 median infectious dose of RSV on day 0. On days +1, +2 and +3 animals were given SP-303, ribavirin or placebo i.p. All animals were killed on day +4 and the lungs from these animals were tested for virus.

[b]Means were compared using the Mann-Whitney U rank sum test; P values are for two tails; no. animals/group = 4; n.s. = not statistically significant.

TABLE 2

Antiviral efficacy of SP-303 administered intraperitoneally (i.p.) to cotton rats experimentally infected with parainfluenza type 3 virus (PIV3)[a]

Daily treatment (mg/kg)	Pulmonary RSV titers (GMT ± S.D.) $\log_{10}$/gm lung	% Reduction virus titer	Significance[b] (Mann–Whitney) (*P*-value)
Experiment 1			
Placebo	4.2 (0.2)	–	–
SP-303 (1)	3.7 (0.3)	68	n.s.
SP-303 (3)	3.3 (0.3)	87	$P=0.05$
SP-303 (10)	3.0 (0.1)	94	$P=0.03$
Experiment 2			
Placebo	4.3 (0.3)	–	–
SP-303 (1)	4.3 (0.3)	0	n.s.
SP-303 (3)	3.3 (0.3)	90	$P=0.03$
SP-303 (10)	3.4 (0.1)	87	$P=0.03$

[a]Cotton rats were inoculated intranasally with approx. 100 median infectious doses of PIV3 on day 0. On days +1, through to +3, animals were injected i.p. once a day with SP-303 or placebo (water). All animals were killed on day +4 and the lungs from these animals were tested for virus.
[b]Means were compared using the Mann-Whitney U rank sum test; *P* values are for two tails: no. animal/group = 4; n.s. = not statistically significant.

Results

Antiviral activity of SP-303 following i.p. administration

Table 1 shows results obtained in three experiments in which cotton rats experimentally inoculated with RSV were administered SP-303 or ribavirin i.p. As indicated, although >75% reductions in pulmonary RSV titers, compared to pulmonary titers in placebo control animals, were observed in all experiments in cotton rats given ≥1 mg SP-303/kg/day, only animals given 10 mg SP-303/kg/day consistently exhibited significant decreases ($P>0.05$) in pulmonary virus. In all of these experiments, cotton rats given 90 mg ribavirin/kg/day (positive controls) had significant reductions in GMPT compared to control animals.

PIV3 levels in lungs of cotton rats experimentally inoculated with this virus and administered single daily doses of SP-303 or placebo i.p. are shown in Table 2. In experiment 1, a significant reduction in PIV3 titer was observed only in the group of animals given 10 mg SP-303/kg/day (94%; $P=0.03$), although animals administered 3 mg of SP-303/kg/day also had markedly lower pulmonary RSV titers than control animals (an 87% reduction; $P=0.06$). In the second experiment, animals given either 3 or 10 mg of SP-303/kg/day i.p. had significantly reduced pulmonary PIV3 titer compared to the pulmonary virus titers in lungs of cotton rats given placebo. In neither experiment, was protection against pulmonary virus infections observed in animals given 1 mg SP-303/kg/day.

TABLE 3

Antiviral efficacy of SP-303 given orally twice daily in divided doses to cotton rats experimentally infected with RSV[a]

Daily treatment (mg/kg)	Pulmonary RSV titers (GMT ± S.D.) $\log_{10}$/gm lung	% Reduction virus titer	Significance[b] (Mann–Whitney) (*P*-value)
Experiment 1			
Placebo	4.5 (0.3)	–	–
SP-303 (0.3)	4.1 (0.3)	60	n.s.
SP-303 (1.0)	4.1 (0.3)	60	n.s.
SP-303 (3.0)	3.8 (0.3)	80	$P=0.03$
Experiment 2			
Placebo	4.4 (0.6)	–	–
SP-303 (1)	4.0 (0.5)	60	n.s.
SP-303 (3)	2.2 (0.5)	99	$P=0.03$
SP-303 (10)	1.9 (0.5)	99.7	$P=0.03$
SP-303 (30)	1.9 (0.4)	99.7	$P=0.03$
Experiment 3			
Placebo	4.1 (0.3)	–	–
SP-303 (10)	3.5 (0.5)	90	n.s.
SP-303 (30)	4.5 (0.5)	75	n.s.
SP-303 (90)	4.7 (0.3)	60	n.s.
Experiment 4			
Placebo	5.1 (0.3)	–	–
SP-303 (10)	4.1 (0.6)	90	n.s.
SP-303 (30)	4.5 (0.5)	75	n.s.
SP-303 (90)	4.7 (0.3)	60	n.s.

[a]Cotton rats were inoculated intranasally with approx. 100 median infectious doses of RSV on day 0. On day +1, through to +3, animals were injected i.p. once a day with SP-303 or placebo (water). All animals were killed on day +4 and the lungs from these animals were tested for virus.

[b]Means were compared using the Mann-Whitney U rank sum test; *P* values are for two tails: no. animals/group = 4; n.s. = not statistically significant.

Antiviral activity of SP-303 administered orally

Several experiments was performed to determine the antiviral efficacy of SP-303 against RSV following oral administration of this compound once daily by gavage. Although some reductions in GMPT were seen in these and similar experiments (data not shown), the reductions were variable, usually not statistically significant and without apparent pattern. Because of these results, a series of experiments were performed in which SP-303 was given orally, twice daily, in divided doses. Data from some of these experiments are shown in Table 3.

As indicated by the data in Table 3, marked reductions in pulmonary RSV titers were observed in some groups of animals given SP-303 twice daily by the oral route of administration. However, no significant virus-inhibitory activity was observed in animals given $\leqslant 1$ or $\geqslant 30$ mg SP-303/kg/day, and although 80 to >99% reductions in RSV titers were seen in cotton rats given 3 or 10 mg SP-303/kg/day, only some of these reductions in virus titer were statistically significant.

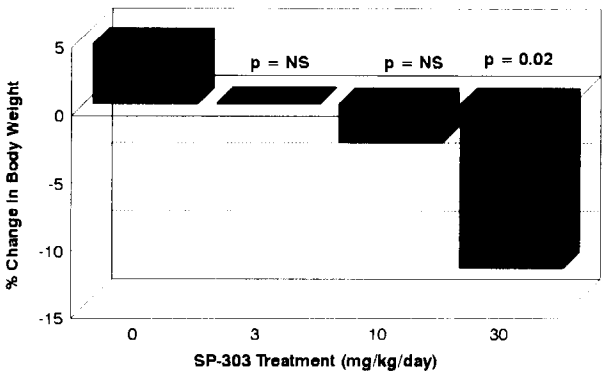

Fig. 2. Percent weight changes in virus-infected cotton rats treated with SP-303 intraperitoneally. Animals were inoculated with respiratory syncytial virus on day 0 and given placebo (water) or 3–30 mg of SP-303/kg once daily on day +1 through to +3. All animals were weighed at the start of the experiment and on the last day of the experiment (day +4, one day after the last inoculation of drug). The *P* values displayed were obtained by comparing the mean percent change in body weight in each group of cotton rats given SP-303 with the mean change in weight in animals given placebo daily. The mean body weights of the animals at the start of this experiment were from 70–100 g.

Effects of SP-303 on cotton rats

Fig. 2 shows the percent change in body weight observed in virus-infected cotton rats injected once daily for 3 consecutive days with 3–30 mg of SP-303/kg. As indicated, animals inoculated with virus on day 0 and given placebo on days +1 through +3, had an average 4.4% increase in weight during the 4 day course of this experiment (animals were harvested on day +4, one day after the last inoculation of SP-303). Such short-term weight gains are the rule in cotton rats less than 100 g (data not shown), but more importantly, this result makes evident the fact that RSV infection does not ordinarily induce loss in cotton rat body weight. In contrast to the weight gain seen in virus infected-placebo-treated animals, cotton rats similarly infected with virus and given 3 mg of SP-303/kg/day averaged only a 0.07% increase in weight, while animals injected daily with 10 mg of this compound/kg/day, had a mean loss in weight of 2.9%. Although neither of these % losses were significant compared to the % weight change in placebo controls using Student's *t* test, cotton rats given 30 mg/kg/day for three consecutive days exhibited a significant mean % weight loss ($P=0.02$) of 12.1%, and 1 of 4 animals in this group died. Similar losses in body weight and mortality were observed in non-RSV-infected cotton rats following administration of comparable doses of SP-303 i.p. Dose of 90 mg SP-303/kg/day were nearly always lethal to cotton rats.

No notable histopathological changes were observed in stained sections of lungs taken from uninfected animals given 30 or 90 mg SP-303/kg/day, nor was the histopathology observed in lungs of uninfected or virus-infected animals treated with SP-303, noticeably different. The lack of pulmonary histopathology attributable to SP-303 treatment suggested that the adverse effects of this compound (i.e., significant weight loss and death) were extrapulmonary.

However, histopathologic studies were not performed on other organs to confirm this.

No significant losses in body weight or mortality were observed in cotton rats given up to 270 mg/kg/day orally by gavage for 3 days. The lungs of these animals, whether uninfected or virus-infected, showed no extraordinary pulmonary histopathology compared to appropriate control animals.

Discussion

This report describes results of experiments testing the antiviral activity of a 2100 Da polyphenolic plant extract, SP-303, in cotton rats. Also described are results of preliminary acute toxicity studies on this compound. In the experiments described, SP-303 reduced pulmonary RSV titers in cotton rats given 1 to 10 mg/kg/day of this compound i.p. (Table 1), and pulmonary PIV3 titers in animals given 3 or 10 mg/kg/day by this route (Table 2). These results were in general agreement with those obtained in in vitro studies (Wyde et al., 1991). Reductions in pulmonary virus titers in animals given these doses generally ranged from 75 to 97% compared to pulmonary titers in control animals given placebo.

Unfortunately, doses $\geqslant 30$ mg of SP-303/kg/day could not be given to cotton rat parenterally. In experiments where $\geqslant 30$ mg of SP-303/kg were given i.p. for 3 or more consecutive days, the injected animals lost significant amounts of weight (Fig. 2) and death of the injected animals was not infrequent. Histopathologic studies of the lungs of SP-303 treated cotton rats suggested that the adverse effects seen were extrapulmonary. More extensive toxicity studies are now being pursued by Shaman Pharmaceuticals.

No evidence of toxicity was observed in cotton rats given SP-303 orally by gavage, even at the maximum doses given (270 mg/kg/day). However, when SP-303 was given by this route of administration, reductions in pulmonary RSV titers were variable and often not significant. Better results were seen when SP-303 was administered orally in divided doses than when given once daily. However, even when given twice daily, significant reductions in pulmonary virus titers were observed only at $\geqslant 1$ or $\leqslant 30$ mg/kg/day. Presumably, the failure of the lower doses of SP-303 to inhibit virus in these experiments was due to insufficient pulmonary drug levels to inhibit virus. It is not known why doses $\geqslant 30$ mg/kg were not efficacious. One conjecture is that aggregation of the SP-303 occurred, interfering with drug adsorption and/or transport. This speculation is based primarily on the observation that precipitation of SP-303 was frequently seen in solutions of this compound containing $\geqslant 30$ mg/ml that were stored at room temperature or at 4°C.

Mode of action studies with SP-303 have been performed by Barnard et al. (1992) suggesting that SP-303 causes an inhibition of penetration of RSV into cells, presumably by binding directly to virus or host cell membranes (or both). In assays performed in our own laboratories, SP-303 has been found to

hemagglutinate human, guinea pig or chicken erythrocytes at concentrations as low as 1 μg/ml, indicating that this compound does indeed bind to cytoplasmic membranes. It is not known if this affinity for membranes is also involved in the toxic manifestations of SP-303 seen in these studies.

SP-303 has now been reported to exhibit selective antiviral activity in tissue culture against several respiratory viruses (Wyde et al., 1991), and to reduce pulmonary RSV titers in experimentally infected monkeys if given 4 h prior to virus (Soike, Zang and Meyerson, 1992). The present studies extend these findings and indicate that this compound has some selective antiviral activity against both RSV and PIV3 when given therapeutically. However, based on % weight loss and mortality, the maximum therapeutic index (T.I.) of SP-303 against RSV when given parenterally, would appear to be 30, and probably is less. Against PIV3, the maximum T.I. was 10. Such a low T.I., would seem to preclude parenteral use of SP-303 in humans, particularly infants, which would comprise the major target population that the compound would be used in.

On the positive side, it may be possible to identify derivatives of SP-303, or related compounds, which may have greater selective antiviral activity. The need to develop more inexpensive and practical mean to treat *Paramyxovirus*-induced infections, should encourage such efforts.

Acknowledgements

This work was supported by National Institute of Allergy and Infectious Disease (Antiviral Research Development Branch) contract NO1-AI-15099.

References

Channock, R.M. and McIntosh, K. (1990) Parainfluenza viruses. In: B.N. Fields (Ed.), Virology, pp. 963–988. Raven Press, New York.

Barnard, D.L., Huffman, J.H., Nelson, R.M., Morris, J.L.B., Gessaman, A.C., Sidwell, R.W. and Meyerson, L.R. (1992) Mode of action of SP-33 against respiratory syncytial virus (RSV). Antiviral Res. Suppl. 1:91 No. 176.

Glezen, W.P., Loda, F.A. and Denny, F.W. (1982) The parainfluenza viruses. In: A.S. Evans (Ed.), Viral Infection of Humans: Epidemiology and Control, pp. 337–349. Plenum Press, New York.

Gruber, W.C., Wilson, S.Z., Throop, B.J. and Wyde, P.R. (1987) Combined immunoprophylaxis and ribavirin therapy of respiratory syncytial virus (RSV) infection in the cotton rat. J. Pediatr. Res. 21, 270–274.

Rhodes, A.J. and Van Rooyen, C.E. (1953) Textbook of Virology, 2nd edit, pp. 66–69. Williams and Wilkins, Baltimore, Maryland.

Soike, K.F., Zhang, J.-Y. and Meyerson, L.R. (1992) Reduction of respiratory syncytial virus (RSV) shedding in African Green monkeys treated with SP-303. Antiviral Res. Suppl. 1:91 No. 89.

Wilson, S.Z., Knight, V., Wyde, P.R., Drake, S. and Couch, R.B. (1980) Amantidine and ribavirin aerosol treatment of influenza A and B infection in mice. Antimicrob. Agents Chemother. 17, 642–648.

Wyde, P.R., Wilson, S.Z., Petrella, R. and Gilbert, B.E. (1987) Efficacy of high dose-short duration ribavirin aerosol in the treatment of respiratory syncytial virus infected cotton rats and influenza B virus infected mice. Antiviral Res. 7, 211–220.

Wyde, P.R., Meyerson, L.R., Ambrose, M.W., Pfeifer, J.P., Voss, T.G. and Gilbert, B.E. (1991) Elucidation of a polyphenolic polymer with antiviral activity against myxo- and paramyxoviruses. Antiviral Res. Suppl. 1:67 No. 45.