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SP-303 Small-particle aerosol treatment of influenza A virus infection in mice and respiratory syncytial virus infection in cotton rats

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Summary

A natural plant product, SP-303, was administered by small-particle aerosol to influenza A/HK virus-infected mice and RSV-infected cotton rats. Aqueous SP-303 at 2 mg/ml in the Collison nebulizer reservoir generated an aerosol with an output of 26 μg/l and a particle size distribution of 1.4 μm $\pm$ 4.6 (MMAD $\pm$ GSD). SP-303 at a dosage of 0.5–9.4 mg/kg per day administered for 3–4 days significantly increased both the rate and duration of survival of mice lethally infected with influenza A/HK virus. SP-303 was toxic to mice at 16 mg/kg per day as indicated by weight loss and a decrease in the duration of survival compared to control animals. From these data, a maximum therapeutic index (T.I.) of 12 was calculated. SP-303 given 3–4 days at dosages of 1.3–9.8 mg/kg per day was effective in reducing the pulmonary titer of RSV in infected cotton rats. However, at the 18.7 mg/kg per day dose a significant weight loss compared to control animals was observed; a T.I. of $\leqslant$14 was estimated. These experiments demonstrate that aerosol administration of SP-303 was effective in the treatment of influenza A-infected mice and of RSV-infected cotton rats.

Aerosol; SP-303; RSV; Influenza virus; Mice; Cotton rats; Ribavirin

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Introduction

SP-303 is a naturally occurring polyphenolic polymer derived from botanical sources with reported in vitro antiviral activity against influenza A and B viruses and respiratory syncytial virus (RSV) (Wyde et al., 1991; Barnard et al., 1992; Wyde et al., 1993b). The compound also has been shown to have antiviral activity against RSV when administered intraperitoneally or orally to RSV-infected cotton rats (Wyde et al., 1993a) or intravenously to RSV-infected African Green monkeys (Soike et al., 1992). However, the therapeutic effect was limited when given by these routes of administration.

In an effort to increase the therapeutic effectiveness of this compound, small-particle aerosol (SPA) administration of SP-303 was tested. Previous studies with ribavirin delivered by SPA to target drug to the infected respiratory epithelium demonstrated the effectiveness of this route (Wyde et al., 1986; Wyde et al., 1987; Gilbert et al., 1992). The present study demonstrates the effectiveness of SPA administration of SP-303 in increasing the rate and duration of survival of mice infected with influenza A virus and in reducing the pulmonary titer of RSV in infected cotton rats.

Materials and Methods

Materials

SP-303 (Lot Batch C, DFS 17–56; Lot Batch D, DFS 17–57/58 and Lot RCB 22–23/25) was provided by Shaman Pharmaceuticals, (San Carlos, CA). Ribavirin was provided by Viratek (Costa Mesa, CA).

Animals

6–8-Week-old (22–28 gm), random-bred, SAS/ICR mice obtained from SASCO, Houston, TX, were used in the influenza virus experiments. The animals were housed in cages covered with barrier filters and fed mouse chow and water ad libitum.

4–8-Week old cotton rats (*Sigmoden hispidus*, 50–100 gm) of either sex were used in the RSV experiments. These animals were obtained from a colony maintained by us. All animals were maintained in cages with barrier filters and fed water and food ad libitum.

Viruses

Influenza A/HK/68 (H3N2) used in these studies was isolated in 1968 from a patient with influenza. The adaptation of this virus to mice and its characteristics were described previously in detail (Wyde et al., 1977). RSV (A2) was obtained from the ATCC (cat. no. VR 1302). Stocks of this virus were prepared by infecting monolayers of HEp2 cells, harvested and pooled as described previously (Wyde et al., 1990).

Small particle aerosol characteristics and treatment

The Collison aerosol generator and ribavirin particle characteristics were as previously described (Knight et al., 1986a; Wilson et al., 1980). The concentration of SP-303 generated in the aerosol and the particle size distribution were determined by using an all-glass impinger and an Andersen sampler, respectively, as previously described (Knight et al., 1986a). Samples from the all-glass impingers were analyzed directly, while those collected by the Andersen sampler were eluted from the filters by soaking the filters in 10 ml of absolute methanol for 1 h. SP-303 was quantitated spectrophotometrically (absorbance at 210 nm) using a standard curve (range 1–20 μg/ml). Mice or cotton rats were placed in sealed plastic cages and exposed to aerosols as previously described (Gilbert and Wyde, 1988; Wyde et al., 1986; Gilbert et al., 1992). The estimated dose of drug retained was calculated from the amount of drug in the aerosol, the minute volume of the animal (Phalen, 1984), a retension factor (0.3 for mice, 0.5 for cotton rats), and the duration of treatment as previously described (Gilbert and Knight, 1986; Wyde et al., 1986).

Virus inoculation

Mice were inoculated with influenza A/HK by small particle aerosol (Wilson et al., 1980; Gilbert et al., 1992). Briefly, a 1:500 dilution of a mouse lung pool of influenza A/HK/68 (H3N2) (1×10^6 $TCID_{50}$/ml) in 0.5% gelatin-MEM was administered by small particle aerosol for 20 min (day 0); the estimated exposure was approximately 2 $TCID_{50}$ of virus/animal. For the next 3 or 4 days (days +1 through +3 or +4, see Fig. 1), animals were treated with SP-303 or ribavirin (positive control) aerosol. Animals were observed for mortality for 21 days.

Cotton rats (day 0) were weighed, anesthetized lightly with ether and inoculated intranasally (i.n.) with approximately 100 median cotton rat infectious doses ($CRID_{50}$) of RSV in 0.1 ml (day 0) (Wyde et al., 1987; Wyde et al., 1990). On day +1 through day +3, animals were exposed to SP-303 or ribavirin (positive control) aerosol. On day +4, the day of maximum RSV pulmonary infection in untreated cotton rats, all animals were killed. The lungs of each animal were removed, weighed and assayed for virus levels as previously described (Wyde et al., 1987).

Virus quantification

Lung homogenates from animals inoculated with influenza A/HK virus were serially diluted (3-fold) using minimal essential medium (MEM) and tested in Madin Darby canine kidney (MDCK) tissue cells using MEM containing trypsin (2 μg/ml) without fetal calf serum (FCS) as described previously (Wyde et al., 1990; Gilbert et al., 1992). After incubation for 5 days at 36°C, 0.05 ml of a 0.5% suspension of chicken erythrocytes was added to each well. Wells exhibiting hemagglutination were considered to be infected with influenza virus.

In assays for RSV, serial 3-fold dilutions of each virus sample were made in

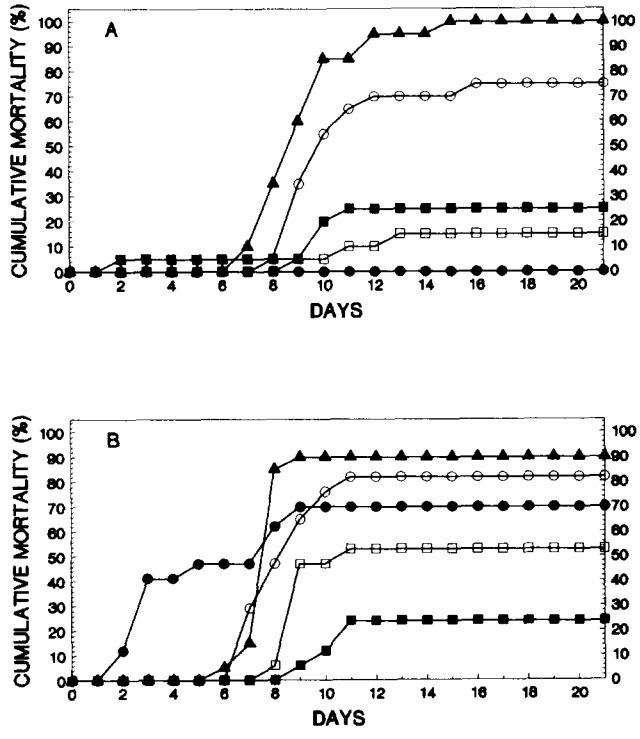

Fig. 1. Effect of SP-303 aerosol treatment on the mortality of influenza A/HK virus-infected mice. Mice ($N = 15$–20 in each group) were exposed to SP-303 aerosol for 2 h, twice daily. In panel A, SP-303 aerosol was administered for 3 days at 0 (▲), 0.5 (○), 1.9 (□) and 9.4 (■) mg/kg per day; ribavirin (●) was administered for 3 days at 43 mg/kg per day. In panel B, SP-303 aerosol was administered for 4 day at 0 (▲), 0.25 (○), 1.0 (□), 4.0 (■) and 16 (●) mg/kg per day.

2% FCS-MEM. Approximately 3×10^3 HEp2 cells were then added to each well. Plates were placed in a 35°C, 5% CO_2 incubator for 7 days. Wells were observed daily for formation of syncytia or other CPE. Mean virus titers (log_{10}/gm of lung tissue) were determined by calculating the means of the last dilutions in replicate rows that contained virus (Wyde et al., 1990; Gilbert et al., 1992).

Statistical analysis

Data analysis was performed using True Epistat™ statistical package from Epistat Services, Richardson, Texas. *P*-Values are based on two-tailed analysis of these data by the Student's *t* test, with ANOVA; Fisher's exact test; or by Life Table Analysis.

Results

SP-303 aerosol particle characteristics

SP-303 resuspended in water at 2 mg/ml was characterized in an aerosol generating system utilizing a Collison nebulizer as previously described (Knight et al., 1986a). The mean output (± standard deviation) after 1 h of aerosolization was 26.0 ± 1.5 μg of SP-303/L of aerosol. The mass median aerodynamic diameter (MMAD) of the aerosol particle was determined to be 1.4 μm with a geometric standard deviation (GSD) of 4.6. An aerosol particle with these characteristics will be distributed throughout the respiratory tract (Knight et al., 1986b; Knight and Gilbert, 1987; Knight et al., 1988a; Knight et al., 1988b). Increasing the concentration of SP-303 in the reservoir of the aerosol generator to increase the amount of drug in the aerosol did not significantly alter these characteristics.

Effect of SP-303 aerosol treatment on influenza A/HK-infected mice

To test the protective efficacy of SP-303 aerosol, mice given a lethal dose of influenza A/HK were exposed to aerosols containing different concentrations of SP-303 for 2 h, twice daily, for 3–4 days. Doses of SP-303 in the range of 0.5–9.4 mg/kg per day were effective in significantly increasing the duration and rates of survival of mice (Fig. 1, Table 1). Ribavirin aerosol, used as a positive

TABLE 1

Effect of SP-303 aerosol treatment on the duration and rate of survival of mice infected with influenza A/HK virus

SP-303 Daily treatment schedule[1]		Rate of survival (No. survivors/total)			Duration of survival[3] (*P* value)
Dosage (mg/kg)	Regimen (No Rx/Time)	Untreated	SP-303-Treated	*P* Value[2]	
0.25	2 × 2 h[4]	2/20	3/17	0.518	0.550
0.5	2 × 2 h	0/20	5/20	0.047[5]	0.012[5]
1.0	2 × 2 h[4]	2/20	8/17	0.023	<0.001
1.9	2 × 2 h	0/16	10/15	0.018[6]	0.005[6]
1.9	2 × 2 h	0/20	17/20	<0.001[5]	<0.001[5]
4.0	2 × 2 h[4]	2/20	13/17	<0.001	<0.001
8.4	1 × 18 h	7/15	13/15	0.023	0.018
9.4	2 × 2 h	0/20	15/20	<0.001[5]	<0.001[5]
16.0	2 × 2 h	2/20	4/17	0.383	0.159

[1]Duration of aerosol treatment was 3 or 4 days starting 24 h after virus inoculation by aerosol. The table represents cumulative data from 4 independent experiments.

[2]Untreated vs. SP-303 treated; Fisher Exact Test, two-tailed.

[3]Untreated vs. SP-303 treated; Life Table Analysis, two-tailed.

[4]Aerosol treatment was extended to 4 days.

[5]Ribavirin (43 mg/kg per day; 2 × 2 h), administered by aerosol as a positive control in these studies, was effective in increasing both the rate and duration of survival ($P < 0.001$; Fisher Exact Test and Life Analysis Table, two-tailed, respectively).

[6]Ribavirin (65 mg/kg per day; 1 × 18 h), administered by aerosol as a positive control in these studies, was effective in increasing both the rate and duration of survival ($P < 0.001$; Fisher Exact Test and Life Analysis Table, two-tailed, respectively).

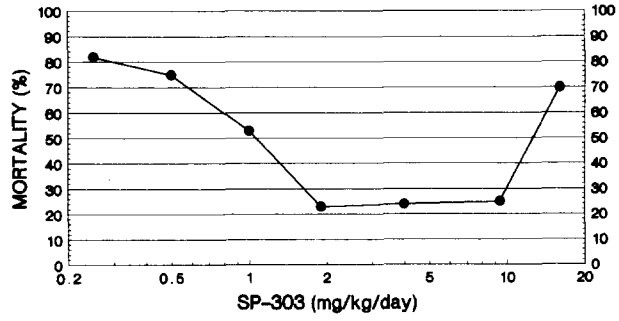

Fig. 2. Effect of the dose of SP-303 administered as an aerosol on mortality in influenza A/HK virus-infected mice. Mortality in untreated mice was 90–100%.

control (Wyde et al., 1986; Gilbert et al., 1992), was effective (Fig. 1A). SP-303 aerosol administered at 0.25 mg/kg per day was not effective (Table 1) and at 16 mg/kg per day was toxic (Fig. 1B, Table 1). Although initially our standard treatment protocol of 18 h of continuous aerosolization was used (Table 1), the shorter treatment regimen of 2 h, twice daily was also effective.

When mortality was used as an indicator of effectiveness, the concentration of SP-303 that reduced mortality by 50% (ED_{50}) was determined to be 1 mg/kg per day (Fig. 2). The median toxic dose (ID_{50}) was estimated to be 12 mg/kg per day, resulting in a therapeutic index (T.I., ID_{50}/ED_{50}) of about 12.

The adverse effects of the drug were manifested as acute toxicity with early mortality beginning on day +2 (Fig. 1B), and by a decrease in mean body weight over 8 days. At the highest dose tested (16 mg/kg per day), it could be seen by the end of the second treatment on day +1 that mice were visibly affected by SP-303 aerosol treatment. This was in contrast to animals exposed to the ribavirin aerosol, who did not exhibit similar distress. Mortality in the SP-303-treated group reached 47% by day +5 (last day of treatment), a period when there was no death in the infected control animals; this was statistically significant ($P < 0.001$). Over the next 5 days, 90% of the control animals died from influenza infection, while only 3 of the remaining 7 mice (43%) in the 16 mg/kg per day group died. Thus, of the animals that survived the initial treatment at 16 mg/kg per day, significantly fewer died than seen overall in the control group ($P = 0.024$).

Animals exposed to the 16 mg/kg per day dose of SP-303 also exhibited a significant decrease in body weight. On day 0, untreated and the 16 mg/kg per day-treated groups weighed 26.3 and 25.4 gm, respectively. Following treatment (day +5), the untreated and treated mice weighed 25.4 and 18.2 gm, respectively; this 7.2 gm difference (28%) was statistically significant ($P < 0.001$). The greater loss in weight in the treated group continued through day +8 after which untreated animals began to resolve their infection and to gain weight. The difference in body weight on day +8 between untreated and treated mice (20.7 vs. 15.1 gm) was statistically significant ($P < 0.001$). The

TABLE 2

Effect of SP-303 aerosol treatment on the pulmonary titer of RSV in cotton rats

SP-303 Daily treatment schedule[1]		RSV Titer[2]			*P* Value[3]	
Dosage (mg/kg)	Regimen (No. Rx/Time)	Untreated	SP-303-Treated	Ribavirin-Treated	SP-303	Ribavirin
0.3	2 × 2 h	3.7 (0.2)	3.7 (0.5)	<2.0 (0)	0.800	<0.001
0.5	2 × 2 h	4.8 (0.4)	4.9 (0.4)	3.9 (0.4)	0.942	0.008
1.3	2 × 2 h	3.7 (0.2)	3.0 (0.3)	<2.0 (0)	0.003	<0.001
2.2	2 × 2 h	4.7 (0.3)	2.7 (0.3)	3.5 (0.4)[5]	<0.001	0.003
2.2	2 × 2 h	4.8 (0.4)	4.2 (0.5)	3.9 (0.4)	0.060	0.008
3.5	2 × 2 h	3.7 (0.2)	3.0 (0.2)	<2.0 (0)	0.006	<0.001
9.8	1 × 18 h	4.3 (0.2)	3.6 (0.2)	3.5 (0.5)[5]	<0.005	0.029
9.8	1 × 18 h	4.7 (0.3)	3.9 (0.4)	3.5 (0.4)[5]	0.02	0.003
10.9	2 × 2 h	4.8 (0.4)	4.1 (0.4)	3.9 (0.4)	0.072	0.008

[1]Duration of aerosol treatment was 3 days starting 24 h after virus inoculation. The table represents cumulative data from 4 independent experiments.

[2]Mean $\log_{10}$ (± S.D.)/gm of lung for cotton rats ($N=4$) determined on day +4 following virus inoculation.

[3]Untreated vs. SP-303 or ribavirin-treated; Student's *t* test on the transformed data, two-tailed.

[4]Unless otherwise noted, ribavirin (50 mg/kg per day) administered by aerosol (2 × 2 h) was used as a positive control.

[5]Ribavirin was administered 1 × 18 h (75.6 mg/kg per day).

other doses of SP-303 tested did not produce a statistically significantly greater loss of weight compared to the untreated mice.

Effect of SP-303 aerosol treatment on RSV-infected cotton rats

When SP-303 aerosol was administered for 3 consecutive days, regimens of 1.3–9.8 mg/kg per day were effective in significantly reducing the pulmonary titer of RSV-infected animals (Table 2). Ribavirin ($\geqslant 50$ mg/kg per day) was effective as a positive control. The shorter-high dose regimen (i.e., 2 × 2 h) was effective and appeared to reduce lung titers as well as the continuous 18 h protocol.

While no overt signs of toxicity due to SP-303 aerosol administration were observed in these experiments, when cotton rats were exposed to 18.7 mg of aerosolized SP-303/kg per day for 3 days (data not shown), there was a significant loss in body weight at day +4. Cotton rats lost on an average 11.4% (9 gm) of their initial body weight ($P < 0.001$).

Discussion

The generation of SP-303 aerosols was readily achieved. This water-soluble antiviral agent produced an aerosol with an output and particle size distribution similar to that of ribavirin, which was employed in these studies as a positive control. Although the MMAD was similar to ribavirin, the geometric standard deviation, an indicator of the heterogeneity of the particle

size, was greater, suggesting that larger particles were being produced. However, the bulk of the aerosol particles were within the size range that would allow deposition throughout the respiratory tract (Knight et al., 1986b; Knight and Gilbert, 1987; Knight et al., 1988a; Knight et al., 1988b).

In vitro, SP-303 is effective at inhibiting influenza virus and RSV replication at lower concentrations than ribavirin (Wyde et al., 1991; Wyde et al., 1993b), although the concentrations that induce cytotoxicity levels are also lower. Similar results were observed in these in vivo studies. SP-303 was effective in protecting mice against a lethal challenge of influenza A virus at a dosage of 0.5–1 mg/kg per day. These doses are considerably less than the minimal aerosol ribavirin concentrations necessary to protect against the same influenza A virus model (10 mg/kg per day, (Gilbert et al., 1992)). SP-303 produced adverse effects at 16 mg/kg per day. The median toxic dose is probably lower since severe weight loss and death probably occur as a result of more subtle effects not measured. Thus, the therapeutic index is probably less than the calculated one of approximately 12.

SP-303 was also effective in reducing RSV infection in cotton rats at a concentration of about 1.3 mg/kg per day. However, a significant weight loss was observed at the highest dose (18.7 mg/kg per day for 3 days). A similar weight loss (and death) in cotton rats has been reported for SP-303 when administered intraperitoneally at $\geqslant 30$ mg/kg per day for 3 or more days (Wyde et al., 1993a). If significant weight loss was used as a parameter of toxicity, a 'T.I.' of about 14 was calculated in the present study. Minimal aerosol ribavirin concentration for similar protection is 10 mg/kg per day (Gilbert et al., 1992). However, the median toxic dose for ribavirin (>90 mg/kg per day) is much higher than the one estimated for SP-303. In contrast to the mice, no mortality was observed in the cotton rats at the dosage of 18.7 mg/kg per day. Moreover, at this highest concentration, the cotton rats appeared unconcerned about the treatment. The difference in response between cotton rats and mice to SP-303, and how this might be manifested in man is not known.

SP-303 administered as an aerosol manifested antiviral activity in both influenza-infected mice and in RSV-infected cotton rats. Whether the therapeutic selectivity of this compound is sufficient for use in humans needs further evaluation. Safety and efficacy studies of SP-303 aerosol in primates seems warranted.

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References

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-303 against respiratory syncytial virus (RSV). Antiviral Res. 17 (Suppl. 1), 138–138.

Gilbert, B.E. and Knight, V. (1986) Biochemistry and clinical applications of ribavirin. Antimicrob. Agents Chemother. 30, 201–205.

Gilbert, B.E. and Wyde, P.R. (1988) Pharmacokinetics of ribavirin aerosol in mice. Antimicrob. Agents Chemother. 32, 117–121.

Gilbert, B.E., Wyde, P.R., Ambrose, M.W., Wilson, S.Z. and Knight, V. (1992) Further studies with short duration ribavirin aerosol for the treatment of influenza virus infection in mice and respiratory syncytial virus infection in cotton rats. Antiviral Res. 17, 33–42.

Knight, V., Gilbert, B.E. and Wilson, S.Z. (1986a) In: T. Stapleton (Ed), Studies with a broad spectrum antiviral agent, pp. 37–56, Royal Society of Medicine Series, New York.

Knight, V., Gilbert, B.E. and Wilson, S.Z. (1986b) In: L.M. de la Maza and E.M. Peterson (Eds), Medical virology V, pp. 445–463, Lawrence Erlbaum Associates, Publishers, Hillsdale.

Knight, V. and Gilbert, B.E. (1987) In: V. Knight and B.E. Gilbert (Eds), Infectious disease clinics of North America volume 1: Antiviral chemotherapy, pp. 441–457, W.B. Saunders, Philadelphia.

Knight, V., Yu, C.P., Gilbert, B.E. and Divine, G.W. (1988a) Estimating the dosage of ribavirin aerosol according to age and other variables. J. Infect. Dis. 158, 443–448.

Knight, V., Yu, C.P., Gilbert, B.E. and Orr, R. (1988b) In: K. Nicholson (Ed), HIV and other highly pathogenic viruses, pp. 69–86. Royal Society of Medicine Services, London.

Phalen, R.F. (1984) Inhalation studies: foundations and techniques, p. 222. CRC Press, Boca Raton.

Soike, K.F., Zhang, J-Y. and Meyerson, L.R. (1992) Reduction of respiratory syncytial virus (RSV) shedding in African Green monkeys treated with SP303. Antiviral Res. 17 (Suppl. 1), 91–91.

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

Wyde, P.R., Ambrose, M.W., Meyer, H.L., Zolinski, C.L. and Gilbert, B.E. (1990) Evaluation of the toxicity and antiviral activity of carbocyclic 3-deazaadenosine against respiratory syncytial and parainfluenza type 3 viruses in tissue culture and in cotton rats. Antiviral Res. 14, 215–226.

Wyde, P.R., Ambrose, M.W., Meyerson, L.R. and Gilbert, B.E. (1993a) The antiviral activity of SP-303, a natural polyphenolic polymer, against respiratory syncytial and parainfluenza type 3 viruses in cotton rats. Antiviral Res. 20, 145–154.

Wyde, P.R., Couch, R.B., Mackler, B.F., Cate, T.R. and Levy, B.M. (1977) Effects of low- and high-passage influenza virus infection in normal and nude mice. Infect. Immun. 15, 221–229.

Wyde, P.R., Meyerson, L.R., Ambrose, M.W., Pfeifer, J.B., Voss, T.G. and Gilbert, B.E. (1991) Elucidation of a polyphenolic polymer with antiviral activity against myxo- and paramyxoviruses. Antiviral Res. Suppl. 1, 67–67.

Wyde, P.R., Meyerson, L.R. and Gilbert, B.E. (1993b) An in vitro evaluation of the antiviral activity of SP-303, an Euphorbiaceae shrub extract, against a panel of respiratory viruses. Drug Devel. Res. 28, 467–472.

Wyde, P.R., Wilson, S.Z., Gilbert, B.E. and Smith, R.H.A. (1986) Protection of mice from lethal influenza virus infection with high dose-short duration ribavirin aerosol. Antimicrob. Agents Chemother. 30, 942–944.

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.