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Comparison of the inhibition of human metapneumovirus and respiratory syncytial virus by ribavirin and immune serum globulin in vitro

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Abstract

Human metapneumovirus (hMPV) is a newly recognized pathogen that like its better-known relative, human respiratory syncytial virus (hRSV), appears to be ubiquitous and an important cause of respiratory disease in diverse subpopulations. No antivirals or vaccines are currently approved for the treatment or prevention of hMPV infections. However, ribavirin is licensed to treat serious hRSV-induced infections in children and immune globulin designed for intravenous administration (IVIG) and palivizumab (Synagis™), a humanized monoclonal antibody preparation, have been utilized as alternatives to vaccines for preventing or reducing the severity of infections caused by this virus. Because both ribavirin and IVIG have broad viral specificities, studies were performed to compare the ability of these two agents to inhibit the replication of hRSV and hMPV in tissue culture-based assays. Two experimental chemotherapeutic agents (i.e. VP14637 and JNJ2408068) and different antibody preparations were included in this testing for comparison. Ribavirin and the IVIG utilized were found to have equivalent antiviral activity against hMPV and hRSV. In contrast, except for antisera specifically raised against hMPV, all of the other materials tested had marked activity only against hRSV.

Keywords: RSV; hMPV; Human metapneumovirus; Antiviral; Respiratory syncytial virus; Ribavirin; IVIG; JNJ2408068

1. Introduction

Human metapneumovirus (hMPV) is a recently elucidated virus that is closely related to human respiratory syncytial virus (hRSV) (van den Hoogen et al., 2001; Peret et al., 2002). Initial epidemiologic studies indicate that like hRSV, hMPV is a significant human respiratory pathogen (van den Hoogen et al., 2001; Boivin et al., 2002; Stockton et al., 2002; Jartti et al., 2003; Freymuth et al., 2003; Greensill et al., 2003) with worldwide distribution (Howe, 2002; Nissen et al., 2002; Peret et al., 2002; Freymuth et al., 2003). Indeed, both viruses appear to have similar clinical manifestations (Boivin et al., 2002; Jartti et al., 2003; Greensill et al., 2003) and seem to affect many of the same subpopulations (Hall, 1998; Ison and Hayden, 2002; Jartti et al., 2003; Pelletier et al., 2002; Stockton et al., 2002), which may be one of the reasons why this virus was not elucidated sooner. No vaccines, chemotherapeutic agents, or antibody (monoclonal or polyclonal) preparations are currently licensed for use to prevent or treat hMPV infections. However, polyclonal antibody preparations with high titers to hRSV (e.g. RSV immunoglobulin intravenous (RSV-IVIG) [RespiGam™]; MedImmune, Inc., Gaithersberg, MD) and one monoclonal antibody (i.e. palivizumab [Synagis™]; MedIimmune, Inc.) are licensed for prophylactic use to prevent hRSV-induced hospitalizations and one chemotherapeutic agent, ribavirin, is approved for use to ameliorate severe hRSV illness in infants. The polyclonal IVIG preparations (Sawyer, 2000) and ribavirin (Fernandez et al., 1986) are known to have broad-spectrum activity and can inhibit different viruses. Thus, studies were initiated to compare the effectiveness of ribavirin and an IVIG preparation to inhibit the replication of hRSV and hMPV in tissue culture-based assays. For comparison, RSHZ19, a humanized monoclonal antibody with similar activity to palivizumab (Johnson et al., 1999), but not approved for clinical use, palivizumab and two experimental compounds with high specific activity against the F protein of hRSV (i.e. VP14637; McKimm-Breschkin, 2000 and JNJ2408068; formerly designated R170591; Andries et al., 2000). R170591, a novel fusion inhibitor with picomolar activity against respiratory

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syncytial virus (RSV) (Andries et al., 2000) were included in the tests. The results of these studies are presented in the following.

2. Materials and methods

2.1. Animals

Male and female cotton rats (*Sigmoden hispidis*) weighing between 50 and 100 g and male Balb/C mice between 20 and 22 g were used to produce hMPV-specific antibodies (see the following). All of the cotton rats utilized were descendants of six pairs of animals obtained in 1984 from the Small Animal Section of the Veterinary Research Branch, Division of Research Services, National Institutes of Health (NIH). The mice were purchased from Charles River Laboratories via the National Cancer Institute Animal Program, Frederick, MD. These animals were housed in the Baylor College of Medicine (BCM) vivarium in cages covered with barrier filters and each was given food and water ad libitum. Blood samples obtained from representative animals housed in these spaces at intervals before or during the course of these experiments were seronegative for adventitious viruses and other rodent pathogens. The antibody production was carried out utilizing NIH and United States Department of Agriculture guidelines and experimental protocols approved by the BCM Investigational Animal Care and Use Committee (IACUC).

2.2. Tissue culture

HEp-2 (human epithelial carcinoma; ATCC CCL23) and LLC-MK2 (Rhesus monkey kidney; ATCC CCL7) tissue culture cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Eagle's minimal essential medium (MEM; Sigma Chemical Co.; cat. no. M4465) supplemented with 10% fetal calf serum (FCS; Summit Biotechnology, Fort Collins, CO; cat. no. FP-200-05), 100 U/ml penicillin (Sigma Chemical Co.; cat. no. P-4458), 100 μg/ml gentamicin sulfate (Sigma Chemical Co.; cat. no. G-1264), 2 mM L-glutamine (Sigma Chemical Co.; cat. no. G7513), and 0.2% sodium bicarbonate (Sigma Chemical Co.; cat. no. S8761) were used to grow both cell types. Similarly supplemented MEM containing 1.0 μg trypsin/ml (Worthington Biochemical Corp., Lakewood, NJ; cat. no. 32C5468) and lacking FCS was utilized to prepare pools of hMPV and in any assay in which hMPV was involved. It was also employed in assays in which hRSV was grown in LLC-MK2 cells. The trypsin-containing medium was utilized in conjunction with hMPV because this protease was required for the replication of this virus in LLC-MK2 cells. Trypsin-containing medium was not required for hRSV replication, but was used when this virus was grown in LLC-MK2 cells to keep variables in different assays to a minimum.

2.3. Viruses

Seed vials of hRSV (Long strain; ATCC VR26) were purchased from the ATCC. The hMPV utilized (CAN97-83) was 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. Working stocks of each virus were prepared by infecting flasks containing the appropriate tissue culture cell line as described previously (Wyde et al., 1993). The only exception was that the trypsin-containing medium described above was used to grow the hMPV virus.

2.4. Virus quantification

Levels of virus in different preparations were determined by serially diluting each sample in duplicate or quadruplicate in sterile 96-well tissue culture plates (Falcon 3072) using half $\log_{10}$ dilutions as described previously (Wyde et al., 1995). The monolayers in the wells of these plates were observed daily and scored for virus-induced cytopathic effects (CPE). Last readings for CPE formation were made when no further progression of virus presence was evident in the titration for at least two successive days (usually Day 7 for hRSV and Day 14 for hMPV). At that time, the wells that were positive or negative for virus-induced CPE in each replicate row were noted. These data, the dilution of virus in each test well and the interpolation method of Karber (Rhodes and Van Rooyen, 1953) were utilized to estimate the amount of virus present in the original suspension. Virus titers were expressed as median tissue culture infectious doses ($\log_{10}$ $TCID_{50}$/ml). In these assays, the minimum detectable virus concentration was 1.8 $\log_{10}$ $TCID_{50}$/ml.

2.5. Chemotherapeutic agents

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, M.W. 244.2; see Fig. 1) was obtained as a powder from ICN Pharmaceuticals (Costa Mesa, CA; cat. no. 196066). On the morning of an experiment, the amount of 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). VP14637, a substituted di-tetrazole-benzhydrylphenol (M.W. 510.52; see Fig. 1) was received suspended in its vehicle (i.e. 85% ethanol, 10% propylene glycol, and 5% water) from Viropharma Incorporated (VP), Exton, PA. Both the drug and carrier were stored at room temperature until utilized. JNJ2408068 ((2[[2-[[1-(2-aminoethyl)-4-piperidinyl]amino]-4-methyl-1*H*-benzimidazol-1-yl]methyl]-6-methyl-3-pyridinol); M.W. 395; see Fig. 1) was supplied by Johnson & Johnson Pharmaceutical Research & Development, Beerse, Belgium. This compound was stored at 4 °C until the morning of an assay. At that

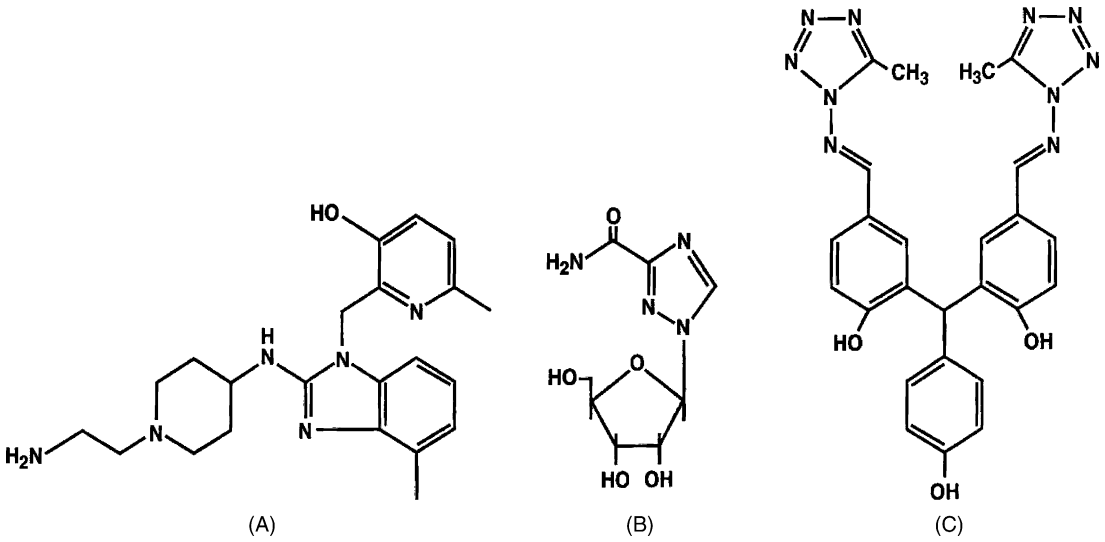

Fig. 1. (A) Chemical structure of JNJ2408068 (2[[2-[[1-(2-aminoethyl)-4-piperidinyl]amino]-4-methyl-1*H*-benzimidazol-1-yl]methyl]-6-methyl-3-pyridinol), (B) structure of ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide); and (C) the structure of VP14637, a substituted di-tetrazole-benzhydrylphenol.

time, the amount of test compound needed was suspended in distilled water and the pH of the resulting suspension was adjusted to be 6.0 using 0.1N hydrochloric acid. Once this was done, the compound was readily soluble and was filtered using a 0.2 μm DynaGard filter. Distilled water adjusted to be pH 6.0 was used as a vehicle control for both ribavirin and JNJ2408068. The VP vehicle was used as the placebo control in all experiments utilizing VP14637.

2.6. Antibody preparations

The IVIG used in these studies, Venoglobulin-S, 5% solution, was purchased from Alpha Therapeutic Corporation, Los Angeles, CA. Palivizumab (Synagis™, MedImmune, Inc., Gaithersberg, MD) was obtained from the Methodist Hospital Pharmacy, Houston, TX. This humanized monoclonal IgG_1 antibody has virus-neutralizing activity against antigenic site A on the fusion (F)-protein of hRSV and thus potentially can prevent infection of both A and B subtypes of hRSV (Russell, 1999). RSHZ19 was obtained from SmithKline Beecham (King of Prussia, PA). Like palivizumab, this antibody is a humanized monoclonal IgG_1 antibody with virus-neutralizing activity against antigenic site A on the F protein of hRSV (Porter et al., 1999). Also utilized in these tests were (1) a mouse monoclonal antibody (Mab) with specific activity to the F0 (70 kDa) and F1 (48 kDa) subunits of the hRSV F protein present on all hRSV strains (A and B; Argene, Inc., North Massapequa, NY; cat. no. 11-042); (2) a polyclonal antiserum to hRSV obtained after bleeding several cotton rats 28 days after inoculating them intranasally (i.n.) with RSV Long; and (3) mouse and cotton rat polyclonal sera with specificity for hMPV that were prepared from cotton rats or mice inoculated intraperitoneally (i.p.) with sucrose-purified hMPV 3 weeks apart as described in the following. All of the antibody preparations were stored at 4 °C until utilized.

2.7. Sucrose purification of hMPV

The hMPV was concentrated as described by Killington et al. (1996) using 50% polyethylene glycol (PEG) to precipitate the virus and ultracentrifugation to pellet this precipitate. The latter was suspended in 20% sucrose in sodium Tris ethylenediaminetetraacetic acid (sucrose–NTE) buffer and layered on top of Beckman polyallomer thin walled ultracentrifuge tubes (14 mm × 89 mm, cat no. 331372) containing sequential layers (top to bottom) of 20, 30 and 45% sucrose in NTE buffer. The tubes were then centrifuged in a swinging bucket rotor (Beckman SW41) for 1 h at 240,000 × *g*. The bands that appeared at the interface between the 30 and 45% sucrose–NTE buffer layer in these tubes following this procedure were collected and portioned in cryovials. These were labeled, snap-frozen using a dry ice and alcohol bath, and then stored in a −70 °C freezer until needed.

2.8. Production of hMPV-specific antibodies

To produce hMPV-specific antibodies, Balb/c mice and cotton rats were injected twice i.p. 3 weeks apart, with 10^6 $TCID_{50}$ of sucrose-purified hMPV mixed with an equal volume of phosphate buffered saline (PBS) containing 10 μg of QS-21 adjuvant (Antigenics, Inc., Framingham, MA). One week after the second inoculation, the animals were bled. The sera that were obtained from these samples were heat-inactivated at 56 °C for 30 min, portioned, labeled, and stored at 4 °C until utilized. They were tested for antibody titer and virus-specificity using a virus-neutralizing antibody assay.

2.9. Virus-neutralizing antibody assay

Heat-inactivated sera were tested for hRSV- or hMPV-specific antibodies in duplicate or quadruplicate using sterile 96-well tissue culture plates (Falcon 3072) as described in detail elsewhere (Wyde et al., 1995) with two modifications. One, LLC-MK2 cells were utilized in tests in which hMPV was used. Secondly, the morning after setting up an assay, the medium in each well of any plate containing hMPV was decanted and the monolayers in them were rinsed with PBS. Two hundred microliters of trypsin-containing medium was then added back to each well and the plates were returned to the 36 °C incubator. The cell monolayers in these plates, as well as those in plates containing hRSV, were observed daily. When ≥70% CPE was evident in the virus-control wells of any plate, all of the wells in that plate were observed and scored for the presence of virus-induced CPE. Titers were expressed as $\log_2$ of the reciprocal of the last dilution of antiserum that completely inhibited virus-induced CPE. The minimum detectable neutralization titer in serum was $1 \log_2/0.05$ ml.

2.10. Assays to determine median cytotoxic drug concentrations

Assays to determine the median cytotoxic concentration (IC_{50}) of ribavirin (4 mg/ml), VP14637 (0.2 mg/ml) or JNJ2408068 (0.1 mg/ml) in HEp-2 and LLC-MK2 cells were performed in quadruplicate or duplicate in 96-well flat bottom tissue culture plate (Falcon 3072) as described previously (Wyde et al., 1993) with the exception that alamar Blue™ (aB; Biosource International, Camarillo, CA; cat. no. DAL1100) was utilized to confirm cell viability instead of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). The assay plates were incubated at 36 °C in a 5% CO_2 incubator until the monolayers in the tissue culture control wells became confluent (usually 48–72 h later). At that time, all of the wells were observed microscopically and scored for drug-induced CPE and estimated confluency of the monolayers. Twenty microliters of aB was then added to each well. This reagent contains an oxidation/reduction (REDOX) indicator and is non-fluorescent and blue in its oxidized state. However, it turns red and fluoresces strongly in its reduced state (excitation and emission wavelengths = 546 and 590 nm, respectively). It has been shown that 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). When the color of the medium present in the tissue culture control wells turned red (usually 4–6 h after addition of the aB), the relative fluorescence in each test and control well was determined using a Fluorolite™ 1000 Microtiter® plate fluormeter (Dynatech Laboratories, Inc., Chantilly, VA). Using these values, the percentage of fluorescence relative to that of the tissue culture control wells and concentration (μM) of ribavirin, VP14637, or JNJ2408068 were noted and entered into the computer using CalcuSyn, (Biosoft, Inc. Ferguson, MO), a Windows™-based program made for dose effect analysis. Utilizing this program, the micromole concentration of each compound that induced a 50% reduction in mean relative fluorescence in one-half of the replicate wells compared to the mean relative fluorescence of the tissue culture control wells (i.e. their IC_{50} value) was obtained.

2.11. Assays to determine the median virus-inhibiting concentration of each compound

Assays evaluating the virus-inhibiting activity of ribavirin (4 mg/ml), VP14637 (0.2 mg/ml), or JNJ2408068 (0.1 mg/ml) against hRSV in HEp-2 cells were performed in quadruplicate or duplicate in 96-well flat bottom tissue culture plate (Falcon 3072) as described previously (Wyde et al., 1993). The test plates were incubated at 36 °C in a 5% CO_2 incubator until the virus-control wells exhibited 70–100% CPE (characteristically conspicuous syncytium formation), usually 5–7 days after the addition of virus to the test wells. At that time, each well was observed, scored for drug and virus-induced CPE and the median concentration (μM) of each compound that totally inhibited virus replication in one-half of the replicate wells (i.e. their EC_{50} values) was determined.

A similar procedure was used when testing these materials for antiviral activity against hMPV or hRSV in assays utilizing LLC-MK2 cells. However, in these tests, 0% FCS-MEM was used as the diluent and maintenance medium and the test materials and vehicles were serially diluted in parallel plates lacking tissue culture cells. The medium in each well of the primary plate (i.e. the plate that contained LLC-MK2 cells) was then removed and the cell monolayers in it were rinsed with PBS. The contents of the wells in the parallel plate were transferred to the primary plate taking care to maintain the proper orientation of the plates. After adding hMPV or hRSV to the primary plate, it was incubated at 36 °C until the virus-control wells exhibited 70–90% CPE. At that time, each well was observed and scored for evidence of virus. However, because the CPE induced by hMPV typically consisted primarily of rounded up cells and minimal syncytium, it could be confused with drug toxicity or rounding up of cells due to overcrowding (see Fig. 2). For this reason, an enzyme linked immunosorbant assay (ELISA) for the detection of hMPV antigens was performed to confirm which wells were positive for this virus (this assay is described in the next paragraph). The data obtained from the ELISA assay were analyzed to determine the mean (μM) concentration of each test compound that completely inhibited replication of hMPV in one-half of the replicate wells. Because the CPE induced by the hRSV in either HEp-2 or LLC-MK2 cells was so distinctive, an ELISA was not required in assays utilizing this virus and the EC_{50} values in this instance could be calculated following visual determination of virus presence or absence. A selective index (S.I.) was calculated for each compound by dividing

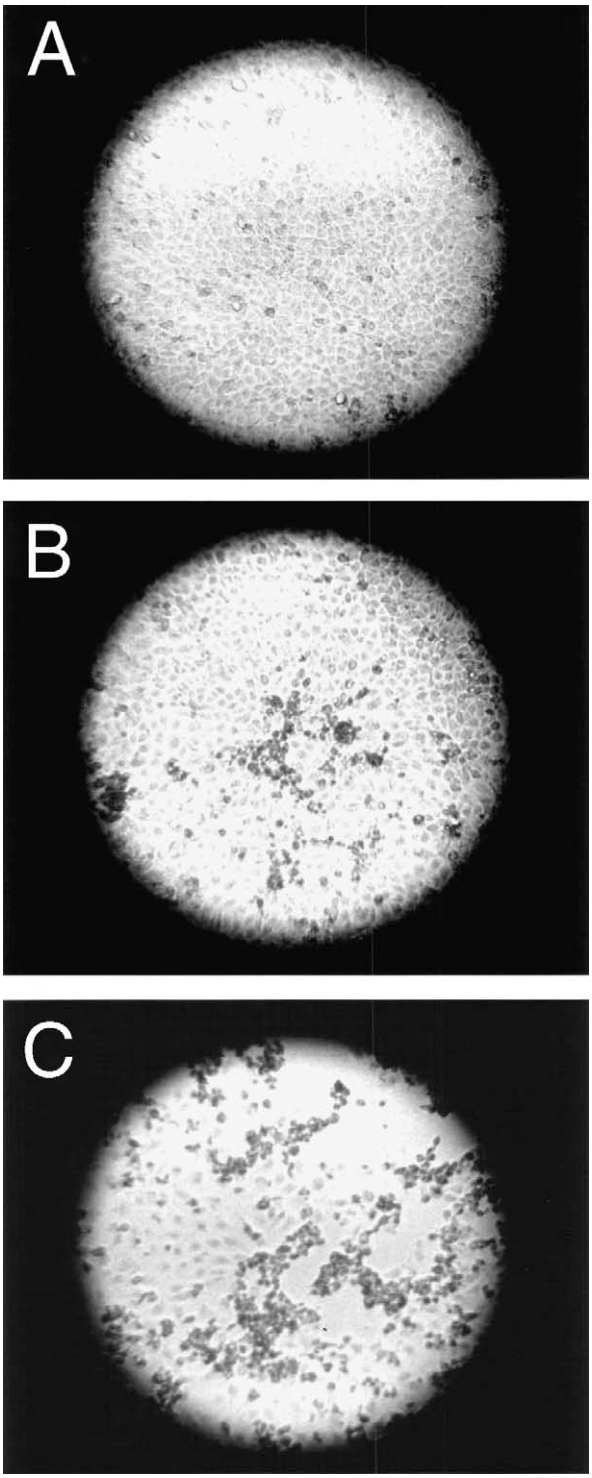

Fig. 2. (A) Uninfected LLC-MK2 cells; (B) LLC-MK2 cells 4 days after infection with human metapneumovirus (hMPV); and (C) these cells 10 days after infection with hMPV.

the IC_{50} value determined for a compound using the alamar Blue assay by the appropriate EC_{50} value obtained by visual observation (hRSV assays) or ELISA (hMPV) assay. For example, the IC_{50} of ribavirin in LLC-MK2 cells determined with alamar Blue was divided by the EC_{50} value of ribavirin against hMPV in LLC-MK2 as determined by the ELISA assay.

2.12. ELISA assay for the detection of hMPV antigens

Omnifix® tissue fixative (An-Con Genetics, Inc., Melville, NY) was added to each well of an EC_{50} assay plate after making visual observations of each monolayer for drug and hMPV-induced CPE. After a 1 h incubation at room temperature, the monolayers were washed three times using a semi-automated plate washer (Labsystems Wellwash model 4 MK 2; Fisher Scientific, Dallas, TX). Two hundred microliters of Superblock® blocking buffer (Pierce Chemical Co., Rockville, IL; cat. no. 37515) was then added to every well. An hour later, the blocking solution was removed and the wells were washed three more times. Mouse antisera with antibodies specific for hMPV (produced as described above) was then diluted 1/1000 in 0.5% Tween 20–PBS and added to each well. After a 60-min incubation, the plates were washed three times and 50 μl of a 1/3000 dilution of horseradish peroxidase (HRP)-conjugated protein A/G (Pierce Chemical Co.; cat. no. 32490) was added to the wells. One hour later, the monolayers were washed three times and 3,3′,5,5′-tetramethyl benzidine (TMB) substrate was added to the wells. When a dark blue color was evident in the virus-control wells, 0.18 M sulfuric acid was added to each well to denature the HRP enzyme and minimize further changes in the color. The optical density (OD) in each well was then determined using a 96-well spectrophotometric plate reader (Molecular Devices, Inc., Palo Alto, CA) set at 450 nM wavelength. The mean OD obtained for the tissue culture control wells exposed to immune antisera + 3 S.D. was determined. The OD values in all other wells greater than this value were considered positive for hMPV and so noted. Generally, one to two more virus-positive wells per row were detected using the ELISA as compared to using visual readings of CPE.

2.13. 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 mean IC_{50} values obtained for ribavirin, JNJ2408068, and VP14637 during testing of these compounds for cytotoxicity in HEp-2 and LLC-MK2 tissue culture cells are shown in Table 1 (column 3). As these values indicate, the LLC-MK2 cells were generally less sensitive to the cytotoxic activity of all three of the test compounds than were the HEp-2 cells. Thus, the mean IC_{50} value obtained for ribavirin was $\geq$4095 μM in LLC-MK2 and only 3071 μM in HEp-2 cells and both JNJ2408068 and VP14637 were more than

Table 1
Comparison of the cytotoxicity of ribavirin, VP14637, and JNJ2408068 in HEp2 and LLC-MK2 cells[a]

Material tested	Cell line	Mean IC_{50} (μM)	Virus tested	Mean EC_{50} (μM)	Selective index
Ribavirin	HEp-2	3071 ± 0	hRSV	58 ± 12	53
Ribavirin	LLC-MK2	≥4095 ± 0	hRSV	88 ± 55	≥47
Ribavirin	LLC-MK2	≥4095 ± 0	hMPV	74 ± 35	≥55
JNJ2408068	HEp-2	238 ± 0	hRSV	0.002 ± 0.001	119,000
JNJ2408068	LLC-MK2	≥2532 ± 0	hRSV	0.001 ± 0.0003	≥2,532,000
JNJ2408068	LLC-MK2	≥2532 ± 0	hMPV	≥2532 ± 0	1
VP14637	HEp-2	4.7 ± 0	hRSV	0.0008 ± 0.004	5875
VP14637	LLC-MK2	56 ± 26	hRSV	0.0006 ± 0.0003	93,333
VP14637	LLC-MK2	56 ± 26	hMPV	≥56 ± 26	≤1

[a] Results shown are from two replicate IC_{50} and EC_{50} assays performed in 96-well tissue culture plates as described in Section 2. 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), hRSV, human respiratory syncytial virus; hMPV, human metapneumovirus.

10× less cytotoxic in the former cell line than in the latter line (≥2532 μM versus 238 μM for JNJ2408068 and 56 ± 26 μM versus 4.7 μM for VP14637). VP14637 appeared to be the most cytotoxic of the three test compounds in both cell lines. However, much of this cytotoxicity may have been due to its vehicle. For example, in HEp-2 cells, this vehicle consistently induced rounding up and death of the test cells of four and five wells up the test plates (i.e. at dilutions that resulted in its 85% ethanol concentration being reduced to between 2.5 and 5%). In most tests, the cytotoxicity induced by the VP vehicle was just one dilution less than that seen with the compound suspended in this vehicle (data not shown). JNJ2408068 did not exhibit any evident cytotoxicity in LLC-MK2 cells, even at the maximum tested concentration (i.e. ≥2532 μM). However, the mean IC_{50} value of 238 μM obtained for this compound in HEp-2 cells indicated that it was somewhat cytotoxic to those cells. Ribavirin was also not cytotoxic to LLC-MK2 cells at the maximum concentration tested (i.e. ≥4095 μM). However, in contrast to the other two compounds, it only slightly inhibited the growth and survival of HEp-2 cells (mean IC_{50} = 3071 μM).

The mean EC_{50} values and selective indices obtained during testing of the three chemotherapeutic agents against hRSV and hMPV are displayed in columns 5 and 6 of Table 1. As the values in column 5 indicate, JNJ2408068 and VP14637 had similar antiviral activity (i.e. there was no more than four-fold differences in their mean EC_{50} values, 0.0006–0.002 μM). Thus, both of these compounds appeared to inhibit hRSV at ≥29,000× lower concentration than ribavirin (EC_{50} ribavirin = 58 ± 12 μM). Because of this activity, the two non-nucleoside compounds had remarkable selective indices against hRSV (i.e. 5875 in HEp-2 cells and >93,000 in LLC-MK2 cells for VP14637 and 119,000 in HEp-2 cells and ≥2,000,000 in LLC-MK2 cells for JNJ2408068). For comparison, the S.I. obtained for ribavirin against hRSV was 53.

In contrast to the marked antiviral activity and extraordinary selective indices obtained for JNJ2408068 and VP14637 against hRSV, neither of these compounds had any detectable selective antiviral activity against hMPV (i.e. the S.I. obtained for each of the compounds was ≤1). However, ribavirin inhibited hMPV in every experiment performed. The mean EC_{50} value obtained in the representative experiments shown was 74 ± 35 μM (S.I. ≥ 55). Most importantly, its antiviral activity against hMPV was equivalent to that seen against hRSV (i.e. 74 ± 35 μM ribavirin versus hRSV compared to 88 ± 55 μM ribavirin versus hMPV).

The ability of different antibody preparations to neutralize hRSV and hMPV is displayed in Table 2. The first three rows of this table compare the virus-neutralizing titers

Table 2
Comparison of the neutralizing antibody titers of different antibody preparations against respiratory syncytial virus and human metapneumovirus[a]

Sera	Species	Type	Specificity	Neutralizing antibody titer (log_2/0.05 ml)	
				RSV Long	hMPV
CRaRSV	Cotton rat	Polyclonal	RSV	5	0
CRaHMPV	Cotton rat	Polyclonal	hMPV	0	6
MoaHMPV	Mouse	Polyclonal	hMPV	0	5
MoaRSVMab	Mouse	Monoclonal	RSV	≥7	0
Synergis	Humanized	Monoclonal	RSV	15	0
RSHZ19	Humanized	Monoclonal	RSV	12	0
IVIG	Human	Polyclonal	None	11	10

Abbreviations: RSV, respiratory syncytial virus; hMPV, human metapneumovirus; CR, cotton rat; a, anti; Mo, mouse; IVIG, human immune serum globulin; 0, undetectable.

[a] The neutralizing antibody assay was performed as described in Section 2 utilizing serial two-fold dilutions.

($\log_2$/0.05 ml) of cotton rat and mouse antisera that were produced by either infecting cotton rats i.n. with hRSV (row 1) or injecting these animals or mice with sucrose-purified hMPV twice 21 days apart as described in Section 2 (rows 2 and 3). As the titers in the last two columns of this table indicate, there was no evident antigenic relationship between the two viruses. Each of these antisera appeared to be specific for the virus that was used to raise them, although each was polyclonal in nature and thus likely contained antibodies specific for most, if not all, of the proteins present in or produced by each of these viruses.

The last four rows of Table 2 presents representative virus-neutralizing titers obtained for three potent monoclonal antibodies specific for epitopes on the F protein of hRSV and an IVIG preparation that has no virus-specificity, but was known to contain significant levels of virus-neutralizing antibodies to hRSV. As the results shown indicate, all three of the monoclonal antibodies inhibited replication of hRSV at relatively high dilutions ($\geq 7\log_2$ to $15\log_2$), but had no apparent activity against hMPV. In contrast to these results, the IVIG was equally effective in neutralizing both viruses.

4. Discussion

Although newly elucidated, it is already apparent that hMPV is a significant human respiratory pathogen. It is also clear that there is a need for agents that can prevent or ameliorate infections caused by this virus. Because of its phylogenetic relationship to hRSV (van den Hoogen et al., 2002), it seemed plausible to begin the search for such interventions with substances that have proven to be successful against hRSV, especially those that are licensed and thus have already been utilized clinically. Even if not optimal, such agents could provide interim relief until more suitable materials are elucidated and developed. With these thoughts in mind, the present studies were undertaken to compare the antiviral activity of ribavirin and a standard IVIG preparation against hMPV and hRSV in tissue culture-based assays.

Initial studies were carried out using HEp-2 cells to evaluate the antiviral potential of ribavirin and two other chemotherapeutic agents against hRSV and LLC-MK2 cells to assess the activity of these compounds against hMPV. These cell lines were utilized because the manifestations of these viruses are optimal in these cells, respectively, an important point in determining viral inhibition endpoints. However, it quickly became apparent that the two cell lines had markedly different sensitivities to the cytotoxic effects of the test compounds. To insure that no similar cellular influence was present in antiviral assays, all three chemotherapeutic compounds were also tested against both viruses using LLC-MK2 cells. A sensitive ELISA assay specific for hMPV antigens was used to confirm visual readings made during the course of these assays and to facilitate distinguishing between drug- and hMPV-induced CPE in the EC_{50} assays utilizing this virus.

As the EC_{50} values in Table 1 make evident, each compound had equivalent antiviral activity against hRSV regardless of which tissue culture cell line was used to test the compound. These values also indicate that both VP14637 and JNJ2408068 were extraordinarily more active against hRSV than ribavirin (i.e. $\geq$29,000×). However, neither VP14637 nor JNJ2408068 had any apparent antiviral activity against hMPV. This finding was not surprising since both of these compounds have been shown to interact specifically with areas on the F protein of hRSV (Pevear et al., 2000. Mechanism and spectrum of activity of a potent inhibitor of RSV replication; and Andries et al., 2000. R170591, a novel fusion inhibitor with picomolar activity against RSV), regions that are not conserved between pneumo (e.g. hRSV) and metapneumo (e.g. hMPV) viruses (van den Hoogen et al., 2002). In contrast, ribavirin, a compound with known broad-spectrum antiviral activity (Sidwell et al., 1972), exhibited similar antiviral activity (i.e. had similar EC_{50} values) against both viruses, regardless of the tissue culture cell line used to perform the assays. This finding would suggest that ribavirin could be useful for treating hMPV infections clinically. However, clinical use of this compound is contentious, (1) because ribavirin is a potential teratogen (Kilham and Ferm, 1977) and thus is a potential health hazard (Bradley et al., 1990; Ito and Koren, 1993); (2) ribavirin treatment is expensive (estimated in 1994 to average US$ 3300 per case; Marquardt, 1995); and (3) there is some question about its use and effectiveness against RSV infections (see Wyde, 1998 for a review of this subject). Despite these problems, ribavirin has been shown to be effective in limiting disease and mortality in immunosuppressed persons infected with hRSV if treatment is started early (Englund et al., 1988; Englund et al., 1997; Sparrelid et al., 1997) and hMPV has already been found to infect this population (Ison and Hayden, 2002; Pelletier et al., 2002). Thus, it is possible that this compound may be of some use in this setting when administered alone or in combination with another compound or materials. For example, it has been reported that ribavirin used in conjunction with IVIG may be more effective in treating hRSV infections in immunosuppressed individuals than using ribavirin alone (Whimbey et al., 1995; DeVincenzo et al., 1996).

Regardless of whether ribavirin can be utilized to treat hMPV infections, the finding that this compound inhibited hMPV is important. For example there are compounds related to ribavirin, EICAR for example, that are more active against hRSV and measles virus (another paramyxovirus) than ribavirin (De Clercq et al., 1991; Balzarini et al., 1993; Wyde et al., 2000). It is possible that these agents may also have greater activity against hMPV and thus merit preclinical testing.

The only other material that consistently inhibited hMPV replication in this study was the standard IVIG preparation. IVIG preparations have been used clinically for prophylaxis

against hRSV and measles virus infections and found to provide measurable protection in clinical trials if the preparation used contained a sufficiently high titer of virus-specific antibodies (reviewed in Ottolini and Hemming, 1997). Indeed, RSV-IVIG, an IVIG preparation that has extraordinary titers to hRSV (Siber et al., 1994), is licensed for prophylactic use against hRSV infections. This material was prepared using only sera that were shown in pretesting to contain high levels of hRSV-specific neutralizing antibodies (Siber et al., 1992). In 1996, after extensive testing, RSV-IVIG was licensed in the United States for use against RSV in select groups of infants with bronchopulmonary dysplasia and/or prematurity (American Academy of Pediatrics Committee on Infectious Diseases, 1997). There seems to be no reason why high-titered serum to hMPV cannot be similarly prepared. However, it should be noted that there are disadvantages associated with RSV-IVIG and IVIG preparations in general. For example, RSV-IVIG is not recommended for use in infants with congenital heart disease, as it was associated with an excess of adverse events in this population (American Academy of Pediatrics Committee on Infectious Diseases, 1997). In addition, it is difficult to administer (i.e. it must be given intravenously), requires large fluid volumes (15 ml/kg) and high-protein load (750 mg/kg) and can interfere with live virus vaccines (e.g. MMR and varicella). Another problem associated with this preparation is its high cost (e.g. >US$ 5000 per season; Thakur et al., 1997; Barton et al., 2001). Indeed, it was these problems that prompted the development of humanized monoclonal antibody preparations (e.g. palivizumab and RSHZ19) to hRSV. However, development of high-titered IVIG preparations to hMPV and the use of IVIG and ribavirin in combination are two approaches that could be undertaken for hMPV until an effective vaccine is developed.

An interesting finding was that the IVIG preparation utilized in these studies contained a relatively high titer of hMPV-neutralizing antibody ($10 \log_2/0.05$ ml; Table 3). Because this preparation was prepared in 1993, this data provides clear evidence that this virus is probably not new but has been circulating for some time.

In summary, although the extent and the populations at risk need to be better defined, hMPV has already been shown to be a medical threat. Presently there are no vaccines, chemotherapeutic agents or immunoglobulin preparations available to prevent or ameliorate infections caused by this virus. The present studies indicate that ribavirin, IVIG preparations, or combinations of these should undergo clinical evaluation and may prove effective until more efficacious agents are developed.

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