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Comparison of the inhibition of human metapneumovirus and respiratory syncytial virus by NMSO3 in tissue culture assays

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Abstract

Human metapneumovirus (hMPV) is a recently elucidated respiratory virus pathogen for which there are no agents currently licensed to prevent or treat infections caused by it. However, NMSO3 has been reported to inhibit replication of human respiratory syncytial virus (hRSV), a virus that is closely related to hMPV, both in vitro in tissue culture cells and in vivo in cotton rats. For this reason, experiments were performed to compare the antiviral activity of NMSO3 against both hRSV and hMPV in tissue culture-based assays. Heparin and ribavirin, two other compounds known to inhibit hRSV, and two other paramyxoviruses, human parainfluenza virus type 3 (PIV3) and measles virus (MV), were included in these tests for comparison. All three compounds significantly inhibited the replication of subtype A and B strains of hRSV and serotypes 1 and 2 hMPV. However, unlike ribavirin, NMSO3 and heparin inhibited only hMPV and hRSV and not PIV3 or MV. Also unlike ribavirin, the activity of the two sulfated molecules was most effective if these materials were present during virus attachment and penetration of host cells. Interestingly, NMSO3, but not heparin, was able to limit secondary infection and spread of both viruses.

Keywords: hMPV; Human metapneumovirus; RSV; Antiviral; NMSO3; Heparin

1. Introduction

Although only elucidated in 2001 (van den Hoogen et al., 2001), human metapneumovirus (hMPV) has already been shown to be a significant human respiratory pathogen (Boivin et al., 2002; Stockton et al., 2002; Jartti et al., 2003; Greensill et al., 2003) with worldwide distribution (Nissen et al., 2002; Peret et al., 2002; Peiris et al., 2003; Freymuth et al., 2003; Ebihara et al., 2003; Boivin et al., 2003). This virus is closely related to human respiratory syncytial virus (hRSV) (van den Hoogen et al., 2001; Peret et al., 2002), causes similar clinical manifestations (Boivin et al., 2002; Jartti et al., 2003; Greensill et al., 2003) and affects many of the same subpopulations as hRSV (Hall, 1998; Ison and Hayden, 2002; Pelletier et al., 2002; Stockton et al., 2002; Jartti et al., 2003). No vaccines, chemotherapeutic agents or antibody preparations are yet licensed for use to prevent or treat hMPV infections. However, results from recent preclinical testing suggest that ribavirin and intravenous immunoglobulin (IVIG) administered alone or in combination may be of some clinical value (Wyde et al., 2003).

NMSO3 is a sulfated sialyl lipid that has been shown to have potent antiviral activity against different laboratory and clinical strains of hRSV in HEp-2 tissue culture cells and in vivo in cotton rats (Kimura et al., 2000). Because of the close phylogenetic relationship between hMPV and hRSV, testing was performed to determine if NMSO3 could also inhibit replication of hMPV. For comparison, hRSV and two other compounds known to inhibit this virus, heparin (Krusat and Streckert, 1997), another sulfated molecule, and ribavirin, a nucleoside analog of guanosine currently licensed for use to treat severe hRSV-induced respiratory disease in infants, were included in most assays. Human parainfluenza virus type 3 (PIV3) and measles virus (MV) were also added to some tests for the same reason. In vivo testing against hMPV was not performed since presently there are no small animal

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models available for evaluating potential antivirals against this virus.

Both NMSO3 and heparin significantly inhibited the replication of different strains of hMPV and hRSV in tissue culture cells. However, unlike ribavirin, neither compound inhibited the other two paramyxoviruses included in these tests, PIV3 or MV. Time of addition and temperature shift studies indicated that both of the sulfated materials were most effective if they were present at times very early in virus replication, most probably during virus attachment and penetration. It was also noted that NMSO3, but not heparin, was able to limit syncytium formation and secondary spread of these viruses.

2. Materials and methods

2.1. Tissue culture

Continuous cultures of HEp-2 (human epithelial carcinoma; ATCC CCL-23), LLC-MK2 (Rhesus monkey kidney; ATCC CCL-7) and Vero (African green monkey, ATCC CCL-81) tissue culture cells were started from vials of these cells purchased from the American Type Culture Collection (ATCC; Manassas, VA). Eagle's minimal essential medium (MEM; Sigma Chemical Co., St. Louis, MO; cat. no. M4465) supplemented with 5% fetal calf serum (FCS; Summit Biotechnology, Fort Collins, CO; cat. no. FP-200-05), 100 μg/ml gentamicin (Sigma Chemical Co.; cat. no. G-1264), penicillin (100 U/ml) and streptomycin (100 μg/ml; Sigma Chemical Co.; cat. no. P-4458), 2 mM L-glutamine (Sigma Chemical Co.; cat. no. G7513) and 0.2% sodium bicarbonate (Sigma Chemical Co.; cat. no. S8761) was used as the growth medium. This same medium, but containing only 2% FCS was utilized to prepare pools of hRSV, PIV3 and MV and in all assays involving these viruses. Because replication of hMPV in LLC-MK2 cells requires cleavage of virus protein, MEM supplemented as above, but with 1.0 μg trypsin/ml (Worthington Biochemical Corp., Lakewood, NJ; cat. no. 32C5468) and no FCS was utilized to prepare pools of this virus and in any assay involving hMPV.

2.2. Viruses

Pools of hRSV Long strain (ATCC VR26) and hRSV strain B Wash/18537/62 (ATCC VR1401; 18537) were prepared using virus purchased from the ATCC. The other strains of hRSV and the PIV3 utilized in these studies were acquired from the Respiratory Pathogens Research Unit (RPRU), Baylor College of Medicine (BCM). The M06 MV tested was isolated in 1993 from a Zambian child hospitalized with measles and sent to us by Dr. Hiroshi Suzuki, Department of Public Health, Niigata University, Niigata, Japan. Pools of hMPV were prepared using low passage levels of isolates CDC 26575 and 26583 obtained from the Centers for Disease Control (CDC), Atlanta, GA, with permission from Dr. Guy Boivin at the Research Center in Infectious Diseases, Regional Virology Laboratory, Laval University, Quebec City, Canada. Another clinical isolate of hMPV, RL Bx, was acquired from Gail Demmler, M.D., Department of Pediatrics-Infectious Diseases, Texas Children's Center, BCM. Subtyping of the hRSV strains were performed by Dr. Larry Anderson at the CDC or by personnel in the RPRU, BCM. The CDC identified the CDC 26583 hMPV as being serotype 1 and the 26575 virus as a serotype 2 hMPV. Dr. Robert Atmar of the RPRU using a reverse transcriptase–polymerase chain reaction (RT–PCR) assay determined that the RL Bx isolate to be have key RNA sequences identical to that of a serotype 1 hMPV. Working stocks of hRSV and PIV3 were prepared by infecting flasks of HEp-2 cells as described previously (Wyde et al., 2003). Stocks of MV and hMPV were also prepared in this same way, except that Vero cells were used to grow the former virus and LLC-MK2 cells and trypsin-containing medium lacking FCS the latter one.

2.3. Chemotherapeutic agents

NMSO3 ($C_{60}H_{112}NO_{23}S_4Na_4$; MW = 1478.7; see Kimura et al., 2000 for structure) was provided by Microbiotix, Inc., Worcester, MA. Heparin (sodium salt) was purchased from the Sigma Chemical Co. (cat. no. 210-6). Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, MW = 244.2) was acquired from ICN Pharmaceuticals (Costa Mesa, CA; cat. no. 196066). On the morning of an experiment, the amount of heparin or ribavirin needed was suspended in sterile water (Baxter Healthcare Corporation, Deerfield, IL; cat. no. 2F7114) and then filter sterilized using a 0.2 μm DynaGard filter (Spectrum Laboratories, Rancho Dominguez, CA; cat. no. DG2M-30-50S). NMSO3 was similarly prepared, except that it was heated to 62 °C to get it completely into solution prior to filtering. Once in solution, it did not come back out.

2.4. Virus quantification

Levels of hRSV in virus pools were determined in sterile 96-well tissue culture plates (Falcon 3072) using serial three-fold dilutions and virus-induced cytopathic effects (CPE) as an endpoint as described in detail previously (Wyde et al., 1995). The monolayers in the wells of these plates were observed daily for CPE until no further development virus presence was evident in the titration for at least two successive days (usually Day 7). Using the final readings and the interpolation method of Karber (Rhodes and Van Rooyen, 1953), an estimation of the amount of virus present in each test suspension was made. The estimates were expressed as median tissue culture infectious doses ($TCID_{50}$; log_{10})/ml and the minimum detectable virus in these assays was 1.8 log_{10} $TCID_{50}$/ml·hPIV3, MV and hMPV were quantified similarly except that Vero cells were used to grow the

MV and LLC-MK2 cells and medium lacking FCS and supplemented with 1 μg trypsin/ml was utilized with hMPV.

2.5. ELISA for the detection of hMPV antigens

An enzyme-linked immunosorbant assay (ELISA) was used to confirm the presence of hMPV in test wells. This assay was performed exactly as described previously (Wyde et al., 2003) except that hMPV-specific antiserum produced in rabbits (discussed in the next paragraph) was utilized as the primary sera. At the end of each test, a 96-well spectrophotometric plate reader (Molecular Devices, Inc., Palo Alto, CA) set at 450 nM wavelength was used to measure the optical density (OD) of the medium in each test well. The mean OD obtained for the tissue culture control wells exposed to the hMPV-specific rabbit antisera + 3 standard deviations (99% confidence limits) was determined. OD values in other wells greater than this value were considered to be positive for hMPV.

2.6. Production of hMPV-specific antibodies for use in the hMPV-specific ELISA

Sucrose purified hMPV virus (CAN97-83; 4.3 μg protein/ml) was inactivated with beta propriolactone and mixed with an equal volume of phosphate buffered saline (PBS) containing 10 μg of QS-21 adjuvant (Antigenics, Inc., Framingham, MA). This mixture was sent to SynPrep Corp., Dublin, CA, where two rabbits were inoculated subcutaneously with this material, twice, 6 weeks apart. Three weeks after the second injection, the two animals were euthanized and exsanguinated. The resulting sera were heat-inactivated at 56 °C for 30 min, portioned, labeled and stored at 4 °C. It was determined that the levels of hMPV-specific neutralizing and ELISA antibody in these sera was 1/256 and ≥1/3000/0.05 ml, respectively. In contrast, both rabbit sera had hRSV-specific neutralizing and ELISA titers <1/4 and <1/10/0.05 ml, respectively.

2.7. IC_{50} assays

Determination of the median cytotoxic drug concentration (IC_{50}) of NMSO3, heparin and ribavirin in HEp-2, Vero or LLC-MK2 cells was carried out using 96-well flat tissue culture plates (Falcon 3072) as described in detail previously (Wyde et al., 2003). The test plates were incubated at 36 °C for 24–48 h in a 5% CO_2 incubator until the monolayers in the tissue culture control wells became confluent. At that time, 20 μl of alamar Blue (aB; Biosource International, Camarillo, CA; cat. no. DAL1100) was added to every well. This reagent contains an oxidation/reduction (REDOX) indicator that is non-fluorescent and blue in its oxidized state, but turns red and fluoresces strongly in its reduced state (excitation and emission wavelengths = 546 and 590 nm, respectively). The reduction of aB occurs in direct proportion to the density and metabolic activity of the cell population to which it is added (VoytikHarbin et al., 1998). In these studies, the relative fluorescence in each test and control well was determined utilizing a Fluorolite™ 1000 Microtiter® plate fluormeter (Dynatech Laboratories, Inc., Chantilly, VA) when the color of the medium present in the tissue culture control wells turned bright red (usually 4–6 h after addition of the aB). The mean fluorescence in each set of wells was then calculated and used to determine the percentage of fluorescence for each test group relative to that of the tissue culture control wells. These values and the corresponding concentration (μg/ml) of NMSO3, heparin or ribavirin were entered into the computer using CalcuSyn (Biosoft, Inc., Ferguson, MO), a Windows™-based program made for dose effect analysis. Using this software, the concentration of each compound that induced a 50% reduction in mean relative fluorescence compared to the mean relative fluorescence of the tissue culture control wells (i.e., their IC_{50} value) was obtained.

2.8. EC_{50} assays

Assays to determine the median efficacious concentration (EC_{50}) of NMSO3, heparin and ribavirin against hMPV, hRSV, PIV3 and MV were performed nearly simultaneously as the IC_{50} assays using procedures described in detail previously (Wyde et al., 2003). The test plates were incubated at 36 °C in a 5% CO_2 incubator until the virus control wells exhibited 70–100% CPE. This was usually 5–7 days after the addition of hRSV, PIV3 or MV and 7–10 days after the addition of hMPV. When the appropriate amount of CPE was apparent in the virus control wells, the cell monolayers in each well were observed and scored for drug-induced cytotoxicity and virus-induced CPE. Because of the unique CPE of hRSV, hPIV3 and MV, the cytotoxicity created by the drugs and the CPE produced by these viruses were usually easily distinguishable and identifiable even at the endpoints in each titration. However, because the CPE induced by hMPV could be confused with drug-induced cytotoxicity, an ELISA for the detection of hMPV antigens (discussed in Wyde et al., 2003 and above) was performed in plates containing this virus. The resulting data were entered into the computer and the mean concentration of each test compound that completely inhibited viral-induced CPE in one-half of the replicate wells (i.e., their EC_{50} value) was determined. The IC_{50} value obtained for each test compound was divided by the appropriate EC_{50} value (for example, the IC_{50} of NMSO3 in HEp-2 cells/the EC_{50} value of NMSO3 against RSV in HEp-2 cells) to obtain a selective index (SI) for each compound.

2.9. Time of addition and temperature shift studies

To help gauge at what steps of virus replication the NMSO3 and heparin were interfering with, time of addition studies were performed in which these compounds were added to monolayers of HEp-2 or LLC-MK2 at the

same time as virus or at intervals up to 8 h later. Four to eight wells were used for each condition. The plates were incubated at 36 °C in a 5% CO_2 incubator until the virus control wells exhibited 70–100% CPE as described above.

Temperature shift assays were performed to gain insight into whether NMSO3 was inhibiting virus attachment and/or penetration. These tests were initiated by placing three replicate test tubes containing suspensions of LLC-MK2 cells and three with HEp-2 cells in ice. After 10 min, hMPV that had been kept in ice was mixed with the contents of the three tubes of LLC-MK2 and similarly chilled hRSV was added to the three tubes of HEp-2 cells. After 90 min with intermittent mixing, cold distilled water (placebo) was added to one of the tubes containing hMPV and LLC-MK2 cells and to one of the test tubes holding hRSV and HEp-2 cells. In the same way, cold NMSO3 (20 μg/ml final concentration) was added to one tube of each set of three tubes and heparin (10 U/ml final concentration) to the last test tube in each set. After a further incubation in ice for 30 min with occasional mixing, a portion of each suspension was removed from each vessel and 0.1 ml samples were added to eight replicate wells of a 96-well tissue culture plates (Falcon 3072). Tissue culture control wells (i.e., wells to which just cells and media were added) were included in each test. These plates were placed in a 36 °C, (5%) CO_2 incubator until the virus control wells (those containing just tissue culture cells, medium and distilled water) exhibited 70–100% CPE. At that time, all of the wells in each assay plate were observed microscopically and scored for virus presence or absence. The visual observations for hMPV were confirmed using the hMPV-specific ELISA.

2.10. Virus foci-inhibition assays

Assays were also performed to determine if the two sulfated compounds could inhibit secondary as well as primary virus infection. In these studies, approximately 30 $TCID_{50}$ of hMPV or hRSV were added to monolayers of LLC-MK2 (hMPV assays) or HEp-2 (hRSV assays) cells and the virus was allowed to absorb for 90 min. During this time, serial two-fold dilutions of distilled water (the diluent used to suspend the heparin and NMSO3), heparin or NMSO3 were made in parallel plates. At the end of the 90 min incubation, the cell monolayers in the primary test plates were washed three times to remove any unadsorbed virus and then the contents of the wells of the parallel plates were transferred to the primary plate taking care to maintain the proper orientation of each pair of plates. Wells containing only cells and medium (tissue culture control wells) and wells with the appropriate virus and tissue culture cells (virus control wells) were included in each assay. The primary test plates were placed in the 36 °C incubator until foci of syncytia (hRSV plates) or infection (hMPV plates) were evident in the virus control wells. At that time, the monolayers in every well were observed and scored for foci indicative of virus infection. The ELISA for the detection of hMPV antigens was utilized to confirm which wells were positive for this virus. From the resulting data, the mean concentration of each test compound that completely inhibited foci formation in one-half of the replicate wells was determined.

2.11. Statistics

Instat, a statistical program designed for IBM compatible computers (version 3, GraphPad Software, Inc., San Diego, CA) was used to calculate all means and standard deviations.

3. Results

The results of experiments performed to compare the cytotoxicity of NMSO3, heparin and ribavirin and their antiviral efficacy against different paramyxoviruses are summarized in Table 1. As the IC_{50} values in column 3 of this table indicate, both NMSO3 and ribavirin exhibited some cytotoxicity in HEp-2 cells (the IC_{50} value of both materials being 750 μg/ml), but none, even at the highest concentration of the compounds tested (i.e., 1000 μg/ml), in the LLC-MK2 cells. No cytotoxic manifestations were observed in either cell line following their exposure to heparin. However, the maximum final concentration of this compound that was tested was only 25 units/ml.

As the EC_{50} values in column 5 of Table 1 indicate, all three test materials inhibited both hMPV and hRSV. Moreover, each had more or less equivalent activity against both of these viruses. Thus, the mean EC_{50} obtained for ribavirin against hMPV was 17 ± 6 μg/ml and 9 ± 7 μg/ml versus hRSV; the EC_{50} determined for NMSO3 against both viruses was 6 μg/ml; and the EC_{50} values ascertained for heparin against hMPV and hRSV were 0.7 ± 0.3 units/ml and 0.8 ± 0.6 units/ml, respectively. Because of its lower EC_{50} values, NMSO3 had higher selective indices than ribavirin (125 and ≥167 for NMSO3 versus hRSV and hMPV, respectively, compared to 84 and ≥59 for ribavirin versus these viruses). Heparin had a SI of ≥31 against hRSV and ≥36 versus hMPV in these tests. In contrast to the nucleoside analog which inhibited all four paramyxoviruses (albeit at different levels), NMSO3 and heparin only inhibited replication of hRSV and hMPV.

All three test compounds equivalently inhibited the different subtype A and B hRSV strains used in these studies as well as both serotypes 1 and 2 human metapneumo-viruses. This equivalence is made evident by the limited range of EC_{50} values and selective indices displayed in each subsection of Table 2. As important, these data indicated that each material had comparable antiviral activity against both hRSV and hMPV.

In experiments in which NMSO3 or heparin were added at different times to the cell monolayers relative to the time that virus was added (see Table 3), no inhibition of virus,

Table 1
Comparison of the cytotoxicity and antiviral efficacy of NMSO3, heparin and ribavirin against different paramyxoviruses[a]

Material tested	Cell line	Mean IC_{50} (μg or U/ml)[b,c]	Virus tested[b]	Mean EC_{50} (μg or U/ml)	Mean selective index
Ribavirin	HEp-2	750 ± 0	hRSV	9 ± 7	84
Ribavirin	LLC-MK2	≥1000 ± 0	hMPV	17 ± 6	≥59
Ribavirin	HEp-2	750 ± 0	PIV3	24 ± 10	31
Ribavirin	LLC-MK2	≥1000 ± 0	MV	110 ± 72	15
NMSO3	HEp-2	750 ± 0	hRSV	6 ± 2	125
NMSO3	LLC-MK2	≥1000 ± 0	hMPV	6 ± 3	≥167
NMSO3	HEp-2	≥750 ± 0	PIV3	≥750 ± 0	1
NMSO3	LLC-MK2	≥1000 ± 0	MV	≥1000 ± 0	1
Heparin	HEp-2	≥25 ± 0	hRSV	0.8 ± 0.6	≥31
Heparin	LLC-MK2	≥25 ± 0	hMPV	0.7 ± 0.3	≥36
Heparin	HEp-2	≥25 ± 0	PIV3	≥25 ± 0	1
Heparin	LLC-MK2	≥25 ± 0	MV	≥25 ± 0	1

[a] The selective indices displayed were obtained by dividing the mean median inhibitory concentration (IC_{50}) obtained for each compound in each tissue culture cell line (column 3) by its respective mean median virus-inhibitory concentration (EC_{50}; column 6). All means were obtained from a minimum of two replicate IC_{50} and EC_{50} assays, performed as described in Section 2.
[b] hRSV: human respiratory syncytial virus; hMPV: human metapneumovirus; PIV3: human parainfluenza virus; MV: measles virus.
[c] The appropriate units for ribavirin and NMSO3 is μg/ml. For heparin, units (U)/ml is the appropriate label.

compared to that seen in the virus control wells, was observed in any of the wells to which these materials were added 60 or 120 min subsequent to virus or in those in which drug had been present but removed just prior to the addition of virus. Marginal, but evident, reductions in virus replication were observed in wells to which heparin was added 30 min after virus (row 6), but not in wells that NMSO3 was inserted at this time (row 11). In contrast to these findings, total inhibition of virus was seen whenever either NMSO3 or heparin were added at the same time as virus.

In temperature-shift studies where ice-cold heparin (10 U/ml final concentration) or NMSO3 (20 μg/ml final concentration) were added to suspensions of cells and virus kept in ice, total inhibition of the replication of both hRSV and hMPV were observed when the suspensions were plated and moved to 36 °C (see Table 4). In contrast, if distilled water was added similarly and the suspensions of cold cells and virus were dispensed into test plates and placed in a 36 °C incubator, both viruses replicated well and produced abundant CPE by days 7–10 of incubation. These findings

Table 2
Comparison of the antiviral efficacy of NMSO3, heparin and ribavirin against different strains of human metapneumovirus and human respiratory syncytial virus[a]

Material tested	Cell line	Virus tested[b]	Subtype or serotype	Mean EC_{50} (μg or u/ml)	Mean selective index
Ribavirin	HEp-2	hRSV Long strain	A	14 ± 3	54
Ribavirin	HEp-2	hRSV 18537	B	9 ± 7	83
Ribavirin	HEp-2	hRSV 37593	A	12 ± 6	63
Ribavirin	LLC-MK2	hMPV RL Bx	1	18 ± 8	56
Ribavirin	LLC-MK2	hMPV 26575	2	9 ± 3	111
Ribavirin	LLC-MK2	hMPV 26583	1	12 ± 4	83
NMSO3	HEp-2	hRSV Long strain	A	6 ± 4	125
NMSO3	HEp-2	hRSV 18537	B	5 ± 2	150
NMSO3	HEp-2	hRSV 37593	A	5 ± 2	150
NMSO3	LLC-MK2	hMPV RL Bx	1	3 ± 1	333
NMSO3	LLC-MK2	hMPV 26575	2	5 ± 2	200
NMSO3	LLC-MK2	hMPV 26583	1	4 ± 1	250
Heparin	HEp-2	hRSV Long strain	A	0.6 ± 0.4	≥42
Heparin	HEp-2	hRSV 18537	B	0.9 ± 0.5	≥28
Heparin	HEp-2	hRSV 37593	A	0.5 ± 0.5	≥50
Heparin	LLC-MK2	hMPV RL Bx	1	0.7 ± 0.3	≥36
Heparin	LLC-MK2	hMPV 26575	2	0.5 ± 0.2	≥50
Heparin	LLC-MK2	hMPV 26583	1	0.4 ± 0.1	≥63

[a] The selective indices displayed were obtained by dividing the mean median inhibitory concentration (IC_{50}) obtained for each compound in HEp-2 or LLC-MK2 tissue culture cells (data not shown) by the mean median virus-inhibitory concentration (EC_{50}) displayed in column 6. All means were obtained from a minimum of two replicate IC_{50} and EC_{50} assays, performed as described in Section 2.
[b] hRSV: human respiratory syncytial virus; hMPV: human metapneumovirus.

Table 3
The effect of the time of addition of NMSO3 and heparin on their antiviral effect against human respiratory syncytial and human metapneumovirus[a]

Material tested	Concentration tested[b]	Time added	Test virus	% CPE[c]		Test virus	% CPE	
				Experiment 1	Experiment 2		Experiment 1	Experiment 2
Distilled water	N.A.	−1 h to T0	hRSV	≥80	≥80	hMPV	≥80	≥80
Distilled water	N.A.	Time 0	hRSV	≥80	≥80	hMPV	≥80	≥80
Distilled water	N.A.	+30 min	hRSV	≥80	≥80	hMPV	≥80	≥80
Heparin	10 U/ml	−1 h to T0	hRSV	≥80	≥80	hMPV	≥80	≥80
Heparin	**10 U/ml**	**Time 0**	**hRSV**	**0**	**0**	**hMPV**	**0**	**0**
Heparin	**10 U/ml**	**+30 min**	**hRSV**	**∼50**	**∼50**	**hMPV**	**∼50**	**∼50**
Heparin	10 U/ml	+60 min	hRSV	≥80	≥80	hMPV	≥80	≥80
Heparin	10 U/ml	+120 min	hRSV	≥80	≥80	hMPV	≥80	≥80
NMSO3	20 μg/ml	−1 h to T0	hRSV	≥80	≥80	hMPV	≥80	≥80
NMSO3	**20 μg/ml**	**Time 0**	**hRSV**	**0**	**0**	**hMPV**	**0**	**0**
NMSO3	20 μg/ml	+30 min	hRSV	≥80	≥80	hMPV	≥80	≥80
NMSO3	20 μg/ml	+60 min	hRSV	≥80	≥80	hMPV	≥80	≥80
NMSO3	20 μg/ml	+120 min	hRSV	≥80	≥80	hMPV	≥80	≥80

[a] Distilled water, heparin or NMSO3 was added to the appropriate test wells at the times (relative to virus addition) stated in column 3. In the wells indicated above as −1 h to T0 (−1 hour to time 0), the distilled water, heparin or NMSO3 were removed just prior to the addition of virus and replaced with medium containing 2% fetal calf serum. The test materials were not removed from any of the other wells. All wells were observed for virus-induced cytopathic effects (CPE) when the monolayers in the virus control wells (i.e., wells containing tissue culture cells + virus and no test agent) exhibited ≥80% CPE.

[b] N.A.: not applicable.

[c] The percentage CPE in each well was estimated based on the approximate amount of the monolayer exhibiting syncytia formation (for wells infected with hRSV) or distinctive rounding up of the cells (for wells infected with hMPV). Bolded rows indicate the conditions that lead to overt reductions in virus-induced CPE compared to the cytopathology seen in the virus control wells.

Table 4
The effect of NMSO3 and heparin on virus penetration of host cells by human respiratory syncytial and human metapneumovirus[a]

Material tested	Concentration tested[b]	Temperature when added (°C)	Test virus	% CPE[c]		Test virus	% CPE[c]	
				Experiment 1	Experiment 2		Experiment 1	Experiment 2
Distilled water	N.A.	0	hRSV	≥80	≥80	hMPV	≥80	≥80
Heparin	**10 U/ml**	**0**	**hRSV**	**0**	**0**	**hMPV**	**0**	**0**
NMSO3	**20 μg/ml**	**0**	**hRSV**	**0**	**0**	**hMPV**	**0**	**0**

[a] Ice-cold human respiratory syncytial (hRSV) or human metapneumo (hMPV) virus was added to plastic test tubes containing similarly chilled suspensions of HEp-2 or LLC-MK2 cells, respectively. After 90 min with occasional mixing, 0.1 ml of each mixture was added to eight replicate wells containing ice-cold distilled water, heparin (10 units (U)/ml final concentration) or NMSO3 (20 μg/ml final concentration). After a further 30-min incubation in the cold, the plates were moved to a 36 °C incubator. All wells in an assay plate were observed for virus-induced cytopathic effects (CPE) when the monolayers in the virus control wells (i.e., wells containing tissue culture cells + virus and no test agent) exhibited ≥80% CPE.

[b] N.A.: not applicable.

[c] The percentage CPE in each well was estimated based on the approximate amount of the monolayer exhibiting syncytia formation (for wells infected with hRSV) or distinctive rounding up of the cells (for wells infected with hMPV). Bolded rows indicate the conditions that lead to overt reductions in virus-induced CPE compared to the cytopathology seen in the virus control wells.

suggested that both NMSO3 and heparin inhibited virus penetration of host cells (and perhaps virus uncoating).

Interestingly, in foci forming assays where the test viruses were allowed to infect monolayers of LLC-MK2 or HEp-2 prior to the addition of test drug (Table 5), NMSO3, but not heparin, was able to completely inhibit hMPV- or hRSV-induced foci of infection if sufficient concentrations of this compound were present. In these assays, the highest (final) concentration of heparin tested was 25 units/ml, or ≥25 times the EC_{50} determined for this compound in the standard EC_{50} test (i.e., 0.5–0.9 U/ml; see Tables 1 and 2). The EC_{50} values obtained for NMSO3 against hRSV and for hMPV in these assays were both somewhat higher than the values obtained for this compound in the standard EC_{50} assay (i.e., 10 ± 3 μg/ml versus hMPV and 18 ± 8 μg/ml versus hRSV in this assay compared to 3–6 μg/ml versus hMPV and 5–6 μg/ml versus hRSV in the standard EC_{50} assay; see Tables 1 and 2).

4. Discussion

Attachment and penetration are steps essential for efficient infection of host cells by most viruses (reviewed in Roizman and Palese, 1996). The former usually requires specific binding between sites on a viral coat protein (viral

Table 5
Comparison of the inhibition of infectious foci produced by human respiratory syncytial and human metapneumovirus viruses by NMSO3 and heparin[a]

Material tested	Cell line	Virus tested	Parameter tested	Mean EC_{50}[b]
Heparin	HEp-2	hRSV Long strain	Syncytia foci[c]	≥10 ± 0 units/ml
Heparin	LLC-MK2	hMPV RL Bx	Infectious foci	≥10 ± 0 units/ml
NMSO3	HEp-2	hRSV Long strain	Syncytia foci	18 ± 8 μg/ml
NMSO3	LLC-MK2	hMPV RL Bx	Infectious foci	10 ± 3 μg/ml

[a] Approximately 100 $TCID_{50}$ of human respiratory syncytial (hRSV) or human metapneumovirus (hMPV) were allowed to adsorb to the test monolayers for 90 min. Each monolayer was then washed and serial two-fold dilutions of heparin or NMSO3 were added in quadruplicate to the test wells. Distilled water (the diluent utilized for the heparin and NMSO3) was added to the placebo-control wells. When the foci of infection were well formed in the virus control wells (those that contained cells, virus and medium only), all of the cell monolayers in each plate were observed for infection foci formation.

[b] EC_{50}: median efficacious concentration (i.e., the concentration of each compound that inhibited infection foci formation 100% in one-half of the replicate wells).

[c] The infectious foci induced by hRSV characteristically exhibited marked syncytium formation while the foci produced by hMPV, although distinctive, generally had minimal or no apparent syncytium formation.

attachment site or antireceptor) and constituents on the host cell surface (virus receptor) and it is often facilitated by the presence of ions, whose role is presumably to reduce electrostatic repulsion between virions and the host cell. Unlike the attachment step, penetration of the host cell by viruses generally requires energy and is temperature-dependent (Dales, 1973). Thus, reducing the temperature to near 0 °C usually inhibits viral penetration of the host cell, but not virus attachment. The former usually occurs almost instantaneously after attachment and for paramyxoviruses it has been shown to involve the fusion of the lipid envelope of the virus with the plasma membrane of the host cell (Lamb, 1993). Because virus attachment and penetration of host cells are so critical for efficient virus infection, these steps have been the target of numerous antiviral candidates (Boehme et al., 1994). A number of these have been sulfated compounds that have been shown to interfere with the interaction(s) of the attachment site on the virus coat proteins and glycosaminoglycan-like receptor moieties on the surface of host cells (e.g., heparan sulfate; WuDunn and Spear, 1989; Sawitzky et al., 1990; Jackson et al., 1996; Bourgeois et al., 1998; Liu and Thorp, 2002). Some of the viruses whose replication has been shown to be inhibited by these materials include *Herpes simplex* (Nahmias and Kibrick, 1964), cytomegalovirus, vesicular stomatitis virus, human immunodeficiency virus (Baba et al., 1988; Di Caro et al., 1999); *Varicella zoster* (Zhu et al., 1995), foot-and-mouth disease virus (Jackson et al., 1996), pseudorabies virus (Ramos-Kuri et al., 1990) and hRSV (Hosoya et al., 1991; De Clercq, 1996).

The studies contained in this report center on NMSO3, a molecule with four sulfate residues that has been reported to markedly inhibit hRSV in vitro and in vivo in cotton rats, but to have marginal or no activity against influenza A, influenza B, parainfluenza type 2 or canine distemper virus (Kimura et al., 2000). The molecule is negatively charged and thus may inhibit the adsorption of hRSV to the cell membrane of host cells by interfering with static electric binding between the viral attachment protein and viral receptors on the host cell membrane. However, other factors may be involved since it has been shown that this molecule can also inhibit virus penetration and syncytia formation as well as virus attachment (Kimura et al., 2000). In those studies, the NMSO3 appeared to be 53 times more active than ribavirin, the only chemotherapeutic currently available to treat hRSV infections. Indeed, it was because of this reported activity, the demonstration that NMSO3 was also effective in vivo and the close phylogenetic relationship between hMPV and hRSV, that the present studies were initiated to determine the ability of NMSO3 to inhibit hMPV.

Heparin was included in the current testing for comparative purposes. It too is a sulfated molecule that has been shown to inhibit RSV replication in vitro (Krusat and Streckert, 1997). Moreover, the mechanism of action by which heparin inhibits hRSV has been well studied. It appears that this compound impairs essential interactions between the G (attachment protein) of hRSV (Feldman et al., 1999) and host cell glycoaminoglycan-like moieties (Martinez and Melero, 2000), most likely heparan (Hallak et al., 2000). This hypothesis is supported by the fact that there is a linear heparin-binding domain within the highly conserved region of the G glycoprotein of hRSV (Feldman et al., 1999). Heparin has also been shown to bind to the other major coat protein of hRSV, the fusion (F) protein (Feldman et al., 2000), which has a role in viral attachment and infectivity (Karron et al., 1997), as well as determining host cell specificity (Schlender et al., 2003). Thus, it appears that heparin may inhibit hRSV replication by interfering with the binding and/or biological activity of both of the major hRSV coat proteins. To our knowledge, no studies have been performed looking at the ability of heparin to inhibit hMPV infections.

The other compound utilized in these studies for comparison, ribavirin, is a nucleoside analog of guanosine with known broad-spectrum antiviral activity (Sidwell et al., 1972). It is thought to inhibit hRSV replication by inhibiting inosine monophosphate dehydrogenase in host cells causing a depletion of intracellular pools of guanosine triphosphate required for viral RNA synthesis (Patterson and Fernandez-Larsson, 1990). It is currently approved for

use to ameliorate severe hRSV infections in infants. Of equal importance, ribavirin was recently shown to equivalently inhibit both hMPV and hRSV replication in tissue culture-based assays (Wyde et al., 2003). Thus it was of great interest to determine if NMSO3 would have equivalent or greater antiviral activity against hMPV than the nucleoside analog.

In fact, in the present studies, based on EC_{50} values and selective indices (see Tables 1 and 2), NMSO3 generally exhibited equivalent activity against hRSV as ribavirin, and equivalent or greater selective antiviral activity against hMPV. On a micromolar basis, NMSO3 (MW = 1478.7) was approximately six times more active than ribavirin (MW = 244.2) against hRSV and at least 12 times more efficacious against hMPV than the guanosine analog. However, regardless of how the calculations are made, it is clear that the differences in inhibition exhibited in the present studies against hRSV by NMSO3 and ribavirin were less than the 53-fold difference in activity previously reported for these compounds (Kimura et al., 2000). The virus-detection assays used in the different studies may account for some of the disparity. In the earlier testing, antiviral activity was determined utilizing either a virus plaque reduction or an ELISA assay for detecting virus antigen, while in the present studies the antiviral assays were performed in 96-well plates and CPE was used as an endpoint. There may also be differences in the purity of the NMSO3 preparations used in the separate studies. Regardless, in the current investigation, NMSO3 appeared to be relatively nontoxic to two tissue culture cell lines (i.e., HEp-2 and LLC-MK2 cells) and consistently inhibited hMPV with selective indices $\geq$150.

The results obtained in the time of addition and temperature shift studies indicate that as they do with hRSV (Kimura et al., 2000; Krusat and Streckert, 1997), both NMSO3 and heparin inhibit hMPV replication by acting at an early step in the replication cycle of this virus (Tables 3 and 4), most likely attachment and penetration. This similarity in apparent mechanism of action and level of antiviral activity seen with NMSO3 and heparin suggests that hRSV and hMPV may have similar or analogous attachment and receptor sites.

NMSO3, but not heparin, also was shown to inhibit the development of infectious foci when it was added after virus adsorbtion (Table 5). These findings indicate that NMSO3 (but not heparin) can inhibit, or at least limit, cell-to-cell spread of hRSV and hMPV by fusion as well as initial infection of host cells from without. Moreover, it may indicate that this compound could have an ameliorating effect if administered subsequent to initial infection. However, the latter may require more compound since as has already been noted, the EC_{50} values obtained in the infection foci-inhibiting assays (e.g., those shown in Table 5) were two to three times the values obtained in the standard antiviral (EC_{50}) assays (see Tables 1, 2 and 5).

Although the elucidation of hMPV is recent, it is already clear that this virus is a significant human respiratory pathogen and that there is a need for agents that can prevent or ameliorate infections caused by this virus. Because of its phylogenetic relationship to hRSV, it is plausible to begin the search for such interventions with substances that have proven to be successful against hRSV. In this vein we have previously shown that ribavirin and a standard IVIG preparation have equivalent activity against hMPV and hRSV. The present studies add another material, NMSO3, to the list of potential compounds that may be of some clinical benefit for preventing or treating infections caused by hMPV. Because of the anticoagulation properties of heparin, this material would not likely be useful as an antiviral against viruses such as hMPV or hRSV. However, it is possible that a derivative or analog of heparin could be. Regardless of whether NMSO3, heparin or a derivative of these materials make it into clinical trials, further testing with these compounds may provide useful insight into the identity of the attachment protein and host cell receptor sites used by hMPV as such testing with heparin has done with hRSV.

Testing of NMSO3 against hMPV in vivo needs to be performed to help determine the potential clinical utility of this molecule. However, currently there are no small animal models available to perform such studies. There is still reason to be optimistic since NMSO3 has been shown to inhibit hRSV replication in cotton rats and in the present studies no significant differences were seen in the inhibition of hRSV and hMPV by NMSO3.

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