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Substituted benzimidazoles with nanomolar activity against respiratory syncytial virus

Koen Andries[a,*], Marc Moeremans[a], Tom Gevers[a], Rudy Willebrords[a], Cois Sommen[a], Jean Lacrampe[b], Frans Janssens[a], Philip R. Wyde[c]

[a] *Johnson and Johnson Pharmaceutical Research and Development, Beerse, Belgium*
[b] *Johnson and Johnson Pharmaceutical Research and Development, Val de Reuil, France*
[c] *Baylor College of Medicine, Houston, TX, USA*

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Abstract

A cell-based assay was used to discover compounds inhibiting respiratory syncytial virus (RSV)-induced fusion in HeLa/M cells. A lead compound was identified and subsequent synthesis of >300 analogues led to the identification of JNJ 2408068 (R170591), a low molecular weight (MW 395) benzimidazole derivative with an EC_{50} (0.16 nM) against some lab strains almost 100,000 times better than that of ribavirin (15 μM). Antiviral activity was confirmed for subgroup A and B clinical isolates of human RSV and for a bovine RSV isolate. The compound did not inhibit the growth of representative viruses from other Paramyxovirus genera, i.e. HPIV2 and Mumps Virus (genus Rubulavirus), HPIV3 (genus Respirovirus), Measles virus (genus Morbillivirus) and hMPV. Efficacy in cytopathic effect inhibition assays correlated well with efficacy in virus yield reduction assays. A concentration of 10 nM reduced RSV production 1000-fold in multi-cycle experiments, irrespective of the multiplicity of infection. Time of addition studies pointed to a dual mode of action: inhibition of virus–cell fusion early in the infection cycle and inhibition of cell–cell fusion at the end of the replication cycle. Two resistant mutants were raised and shown to have single point mutations in the F-gene (S398L and D486N). JNJ 2408068 was also shown to inhibit the release of proinflammatory cytokines IL-6, IL-8 and Rantes from RSV-infected A549 cells.
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1. Introduction

Respiratory syncytial virus (RSV), a Paramyxovirus, is an important cause of respiratory tract infection in infants, young children and adults. RSV infection is responsible for a large proportion of hospitalisations of infants in the winter months and consumption of significant health care resources. Significant morbidity is associated with RSV bronchiolitis, and a link to development of asthma has been proposed (Welliver, 1995). Although the importance of RSV among young children is well recognized, it has recently been appreciated that more than 78% of RSV-associated underlying respiratory and circulatory deaths occur among persons aged 65 years or older (Thompson et al., 2003).

The options to prevent or treat RSV infections are limited. Prophylactic administration of palivizumab, a monoclonal antibody binding the respiratory syncytial virus fusion protein, has been shown to significantly decrease the incidence of RSV-related hospitalisations among high-risk children (The IMpact-RSV Study Group, 1998). There are questions concerning the efficacy and safety of using ribavirin, the only licensed antiviral for treatment of RSV infections (Anonymous, 1996). Development of new and better antivirals is therefore a priority. Several small molecules with potent anti-RSV activity have recently been discovered. While some of these also affect the replication of other viruses (Kimura et al., 2000), others have been described as specific inhibitors of RSV (Huntley et al., 2002; McKimm-Breschkin, 2000; Watanabe et al., 1998) and these usually target the fusion protein of the virus. The current report describes the discov-

* Corresponding author.

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ery and antiviral properties of a new family of potent RSV inhibitors.

2. Materials and methods

2.1. Viruses and cells

Human (strain LO) and bovine RSV, human parainfluenza viruses 2 and 3 (HPIV2 strain Greer and HPIV3 strain C243), human rhinoviruses 2 and 14 (HRV2, HRV14), Mumps Virus (strain Jones), Measles virus (strain Edmonston), and pneumonia virus of mice (PVM, strain 15) were provided by the American Type Culture Collection, Manassas, VA, USA. Influenza viruses (A/Taiwan/1/86 [H1N1], A2/Japan/305/57 [H2N2], A/Virginia/88 [H3N2], B/Hong Kong/5/72) were obtained from F.G. Hayden, Charlottesville, USA. Human RSV was grown in HeLa/M, HEp-2 or A549 cells, HRV in HeLa/M cells, bovine RSV in MDBK cells, influenza viruses in MDCK cells, mumps and HPIV3 virus in LLC-MK2 cells, Measles virus and HPIV2 in Vero cells and HIV in MT-4 cells. Virus stocks were stored at −70 °C. Cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 5% foetal calf serum (FCS), 2% sodium bicarbonate and 2 mM L-glutamine. Clinical isolates of RSV were obtained from A.M. Van Loon, University of Utrecht, The Netherlands, and G. Ieven, University of Antwerp, Belgium. These were also grown in HeLa/M cells. Human metapneumovirus (hMPV) was obtained from Dr. Guy Boivin, Research Center in Infectious Diseases, Regional Virology Laboratory, Laval University, Quebec City, Canada. This virus was grown in LLC-MK2 (rhesus monkey kidney; ATCC CCL7) cells in MEM supplemented as described above except that foetal calf serum was not added and the medium was supplemented with 0.5 mg/ml Worthington trypsin (Worthington Biochemical Corp., Lakewood, NJ; cat. no. 32C5468).

2.2. Compounds

Substituted benzimidazoles such as JNJ 2408068 (2-[[2-[[1-(2-aminoethyl)-4-piperidinyl]amino]-4-methyl-1*H*-benzimidazol-1-yl]methyl]-6-methyl-3-pyridinol) (Fig. 1, MW 395, formerly R170591, Andries et al., 2000) were synthesized in the Johnson and Johnson (Pharmaceutical Research and Development) laboratories by methods described elsewhere (Janssens et al., WO 01/00611, WO 01/00612, WO 01/00615). Compounds were dissolved in dimethylsulfoxide (DMSO) at 20 mM and diluted in growth medium to achieve the final concentration needed. Ribavirin was acquired from Sanico (Turnhout, Belgium) and palivizumab from Medimmune (Gaitersburg, MD, USA).

2.3. Cytotoxicity assay

To assess the cytotoxicity of JNJ 2408068, HeLa/M cells were seeded in 6-well plates in a volume of 2 ml of MEM at a concentration of 125,000 or 800,000 cells per well. After 24 h incubation at 37 °C in a humidified 5% CO_2 atmosphere, the growth medium was replaced by test solutions (fresh growth medium with or without JNJ 2408068). The number of living cells present in triplicate cultures was determined at the time of drug addition (day 0), and days 1, 2 and 3. At these time points, cells were rinsed twice using Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and then trypsinized, pooled, centrifuged for 5 min at 3000 rpm, resuspended in medium and counted in triplicate using a Coulter Counter.

2.4. Antiviral activity

For all viruses listed, a tetrazolium-based colorimetric method, the MTT assay (Mosmann, 1983) was used to determine EC_{50}s reflecting antiviral activities.

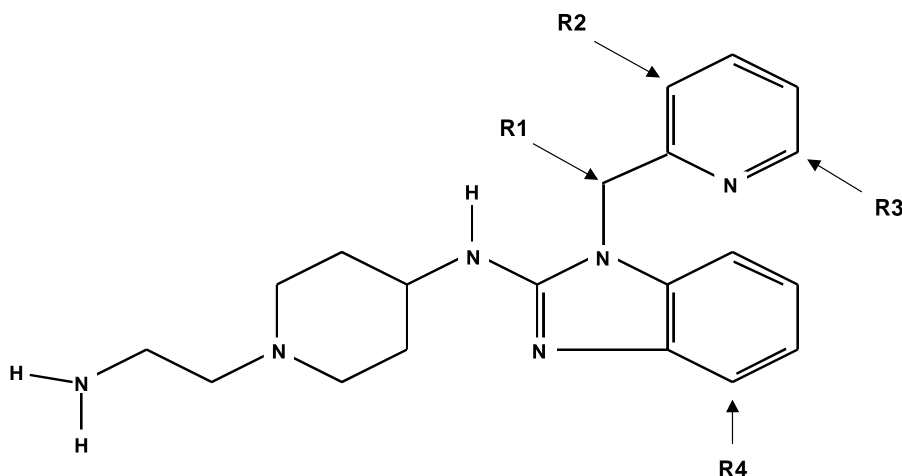

Fig. 1. Chemical scaffold of the substituted benzimidazole series.

Flat-bottom, 96-well plastic microtiter trays were filled with MEM. Stock solutions of compounds were added to a series of triplicate wells so as to allow simultaneous evaluation of their effects on virus- and mock-infected cells. Serial five-fold dilutions were made directly in the microtiter trays using a customised robot system (Zymark Corp., Hopkinton, MA). Virus- and mock-infected controls were included in each test. Approximately 100 median tissue culture infectious doses ($TCID_{50}$) of virus were added to two of the three rows, and medium without virus to the third row. After 2 h incubation at 37 °C in a 5% CO_2 atmosphere, a suspension (4×10^5 cells/ml) of susceptible cells was added to all wells in a volume of 50 μl. Cultures were further incubated at 37 °C, except for HRV (33 °C). Four to seven days post-virus exposure, the viability of mock- and virus-infected cells was quantitated spectrophotometrically using the MTT method. The EC_{50} was defined as the 50% inhibitory concentration for cytopathic effect and CC_{50} as the 50% concentration for cytotoxicity.

To assess whether the antiviral effect was cell-dependent, an EC_{50} for cytopathic effect (CPE) inhibition was also determined in HEp-2 and A549 cells using the method described above. Plaque assays were performed in Vero cells.

Activity against PVM was assessed using a limiting dilution reverse transcriptase-polymerase chain reaction (RT-PCR) assay. RNA was extracted from cell culture supernatants. A region encoding the attachment protein of PVM (forward primer: TTT TCA CAT CAA ACC ACA AGG CC, nucleotides 256–278; reverse primer: TAG CCT GTT GAC CTC CAA GCT CT, nucleotides 797–819) was amplified using the Titan One Tube RT-PCR system (Roche, Mannheim, Germany). Samples were analysed with agarose electrophoresis.

2.5. Virus yield reduction assay

RSV (Long strain) was pre-incubated with JNJ 2408068 at 1–1000 times the EC_{50} for 60 min and then added to HeLa/M cells at a multiplicity of infection (MOI) of approximately 1 pfu per cell. After 1 h adsorption at 37 °C, inoculum was removed, cells were washed three times and fresh maintenance medium containing identical concentrations of JNJ 2408068 was added. Extracellular and cell-associated (cells washed three times) virus yields were determined by plaque assay after different incubation times (1, 2, 3 and 4 days post-virus exposure) at 37 °C. Separate experiments assessed the effect of lowering the MOI from 1 to 0.01, and the effect of delaying exposure of the drug until completion of the adsorption period. The data represent results obtained in experiments that were shown to be reproducible in at least two occasions.

2.6. Mechanism of action

2.6.1. Correlation between inhibition of fusion and inhibition of virus production

Seven benzimidazole derivatives (see Fig. 1, Table 1) were incubated for 60 min with RSV (Long strain) at fixed concentrations (0.1 or 1 μM) in MEM containing 5% FCS and 2% HEPES buffer at 37 °C. The virus–drug mixtures were inoculated into HeLa/M cells, the same concentration of compound was added and the extracellular virus yield was determined at 2 days post inoculation by plaque assay.

2.6.2. Effect of time of addition

JNJ 2408068 at 100 times the EC_{50}, palivizumab at 10 times the EC_{50} and ribavirin at 13 times the EC_{50} (the maximal non-cytotoxic dose of ribavirin) were added at different times (−60, 0, 30, 60 min, and 3, 6, 9, 12, 18, 24, 36 and 48 h) post-virus exposure of parallel cultures of HeLa/M cells with human RSV (Long strain) at an MOI of 1. After 60 min of incubation at 37 °C, cells were washed to remove unadsorbed virus and medium with the same drug concentration (if any) was added. The experiment was terminated when syncytia formation was complete in control cultures (48 h post inoculation). The extracellular virus yields of culture supernatants were determined by plaque-titration.

In a second set of experiments, we studied the effect of first adsorbing the virus at 4 °C and then adding the compound, with or without a temperature shift. RSV binds to cells both at 4 and 37 °C but fusion occurs only when the temperature is raised above 18 °C (Srinivasakumar et al., 1991). Virus was first concentrated on a sucrose cushion (1.45 M, 4 h, 7500 × *g*) and then purified by ultra centrifugation on a discontinuous sucrose gradient (30–45–60%). Gradient purified virus at an MOI of 1 was allowed to ad-

Table 1
Antiviral activities of selected substituted benzimidazoles

JNJ No.	R1	R2	R3	R4	EC_{50} (nM)	pEC_{50}	Log yield reduction 100 nM	Log yield reduction 1000 nM
JNJ 1789008	H	H	H	H	398	6.4	0.1	1.1
JNJ 4977648	H	H	Me	H	158	6.8	0.1	1.4
JNJ 4688840	OEt	H	H	H	63	7.2	0.2	2.3
JNJ 4955808	OEt	H	Me	H	13	7.9	1.7	3.4
JNJ 4841278	H	OH	H	H	2.5	8.6	2.7	4.3
JNJ 4749914	H	OH	Me	H	0.40	9.4	4.1	4.7
JNJ 2408068	H	OH	Me	Me	0.16	9.8	4.4	4.6

The EC_{50}s (nM) and effects on virus yield reduction for human RSV were assayed (R1 to R4 refer to substituents on the scaffold of Fig. 1). The pEC_{50} is the negative logarithm of the EC_{50} (e.g. for 1 nM the pEC_{50} is 9, for 10 nM the pEC_{50} is 8).

sorb onto HeLa/M cells at 4 °C for 1 h. In some cultures, 16 nM of JNJ 2408068 ($100 \times EC_{50}$) was added at 4 °C for 1 h at different times during adsorption, and cells were subsequently incubated at 37 °C for 24 h and processed as described below. In other cultures, adsorption was performed at 4 °C for 1 h, unbound virus was removed by washing and cells were warmed to 37 °C for 0, 7.5, 15, 30, 60, 120 or 240 min before addition of 16 nM of JNJ 2408068. After addition of JNJ 2408068, cells were cooled again to 4 °C for 1 h before further incubation at 37 °C. At 24 h post-infection, cells were lysed for 5 min at room temperature in phosphate buffered saline (PBS) supplemented with 1% NP-40, 1 mM EDTA and a cocktail of protease inhibitors. The lysate was centrifuged at $230 \times g$ for 5 min at 4 °C and the supernatant was stored at −70 °C until analysed using an enzyme-linked immunosorbent assay (ELISA). For analysis, microtiter wells were first coated overnight at 4 °C with 200 μl of palivizumab at 10 μg/ml in 20 mM carbonate buffer, pH 9.6, then blocked with 250 μl of 10% horse serum (HS) in PBS. After removal of the blocking solution, 100 μl of two-fold dilutions of the samples in incubation buffer (IB, PBS-0.05% Tween 20, 4% HS) was added, the plates were incubated for 1 h at 37 °C and then washed 4× with 250 μl washing buffer (WB, PBS-0.05% Tween 20). Thereafter 100 μl of MAB858-1 (Chemicon, CA, USA), a mouse monoclonal RSV F protein AB (diluted 1/3200 in IB), was added and the plates were incubated for 1 h at 37 °C. After rinsing the wells 4× with WB, 100 μl of a peroxidase labelled goat anti-mouse antibody (AB) (diluted 1/4000 in IB) was added and the plates were incubated at 37 °C for 1 h. Thereafter the washing procedure was repeated and 100 μl of the chemiluminescent substrate solution (Roche, Mannheim, Germany) was added. After 5 min, the signal was measured with a chemiluminescence reader (Labsystems, Helsinki, Finland). A dilution series from a cell-derived viral stock was used to generate a standard curve.

2.6.3. Virus inactivation

Virucidal and binding properties of JNJ 2408068 and palivizumab were studied by incubation of approximately 10^7 plaque forming units (pfu)/ml RSV (Long strain) with or without approximately 100 times the EC_{50} of JNJ 2408068 (16 nM) or palizivumab (6 μg/ml) for 0, 1, 12 and 24 h at 37 °C. After these incubation periods, the reversibility of the binding between the virus and the compound was assessed by making 10-fold dilutions of the virus–drug mixtures until non-inhibitory concentrations of free compound (as established by the EC_{50} test) were obtained. These dilutions were plaque-titrated in Vero cells for remaining infectious virus. Each result represents the average of three replicates.

2.6.4. Inhibition on viral RNA and protein synthesis

The effect of adding JNJ 2408068 early in the cycle on plus strand RNA synthesis was assessed by RT-PCR. Gradient purified RSV (Long strain) was diluted to 10^5 pfu/ml, pre-incubated with drug or solvent for 1 h at 37 °C and inoculated into confluent monolayers of HeLa/M cells (MOI 0.1). After adsorption for 1 h at 37 °C, the inoculum was replaced by medium with the same drug concentration and cultures were incubated for 18 h at 37 °C. Cells were then rinsed with PBS and RNA was extracted with TRIzol (Life Technologies, MD, USA). Purified RNA was dissolved in DEPC water and stored at −70 °C. RNA was reverse transcribed at 42 °C with RT Expand (Roche, Mannheim, Germany) using a primer complementary to plus strand RNA and tagged (Schoenike et al., 1999). The resulting cDNA was amplified with Expand High Fidelity (Roche, Mannheim, Germany) using an RSV-specific primer, complementary to minus strand RNA, and a tag primer. Viral-specific regions of the primers were designed on basis of the sequence coding for the nucleocapsid region. The sequence of the primers was as follows: tagged-antisense primer, TCG CAG ACC AAT ACG CAT GAC TCA CCC CTG CTG CTA ATT TAG TTA TTA C; sense primer, GGC TCT TAG CAA AGT CAA GTT G; tag (Schoenike et al., 1999), TCG CAG ACC AAT ACG CAT GAC TCA. PCR consisted of a 2 min denaturation at 95 °C, 25 cycles of 95 °C for 30 s, 48 °C for 30 s and 68 °C for 45 s. After extension at 68 °C for 7 min the samples were stored at 4 °C. All amplicons were detected with ethidium bromide after electrophoresis in a 2% gel.

The effect of adding JNJ 2408068 early in the cycle on viral protein synthesis was assessed by metabolic labelling of viral proteins with ^{35}S-methionine-cysteine. Virus (10^7 pfu/ml, Long strain) was pre-incubated with drug or drug solvent for 1 h at 37 °C and then inoculated on HeLa/M cells for 1 h at 37 °C. After adsorption, medium supplemented with drug or solvent was added for 18 h at 37 °C and then pulse labelled for 2 h using medium supplemented with dialysed FCS (Life Technologies, MD, USA) and 10 μCi/ml ^{35}S-methionine-cysteine (Amersham Pharmacia Biotech, Buckinghamshire, England). After labelling, cells were lysed for 3 min in ice-cold SDS-electrophoresis sample buffer supplemented with 1 mM PMSF and 1 mM EDTA, boiled for 5 min and then stored at −20 °C until SDS–PAGE on a 12% gel. Metabolically labelled proteins were visualised with autoradiography.

2.6.5. Resistance studies

RSV (Long strain) at an MOI > 1 was exposed to JNJ 4955808 or JNJ 2408068 at concentrations approximately 1000 times their EC_{50}s, before and during three passages in HeLa/M cells. At the end of each passage (lasting 3 days each), virus from one or two end dilutions showing CPE was harvested by freezing and thawing and used to initiate a subsequent passage. The resulting isolates were cloned and the genes coding for F-, G-, and SH-protein were amplified with RT-PCR. The amplicons were sequenced with an ABI PRISM 3700 DNA analyser. The extent of resistance and cross-resistance was assessed using the antiviral assay described above.

2.7. Inhibition of cytokine release in alveolar epithelial cells

RSV (Long strain) was grown on HEp-2 cells and purified by ultracentrifugation on a sucrose gradient (Mbiguino and Menezes, 1991). A549 cells were seeded in 6-well plates at day −1 in 2 ml of growth medium at a concentration of 800,000 cells per well. After 24 h, when 80% confluent, cells were inoculated with virus at an MOI of 1 in the presence of drug ($100 \times EC_{50}$), palivizumab (6 μg/ml) or drug-solvent. Supernatants were harvested at 0, 3, 6, 12 and 24 h post-infection and were stored at −20 °C until analysis. IL-6 was measured with specific ELISA (R&D, Minneapolis, USA), the sensitivity of the assay was 0.2 pg/ml. Cell lysis was determined by measuring lactic dehydrogenase (LDH) release using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, MD, USA).

3. Results

3.1. Cytotoxicity

Cytostatic or cytotoxic effects were determined by assessing the effect of JNJ 2408068 on logarithmic cell growth. The HeLa/M cell number for untreated controls increased approximately 10-fold compared to day 0. A JNJ 2408068 concentration of 100 μM reduced cell growth by 38%, and lower concentrations of the drug were not inhibitory for cell growth. The CC_{50} of JNJ 2408068 for logarithmic cell growth at 37 °C was therefore >100 μM. Under the conditions of the antiviral screening assay (subconfluent HeLa/M cells, 7 days at 37 °C), the CC_{50} was for JNJ 2408068 was 400 μM. The CC_{50} of compounds shown in Table 1 was also greater than 100 μM in each case. Cytotoxicity in other cell lines was studied in less detail, but observations in BHK-21, MT-4 and MDCK cells did not suggest increased cytotoxicity for other cell lines.

3.2. Antiviral activity

A remarkably active substituted benzimidazole derivative, JNJ 1789008 (Fig. 1, Table 1), was identified as a lead molecule by screening a representative subset of a 130,000 compounds library against the Long strain of RSV. We embedded the lead structure JNJ 1789008 into a general formula (Fig. 1) to optimise the antiviral activity and recognized several possibilities for chemical modification: the amino ethyl group, the piperidine ring system, the bridge modification R1 between the benzimidazole and pyridine rings and the substitution pattern on these ring systems (substituents R2, R3 and R4) (Table 1). Modifications of the amino ethyl group or the piperidine system did not substantially affect the antiviral activity, while introduction of an ethoxy-methylene bridge (R1 = OEt) in JNJ 4688840 instead of a methylene bridge (R1 = H) in JNJ 1789008 significantly enhanced the anti-RSV activity. Substitution on the pyridine ring, particularly on position three and six, are crucial for improvement of the antiviral activity as was illustrated by the introduction of a methyl on position six and a hydroxy group on position three (compare JNJ 4977648, JNJ 4955808 and JNJ 4749914 with the desmethyl derivatives JNJ 1789008, JNJ 4688840 and JNJ 4841278, respectively). The crucial introduction of a 3-hydroxy substituent (R2 = OH) led to a hundred-fold improvement in anti-RSV activity, as demonstrated with JNJ 1789008 and its hydroxylated analogue JNJ 4841278. The simultaneous substitution of the three and the six position resulted in additional benefits as illustrated by comparing the 3-hydroxy-pyridine derivative JNJ 4841278 to the 3-hydroxy-6-methyl-pyridine JNJ 4749914. Extra substituents on the benzimidazole ring system, in particular on position seven ($R4 = CH_3$), resulted in only a minor amelioration of the antiviral activity as illustrated by JNJ 2408068 compared to JNJ 4749914.

JNJ 2408068, the compound that was selected for further evaluation, inhibited 50% of the syncytium formation in HeLa/M cells at concentrations as low as 0.16 nM (0.063 ng/ml), compared to 15 μM (3.9 μg/ml) for ribavirin, and 0.32 nM (0.047 μg/ml) for palivizumab, which were included as positive controls. Selectivity indexes ranged from >251 for the lead compound JNJ 1789008 to >100,000 for the selected compound JNJ 2408068.

To determine if the anti-RSV activity was cell-line-dependent, JNJ 4749914 and JNJ 2408068 were also evaluated for inhibition of RSV (strain Long) in A549 cells and HEp-2 cells, two human respiratory epithelial cell lines. Inhibition of virus replication (as assessed by the development of CPE) was comparable to that found in HeLa/M cells for both compounds (differences less than three-fold).

3.3. Antiviral spectrum

To determine if the anti-RSV activity was viral strain-specific, the inhibitory spectrum of JNJ 2408068 against typed clinical isolates of RSV was determined in HeLa/M cells. The EC_{50}s for five isolates from subgroup A ranged from 0.16 to 1.3 nM (median 0.50 nM), and those for five different isolates from subgroup B ranged from 0.32 to 4.47 nM (median 0.48 nM).

Replication of bovine RSV was also inhibited at similar concentrations. However, as assessed by semi-quantitative RT-PCR, no activity could be identified against Pneumonia Virus of Mice, a virus belonging to the same Paramyxovirus genus (genus pneumovirus). Both compounds were also unable to inhibit the growth of representative viruses from other Paramyxovirus genera, i.e. HPIV2 and Mumps Virus (genus Rubulavirus), HPIV3 (genus Respirovirus), and Measles virus (genus Morbillivirus). JNJ 2408024 had no detectable inhibitory activity against MPV. Its selective index (SI; determined by dividing the CC_{50} for LLC-MK2/EC_{50} [200 μg] by the EC_{50} obtained for JNJ 2408024 against hMPV [>200 μg]) was <1. Members of the Orthomyx-

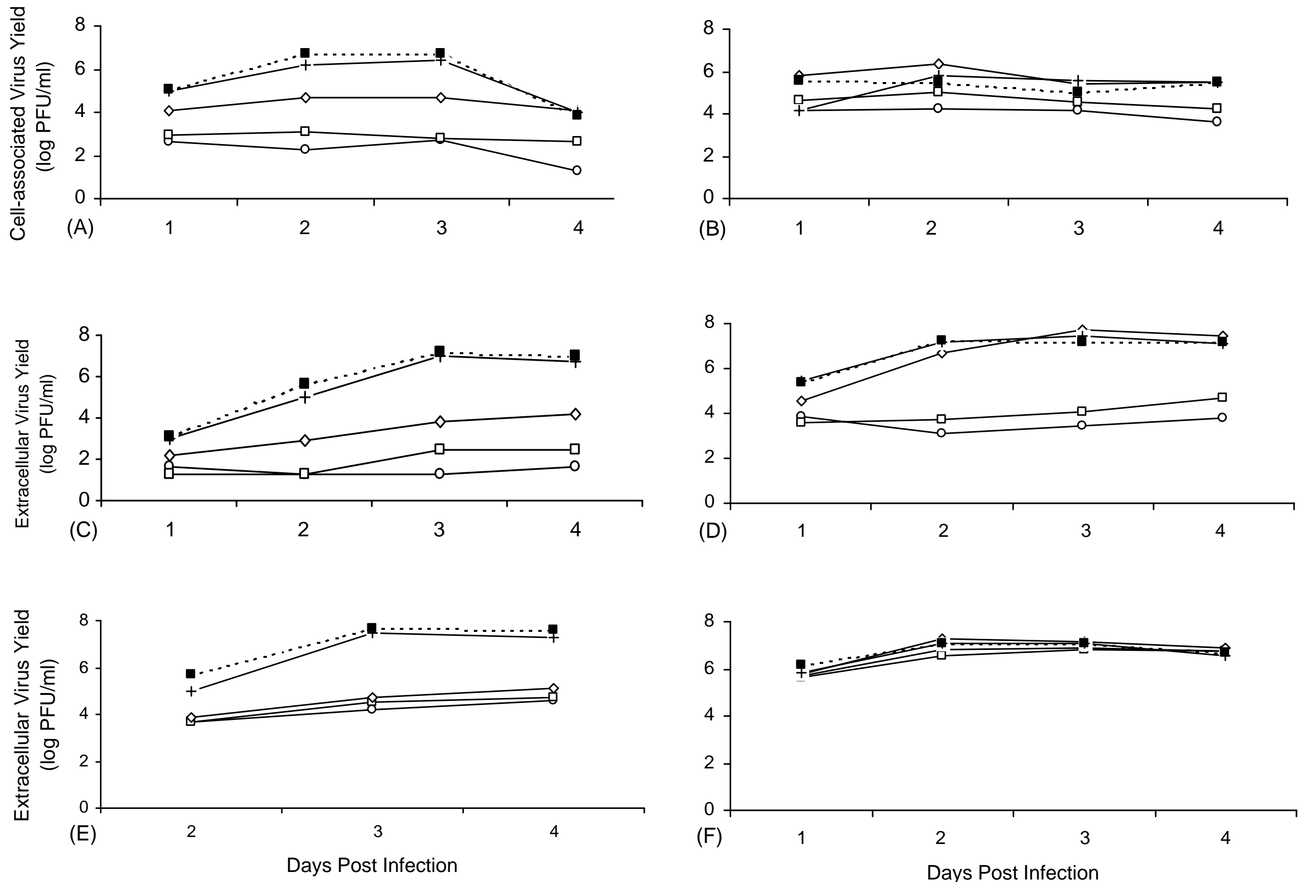

Fig. 2. Effects of JNJ 2408068 on human RSV yield. HeLa/M cells were infected with the human RSV, pre-incubated with the appropriate concentration (controls (■), $1 \times EC_{50}$ or 0.16 nM (+), $10 \times EC_{50}$ (◇), $100 \times EC_{50}$ (□), $1000 \times EC_{50}$ (○)) of antiviral compound (Fig. 2A–D) or treated with JNJ 2408068 at 6 h post inoculation (Fig. 2E and F). Cells were incubated for 48 h at 37 °C, frozen and thawed, and the virus yield was plaque-assayed. (A) Low MOI, cell-associated virus, (B) high MOI, cell-associated virus, (C) low MOI, extracellular virus, (D) high MOI, extracellular virus, (E) low MOI, extracellular virus, late addition, (F) high MOI, extracellular virus, late addition.

oviridae (several influenza virus strains), Retroviridae (HIV strain LAI), Picornaviridae (HRV2, HRV14), Herpesviridae (HSV2 strain 196) were also insensitive.

3.4. Virus yield reduction assay

The effect of different concentrations of JNJ 2408068 on cell-associated and extracellular virus yield in multiple rounds of replication was determined in 6-well plates, inoculated with human RSV at a low (Fig. 2A, C and E) or high MOI (Fig. 2B, D and F). In a first set of experiments, JNJ 2408068 was added to the virus before virus inoculation. In experiments using a low MOI (0.01), a drug concentration exceeding the EC_{50} 10-fold resulted in a 100-fold reduction of both cell-associated and extracellular virus titers (Fig. 2A and C). Concentrations 10–100-fold higher reduced the virus yields even further (1000–10,000-fold). When HeLa/M cells were infected with 100-fold higher MOI's, virus controls peaked 1 day earlier, and about 100 times the EC_{50} was needed to reduce the extracellular virus yield 1000-fold (Fig. 2D). Cell-associated virus titers peaked even earlier and in this case significant reductions in virus titers were only obtained if 1000 times the EC_{50} was used (Fig. 2B).

To assess the effect of a delay in treatment of virus-infected cultures, JNJ 2408068 was also added at 6 h after the inoculation of RSV, after the sensitive stage had passed in the first round of replication. In this case, significant reductions of extracellular virus yields could still be obtained, but only if cultures had been infected with a low MOI (Fig. 2E and F).

3.5. Mechanism of action

3.5.1. Correlation between inhibition of fusion and inhibition of virus production

Seven different benzimidazole derivatives were incubated for 60 min with RSV at concentrations of 0.1 and/or 1 μM in MEM containing 2% FCS at 37 °C. The virus–drug mixtures were inoculated into HeLa/M cells, which were then incubated for 48 h. The reductions in extracellular virus yield as compared to virus controls were determined using plaque assay and plotted against the log EC_{50}. The median values of two to five separate assays are presented (Fig. 3).

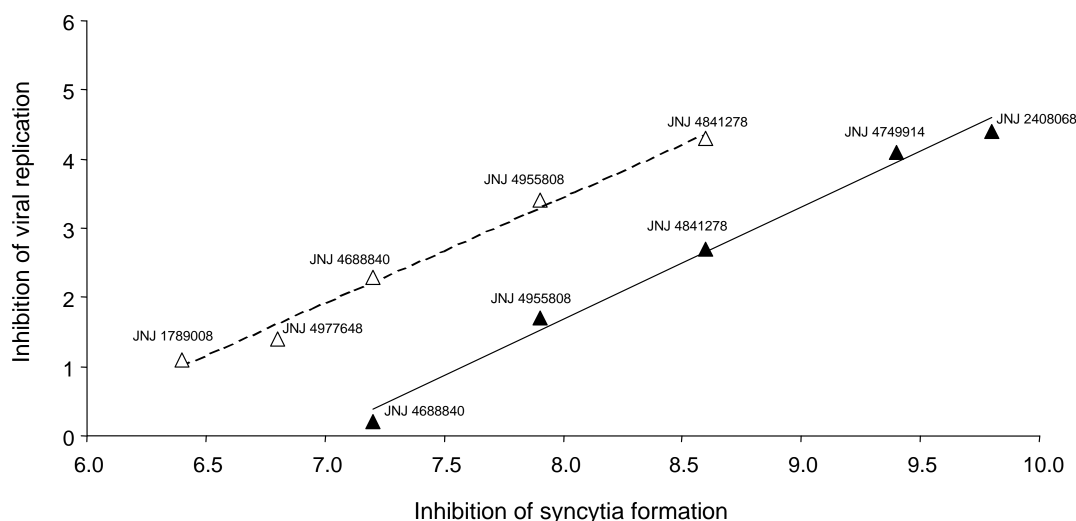

Fig. 3. Correlation between inhibition of syncytium formation and inhibition of viral replication. Seven benzimidazole derivatives were incubated with RSV at concentrations of 0.1 μM (full line) and/or 1 μM (dotted line) and inoculated into HeLa/M cells, which were then incubated for 48 h. The log reduction in extracellular virus yield as compared to virus controls (*Y* axis) was determined using plaque assay and plotted against the pEC_{50} (*X* axis). pEC_{50} is the negative logarithm of the EC_{50} (e.g. for 1 nM the pEC_{50} is 9, for 10 nM the pEC_{50} is 8). Less active compounds (with lower pEC_{50}s, left side of the *X* axis) were tested at lower multiples of their EC_{50}s and produced lower reductions in virus yield than compounds with higher log EC_{50}s.

Compounds with lower log EC_{50}s (left side of the *X* axis) were tested at low multiples of their EC_{50}s and produced lower reductions in virus yield than compounds with higher log EC_{50}s. The correlation between inhibition of CPE and inhibition of virus production (r^2) reached 0.96 for both 0.1 and/or 1 μM.

The ratio of the used concentrations to the EC_{50} can also be plotted in the *X* axis. This reveals the correlation between the multiple of the EC_{50} used on the one hand (*X* axis) and the reduction in virus yield on the other hand (*Y* axis). From Fig. 3 one can estimate that a concentration approximately 100 times the EC_{50} results in an approximately 1000-fold reduction of the extracellular virus titer.

3.5.2. *Effect of time of addition of JNJ 2408068 on virus inhibition*

To learn more about the mechanism of anti-RSV activity of JNJ 2408068, compound at 100 times the EC_{50} was added at different times post-virus exposure of parallel cultures of HeLa/M cells with human RSV at an MOI > 1. Ribavirin and palivizumab were included as controls. Development of syncytia reached 100% at 48 h post-virus exposure in the virus controls. At this time point, supernatant fluid was collected and the extracellular virus yield was determined by plaque-titration. As shown in Fig. 4A, JNJ 2408068 was effective in inhibiting RSV replication if added before the 1 h virus adsorption period. When JNJ 2408068 was added after virus adsorption had been allowed to proceed at 37 °C, RSV replication was not inhibited. Similar results were obtained when palivizumab was used. In contrast, ribavirin was still effective in suppressing virus replication when added up to 6 h after inoculation.

Interestingly, the time-dependence for inhibition of syncytia formation was very different from the one described above, in that both palivizumab and JNJ 2408068 could be added as late as 6 and 12 h post inoculation, respectively to prevent syncytium formation. Ribavirin, on the other hand, was less effective in inhibiting syncytia formation if added at 12 h post inoculation (results not shown).

When virus was adsorbed in the cold and JNJ 2408068 was added before cells were warmed to 37 °C, viral fusion protein synthesis as assessed by ELISA was blocked completely (Fig. 4B). Adding the antiviral at the beginning or at the end of the adsorption period had a similar effect. Incubation of infected cells for 7.5 min at 37 °C before addition of compound resulted in a ∼15-fold increase in F-protein synthesis. A further gradual increase in fusion protein synthesis was observed when the antiviral was added at 15, 30, 60, 120 and 240 min after warming the cells to 37 °C. Similar data were obtained upon analysis of viral RNA (data not shown).

3.5.3. *Virus inactivation*

Incubation of 10^7 pfu/ml RSV with a concentration of JNJ 2408068 exceeding the EC_{50} 100-fold (16 nM) for 0, 1, 12 and 24 h at 37 °C did not result in a reduction of viral infectivity (results not shown), suggesting that JNJ 2408068 has no virus inactivating properties and that any binding between RSV proteins and JNJ 2408068 is fully reversible by dilution.

Incubation of 10^7 pfu/ml RSV with a concentration of palivizumab exceeding the EC_{50} 127-fold (6 μg/ml) for 0, 1, 12 and 24 h at 37 °C resulted in the following reductions of viral infectivity: −1.3 log pfu after 1 h, −3.4 log pfu after 12 h, and −3.6 log pfu after 24 h.

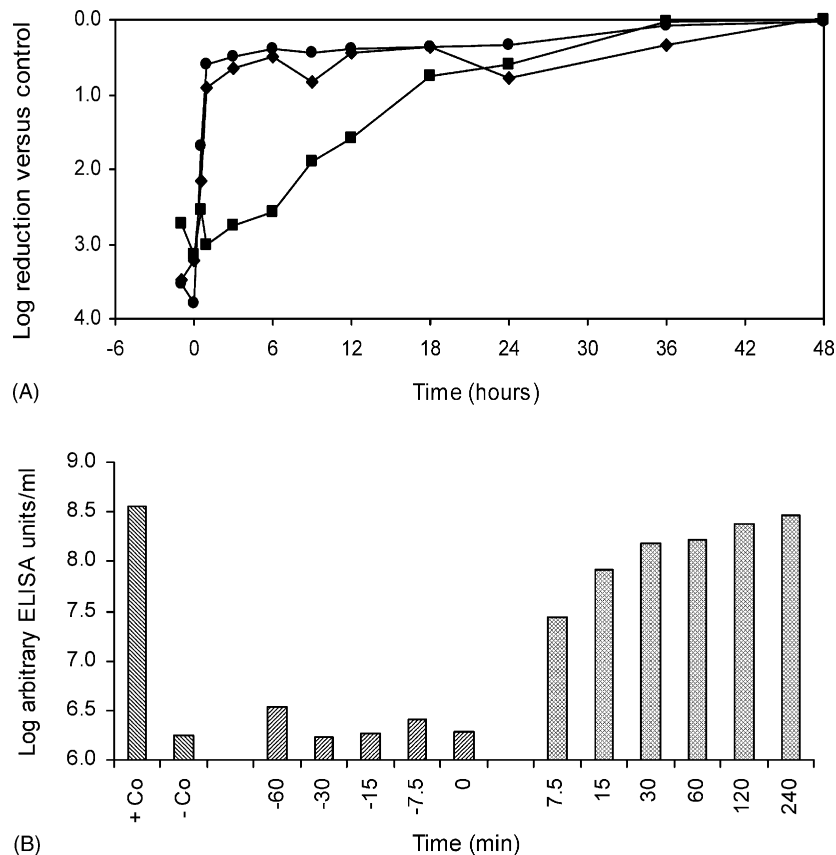

Fig. 4. Time-dependent drug of addition effects. (A) HeLa/M cells were infected with human RSV (MOI > 1) JNJ 2408068 ($100 \times EC_{50}$ (●)), ribavirin ($13 \times EC_{50}$ (■)) or palivizumab ($10 \times EC_{50}$ (◆)) were added at the indicated times. Extracellular virus yield was determined by plaque assay after 48 h incubation. (B) Virus was first adsorbed in the cold and JNJ 2408068 was added at different times before and after warming cells to 37 °C. Viral fusion protein synthesis was assessed by ELISA after 48 h of incubation. (▧) Positive and negative control; (▨) addition of JNJ 2408068 before raising temperature to 37 °C; (▩) addition of JNJ 2408068 after raising temperature to 37 °C.

3.5.4. Inhibition on viral RNA and protein synthesis

To study the effect of early addition of JNJ 2408068 on viral RNA synthesis, HeLa/M cells were inoculated with gradient purified and JNJ 2408068 (0.01, 0.1 and 1 μM) treated virus. After 18 h incubation at 37 °C the viral RNA present in the cells was analysed with plus strand-specific PCR. Viral RNA could not be detected with the used RT-PCR procedure immediately after inoculation (negative controls). A distinct signal was obtained in the positive controls and a concentration-dependent reduction in viral RNA in the compound treated samples.

Two additional proteins could be detected in the virus-infected cells upon comparison with mock-infected controls, one with a MW of approximately 40 kDa and the second with a MW of approximately 30 kDa. Addition of JNJ 2408068 during the adsorption period prevented the accumulation of both proteins.

3.5.5. Resistance studies

Exposure of RSV (Long strain) to JNJ 4955808 at a high selection pressure (10 μM, i.e. approximately 1000 times the EC_{50}) during three passages in HeLa/M cells resulted in the isolation of a resistant virus isolate coded R1LO. A second isolate was obtained using JNJ 2408068 (three passages at 100 nM, coded R2LO). Sequencing of the F, G and SH proteins of these isolates resulted in the identification of single point mutations in the F protein: S398L in R1LO, and D486N in R2LO. The EC_{50} of R1LO increased >10,000-fold for R139304 and 500-fold for JNJ 2408068. For the R2LO isolate, the EC_{50} increased 50,000-fold for both drugs.

A third mutant (R6LO) was raised to palivizumab and again a single point mutation (K272M/T) was observed in the F protein of virus isolated in the third passage. The EC_{50} for this isolate increased 300,000-fold. No cross-resistance was observed between ribavirin, JNJ 2408068 and palivizumab.

3.6. Inhibition of cytokine release in alveolar epithelial cells

The effect of JNJ 2408068 on cytokine production and release was studied in A549 cells. A549 cultures were infected with RSV preincubated with drug or drug solvent for 1 h at 37 °C. After adsorption, the inoculum was replaced with medium, supplemented with drug or drug solvent. IL-6 was measured in culture supernatants harvested at 0, 3, 6, 12

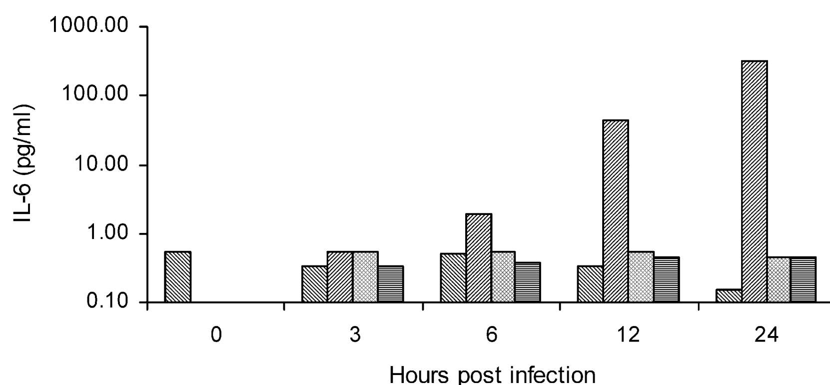

Fig. 5. Effect of JNJ 2408068 and palivizumab on RSV induced IL-6 release from A549 cells. A549 cultures were infected with RSV preincubated with drug or drug solvent for 1 h at 37 °C. After adsorption, the inoculum was replaced with medium, supplemented with drug or drug solvent. IL-6 was measured in culture supernatants harvested at 0, 3, 6, 12 and 24 h post-infection. (▧) Control; (▨) RSV; (▩) RSV + JNJ 2408068; (▤) RSV + palivizumab.

and 24 h post-infection. As shown in Fig. 5, the inoculation of A549 cells with replicative RSV resulted in an increased, as compared to uninfected control cells, concentration of IL-6 in the culture supernatant. An increase of IL-6 could be observed at 6, 12 and 24 h post-infection. Preincubation with JNJ 2408068 or palivizumab prevented the increased release of the cytokine. Similar data were obtained for IL-8 and Rantes (data not shown). Measurement of LDH indicated that less than 1.5% of the cells were lysed in both the control and the infected cells, indicating that the increased release of cytokines, could not be ascribed to cell lysis. Neither JNJ 2408068 nor palivizumab had an effect on the limited increase of IL-8 or IL-6 secretion induced by binding of heat- or UV-treated, non-replicative virus (data not shown).

4. Discussion

The described substituted benzimidazoles represent a new class of anti-RSV drugs with significant in vitro efficacy. EC_{50}s in several human cell lines (HeLa/M, HEp-2 and A549) are significantly lower than those for ribavirin. Cytotoxicity studies with JNJ 2408068 indicated that 100 μM was not cytotoxic in various cell lines.

JNJ 2408068 is especially active against members of the genus pneumovirus. Within this genus, bovine and human RSV are both very sensitive, while the somewhat genetically distinct pneumovirus of mice was not. Both human RSV subgroups A and B appear susceptible, as the range of EC_{50}s from clinical isolates of both groups was similar. Members of other Paramyxoviridae genera (Morbillivirus, Rubulavirus, Respirovirus, Metapneumovirus) were not sensitive, nor other viruses that are important pathogens of the upper respiratory tract, such as influenza viruses and human rhinoviruses.

The effect of JNJ 2408068 on cell-associated and extracellular virus yield in multiple rounds of replication was studied upon infection at a low or a high MOI. When using a low MOI, cell-associated virus peaked before extracellular virus, and the effect of the drug on the extracellular virus yield was more pronounced (Fig. 2A and C). This suggests that taking lung washings during in vivo experiments may be more sensitive than studying tissue (lung) homogenates. When a higher MOI was used for infection, cell-associated and extracellular virus yield peaked earlier, and higher concentrations of drug were needed to reduce the yield significantly (Fig. 2B and D).

When drug was added 6 h after the inoculation of RSV, after the sensitive stage had passed in the first round of replication (see time of addition experiment), significant reductions of extracellular virus yields were only seen if cultures had been infected with a low MOI (Fig. 2E and F), indicating that it is harder to achieve antiviral efficacy in case a significant number of cells is already infected at the time drug is added.

The observed correlation between inhibition of fusion and inhibition of virus replication (Fig. 3) suggests that these two properties are causally related. This is an interesting observation because earlier benzimidazole derivatives have been described where such correlation was absent (Dubovi et al., 1980). Within the current series of benzimidazoles, it appears that a concentration approximately 100 times the EC_{50} is sufficient to reduce the extracellular virus yield by 3 logs, at least in HeLa cells.

The time of addition study (Fig. 4A) and the experiments on viral RNA and viral protein synthesis all suggest that JNJ 2408068 has an effect on an early event in the replication cycle of human RSV. The time of addition profile of palivizumab was similar to that of JNJ 2408068 in that a 90% reduction of virus production was observed when compound was added within 30 min after the start of the experiment. In contrast, ribavirin could be added up to 6 h, but not 9 h, after the start of the replication cycle (Fig. 4A). Interestingly, the time dependency for inhibition of syncytia formation was different, at least in case of palizivumab and JNJ 2408068. Both compounds could be added up to 6 h post-virus exposure and still inhibit syncytium formation by at least 50%. These findings point to a dual mode of action:

inhibition of virus–cell fusion early in the infection cycle and inhibition of cell–cell fusion at the end of the replication cycle resistance. An effect on virus adsorption, as described for another RSV fusion inhibitor (Razinkov et al., 2001), was not apparent from the data in Fig. 4B. The time of addition studies in the cold suggest that the compound can bind to the pre-hairpin conformation of RSV. The virus inactivation studies indicate that any binding on extracellular virus particles is fully reversible. For palivizumab, binding to extracellular virus particles is not reversible by dilution.

The gp41/120 glycoprotein of HIV structurally resembles the RSV F protein including a predicted coiled coil structure in and around the fusion domain. Peptide inhibitors of this fusion protein have been described for both RSV and HIV (Lambert et al., 1996). Although JNJ 2408068 has no activity on the replication of HIV, the similarity in target between the two viruses suggests that it may be possible to identify small molecule inhibitors of the fusion event of HIV.

The isolation of escape mutants with point mutations in the fusion protein of RSV supports the suggestion that the antiviral activity is caused by an inhibition of the fusion process. Reverse genetics experiments could confirm this hypothesis. A model of the structure of the fusion protein has recently been described and the positions of the point mutations obtained by JNJ 2408068 are discussed (Smith et al., 2002). While residue 486 was not included in the model, residue 398 lies N-terminal to the immunoglobulin-like B-sandwich domain and is found surface exposed on the rim of an axial channel at the junction between two F protein monomers.

The high level of resistance of the isolated escape mutants is a cause of concern. On the other hand, highly resistant escape mutants can also be isolated in the presence of palivizumab, a drug that is successfully used for prophylaxis of RSV in high risk babies. No cross-resistance between the two agents was observed. The moderate replication levels of RSV in patients may decrease the chances for a break-through mutant to emerge.

The effects of RSV on airway inflammation may be at least partly mediated by production of proinflammatory cytokines in infected airway epithelium (Noah and Becker, 1993). Infection of the lung epithelial cell line A549 by RSV results in the elevated synthesis of multiple cellular cytokines, including a number of interleukins (Bitko et al., 1997). In A549 cells, a pulmonary type II-like epithelial cell line, membrane-based cDNA macroarrays and high-density oligonucleotide probe-based microarrays identified RSV inducible expression of several CC, CXC, and CX(3)C chemokines (Zhang et al., 2001).

JNJ 2408068 was able to concomitantly reduce the levels of inflammatory cytokines, IL-6, IL-8 and Rantes. The inhibition was specific for virus infection, since the compound had no effect on the cytokines produced by uninfected cells. The inhibition of the release of these cytokines is of course an indirect consequence of the inhibition of replication of RSV, but it does suggest symptoms caused by cytokines can be reduced to the extent these are caused by continuing virus production. In a recent study of nasal lavage fluid collected from volunteers experimentally infected with influenza A, a cascade of cytokine responses was documented that implicate their involvement not only in disease resolution but also in symptom formation. Specifically, interleukin IL-6, interferon alpha, and tumour necrosis factor alpha levels increased and peaked by days 2 or 3, while IL-8 levels peaked later. These responses correlated significantly with the magnitude and kinetics of viral replication, mucus formation, and disease symptoms (Hayden et al., 1998). In a subsequent study, it was shown that treatment with a neuraminidase inhibitor prevented the infection and abrogated the local cytokine and chemokine responses (Hayden et al., 1999).

The extraordinary selective antiviral activity seen in these studies has lead to testing of JNJ 2408068 in cotton rats (Wyde, in press). In this in vivo testing, 0.39 mg JNJ 2408068/kg administered just once by small droplet aerosol 24 h prior, or subsequent, to virus administration, significantly inhibited replication of both A and B subtype RSV strains in the lungs of cotton rats without any evidence of toxicity. Thus, the results obtained in vivo support those obtained in tissue culture, and both strongly encourage further evaluation of this compound and related substituted benzimidazole compounds.

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