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Short duration aerosols of JNJ 2408068 (R170591) administered prophylactically or therapeutically protect cotton rats from experimental respiratory syncytial virus infection

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Abstract

Cotton rats exposed to continuous small droplet aerosols of 2[[2-[[1-(2-aminoethyl)-4-piperidinyl]amino]-4-methyl-1*H*-benzimidazol-1-yl]methyl]-6-methyl-3-pyridinol (JNJ 2408068) or its hydrochloric salt for only 15 min, one day prior to virus inoculation or one day after, were significantly protected from pulmonary respiratory syncytial virus (RSV) infection compared to control animals similarly infected but exposed to aerosols of placebo at these times. No evidence of toxicity was seen in any of these animals or in cotton rats administered 10 times the minimum cotton rat efficacious dose (i.e. 10×0.39 mg of active compound per kilogram of body weight) for four continuous days. The marked selective antiviral activity observed in the cotton rats mirrored that seen for these compounds in cytotoxicity and antiviral assays performed against RSV in vitro. Plasma kinetics and tissue distribution of JNJ 2408068 in cotton rats following inhalation were determined in separate experiments performed using conditions similar to those utilized in the in vivo efficacy studies. The data from these experiments indicated that significant levels of the test compound were delivered to the lungs of exposed animals, but that extrapulmonary distribution was limited.

Keywords: RSV; Antiviral; Cotton rats; Respiratory syncytial virus; Aerosol; JNJ 2408068

1. Introduction

Respiratory syncytial virus (RSV) is a leading cause of serious lower respiratory tract infections in infants and children under two years of age (Hall and McCarthy, 2000). It is also a major cause of significant morbidity and mortality in certain immunosuppressed populations (Couch et al., 1997) and the elderly (Agius et al., 1990; Fleming and Cross, 1993; Falsey et al., 1995; Thompson et al., 2003). No vaccines are currently licensed for prevention of RSV infections. However, palivizumab (Synagis™; MedImmune, Inc., Gaithersberg, MD), a humanized monoclonal antibody, and immunoglobulin (IG) preparations with high neutralizing antibody titers to RSV, e.g. RSV-IVIG (RespiGam™; MedImmune, Inc.) are licensed for this use. Unfortunately, these preparations are expensive and this has lead to questions about their relative cost-benefit ratio (Thakur et al., 1997; Hashmi et al., 2000; Lofland et al., 2000; Numa, 2000; Barton et al., 2001). The only chemotherapeutic available to treat RSV infections, ribavirin, is restricted to use in high risk or severely ill infants (Committee on Infectious Diseases, 1993) and like the antibody preparations is expensive (Marquardt, 1995). Moreover, it is a potential mutagen (Hoffmann et al., 1987; Krilov, 2002). Because of the problems associated with the different agents currently licensed for use against RSV, efforts have continued to try and identify new materials to prevent or ameliorate RSV infections.

This report summarizes results of studies performed in cotton rats to evaluate the toxicity and antiviral activity against RSV of 2[[2-[[1-(2-aminoethyl)-4-piperidinyl]amino]-4-methyl-1H-benzimidazol-1-yl]methyl]-6-methyl-3-pyridinol (JNJ 2408068; formerly designated R170591) and its hydrochloric salt. Both compounds were elucidated by scientists at Johnson & Johnson Pharmaceutical Research & Development, Beerse, Belgium during testing for potential RSV inhibitors using tissue culture assays. Stud-

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ies there showed that these low molecular weight (MW of JNJ 2408068 = 395) benzimidazole derivatives have a dual mode of action (i.e. they inhibit virus–cell fusion early in the infection cycle and cell–cell fusion at the end of the replication cycle) and a low median efficacious concentration (EC_{50}; e.g. 0.16 nM against some RSV laboratory strains; Andries et al., 2000; Andries et al., in press). In the tests performed for this report, both forms of JNJ 2408068 significantly inhibited replication of RSV A and B subtypes in the lungs of cotton rats without any evidence of toxicity. Of particular interest, a single 15-min exposure of test animals to a small droplet aerosol (sda) of either material one day prior to, or one day after virus, significantly protected them from pulmonary RSV infection. The plasma kinetics and tissue distribution of JNJ 2408068 in cotton rats following inhalation were determined in separate experiments performed under conditions similar to those used in the in vivo efficacy studies. The data obtained during these studies indicated that significant levels of the test compound were delivered to the lungs of exposed animals, but that extrapulmonary distribution was limited.

2. Materials and methods

2.1. Animals

Fifty to hundred grams of cotton rats (*Sigmoden hispidis*) of either sex were used in these studies. All were from the Baylor College of Medicine (BCM) colony. These animals were housed in the BCM vivarium in cages covered with barrier filters and given food and water ad libitum. Blood samples obtained from representative animals during the course of these studies were seronegative for RSV, adventitious viruses, and other rodent pathogens.

2.2. Tissue culture

HEp-2 (human epithelial carcinoma; American Type Culture Collection (ATCC), cat. no. CCL23) cells were used to prepare working stocks of RSV and to titer levels of virus in virus pools and lung lavage fluids (LF). Eagle's Minimal Essential Medium (MEM; Sigma Chemical Co., cat. no. M4465) supplemented with 10% fetal calf serum (FCS; Summit Biotechnology, cat. no. FP-200-05), 500 U penicillin/ml (Sigma Chemical Co., cat. no. P-4458), 50 μg streptomycin sulfate/ml (Sigma Chemical Co., cat. no. P-4458), 2 mM L-glutamine (Whittaker Bioproducts, Inc., cat. no. 17-605A) and 0.2% sodium bicarbonate (Sigma Chemical Co., cat. no. S8761) was used grow these cells.

2.3. Viruses

The two RSV subtype A strains (RSV A2 (ATCC cat. no. VR1302) and Long (ATCC cat. no. VR26)) and one RSV subtype B strain (18537 (ATCC cat. no. VR1401)) utilized in these studies were originally obtained from the ATCC. Working stocks of each were prepared by infecting flasks of HEp2 cells as described previously (Wyde et al., 1995).

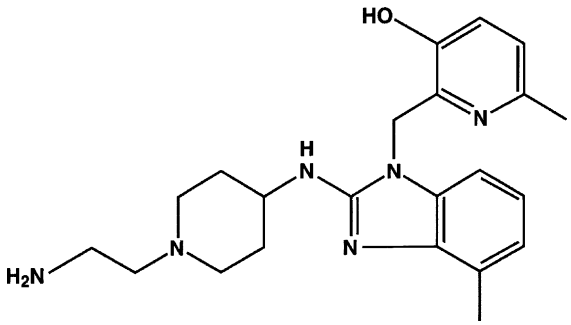

Fig. 1. Structure of JNJ 2408068.

2.4. Virus quantification

RSV levels in virus pools and LF 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). Median virus titers were calculated using the method of Karber (Rhodes and Van Rooyen, 1953) and expressed as $\log_{10}$ median tissue culture infectious doses $TCID_{50}$/ml (virus pools) or $\log_{10}$ $TCID_{50}$/g lung (LF). The minimum detectable virus concentration for virus pools in these assays was 1.8 $\log_{10}$ $TCID_{50}$/ml. For lung fluids, the minimal detectable virus concentration was 2.8 $\log_{10}$ $TCID_{50}$/g lung.

2.5. Compounds

Johnson & Johnson Pharmaceutical Research & Development, Beerse, Belgium provided both the free base (MW 395; see Fig. 1 for structure) and hydrochloric salt of JNJ 2408068 used in these studies. Ribavirin, used as a control in some experiments, was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA; cat. no. 196966). Just before use, the amount of each material needed was weighed out, suspended in distilled water (Baxter Healthcare Corporation; Deerfield, IL; cat. no. 2F7114) and then filter sterilized using a 0.2 μm DynaGard filter (Spectrum Laboratories, Inc., Rancho Dominguez, CA; cat. no. DG2M-30-50S). The only difference seen between the free base and hydrochloride salt of JNJ 2408068 in these studies was that the pH of the water had to be adjusted to be 6.0 before the former would go into solution.

2.6. Antiviral studies in cotton rats

Experiments evaluating the antiviral activity of JNJ 2408068 in cotton rats were carried out using prophylactic or therapeutic drug administration schedules. In experiments utilizing therapeutic drug regimens, the animals

were lightly anesthetized with Isoflurane (Abbot Laboratories, North Chicago, IL), weighed and then inoculated intranasally (i.n.) with approximately 100 median cotton rat infectious doses ($CRID_{50}$; approximately 10^4 $TCID_{50}$) of RSV in 0.1 ml. At the desired time, the appropriate drug or placebo (Baxter Healthcare distilled water) was administered as a continuous sda as described previously (Wilson et al., 1980; Knight and Gilbert, 1988) with the exceptions that an Aero-Mist nebulizer (CIS-US, Inc., Bedford, MA, cat. no. CA-209) was used to generate the aerosols and in most experiments the duration of each was for only 15 min. In experiments using a prophylactic drug administration schedule, drug or placebo was administered to the cotton rats for 15 min starting at a designated time prior to virus inoculation. Then on Day 0 (by convention, always the day of virus inoculation), these animals were inoculated i.n. with approximately 100 $CRID_{50}$ of RSV in 0.1 ml.

In all in vivo experiments, regardless of the drug administration schedule utilized, the cotton rats were sacrificed on Day 4 after virus inoculation using CO_2 to rapidly asphyxiate the animals. Day 4 was chosen because with the dose of virus used in these experiments, this is the day that maximum RSV pulmonary titers are usually seen in untreated cotton rats. At that time, the lungs of the sacrificed animals were removed, rinsed in sterile phosphate buffered saline (PBS; pH 7.2) and weighed. Each set of lungs was then transpleurally lavaged using 3 ml of 2% FCS–MEM as described in detail previously (Wilson et al., 1980).

The estimated dosage of JNJ 2408068 per kilogram of body weight delivered to the cotton rats in 15 min by sda was calculated as described previously (Wyde et al., 1987). In experiments using 75 g cotton rats, 5 mg active compound per milliliter in the delivery reservoir and the Aero-Mist nebulizer, it was estimated that a single sda of JNJ 2408068 administered for 15 min resulted in each animal receiving approximately 394 μg of drug per kilogram (0.39 mg/kg).

2.7. Drug carryover experiments

Because of the high specific activity of the JNJ 2408068, the possibility existed that residual drug present in the lungs of test animals at the time of harvest could be transferred ("carried over") to test plates and inhibit virus in vitro during assays performed to measure lung virus titers. This possibility was investigated by exposing one cotton rat for 15 min to an sda of distilled water and nine other animals for 15 min to an aerosol of JNJ 2408068 (aerosol delivery reservoir concentration = 5 mg/ml). The next day the cotton rat that was exposed to distilled water and three of the animals that were exposed to the test compound were sacrificed. The lungs of these animals were removed, lavaged and added undiluted in duplicate to the first wells of a 96-well assay plate. These samples were then serially diluted in duplicate up the plate using a two-fold dilution scheme. RSV (shown by back titration to have a titer between 10 and 30 $TCID_{50}$) was then added to each well in the plate that contained LF and several that did not. After seven days of incubation in a 36 °C, 5% CO_2 incubator, each well of the test plate was observed for RSV-induced syncytia. On each of the next two days, three more of the animals exposed to the test compound were sacrificed and their lungs were tested for the presence of biologically active drug as was done with the animals sacrificed on Day +1. The results of this experiment are shown in Table 1.

As zeros in the last column of Table 1 indicate, RSV replication was not inhibited in any of the wells containing LF obtained from placebo control animal or those obtained from treated animals ≥48 h after drug delivery. In contrast, overt inhibition of RSV was seen two- to three-wells up the plate in all of the test rows that contained dilutions of LF obtained from animals sacrificed approximately 24 h after they were exposed to either of the test compounds. These results indicated that active test compound that could interfere with in vitro assays was indeed present in LF of cotton rats treated for 15 min with aerosols of JNJ 2408068 generated from aerosol delivery reservoirs containing 5 mg active compound per milliliter for at least 24 h but not 48 h or after.

2.8. Toxicity studies in cotton rats

Animal toxicity experiments were performed using uninfected cotton rats that were exposed once daily for four consecutive days to a 15 min sda containing 10 times the maximum dosage of JNJ 2408068 utilized in antiviral studies (i.e. 10×0.39 mg active compound per kilogram of body weight). These cotton rats were sacrificed on Day 5, one day after stopping the last treatment. Because the animals in the antiviral studies usually received only one 15 min treatment, the cotton rats in these studies were given approximately 40× more compound than the animals involved in the antiviral studies.

The toxicity studies were limited to: (1) observing the test animals for morbidity, mortality, diarrhea, irritability, or other unusual behavior over the five-day test period; (2) assessing the effects of the test compounds on weight gain during this period; (3) comparing the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine and blood urea nitrogen (BUN) in the sera of treated and untreated cotton rats; and (4) comparing the pulmonary histopathology in hematoxylin and eosin (H&E) stained sections of lungs obtained from treated animals at the conclusion of each experiment (i.e. on Day 5) with that seen in similarly processed and stained sections of lung obtained from uninfected, untreated control animals. In these last studies, the lungs from both groups of animals were collected, rinsed in PBS (pH 7.4) and placed in Histochoice fixative (Amresco, Solon, OH, cat. no. H120-1L) overnight. They were then dehydrated and embedded in low-melting point paraffin and sectioned at 5 μm thickness. After rehydration, the resulting sections were stained with H&E and observed in a blinded manner for overt changes in the lungs (e.g. thickening of the alveoli walls, the presence of edema

Table 1
Comparison of the inhibition of respiratory syncytial virus replication induced by lung lavage fluids collected from cotton rats at different times after these animals were exposed to small droplet aerosols of JNJ 2408068 for 15 min[a]

Lung no.[b]	Treatment	Day post-Rx LF collected	Dilution scheme	Amount RSV added to wells[c]	No. of wells RSV CPE inhibited
1	dH_2O	1	1:2	10 $TCID_{50}$	0/0
2	JNJ 2408068	1	1:2	30 $TCID_{50}$	2/3
3	JNJ 2408068	1	1:2	10 $TCID_{50}$	2/2
4	JNJ 2408068	1	1:2	10 $TCID_{50}$	2/2
5	JNJ 2408068	2	1:2	30 $TCID_{50}$	0/0
6	JNJ 2408068	2	1:2	10 $TCID_{50}$	0/0
7	JNJ 2408068	2	1:2	10 $TCID_{50}$	0/0
8	JNJ 2408068	3	1:2	30 $TCID_{50}$	0/0
9	JNJ 2408068	3	1:2	10 $TCID_{50}$	0/0
10	JNJ 2408068	3	1:2	10 $TCID_{50}$	0/0

[a] Uninfected cotton rats were exposed once for 15 min to aerosols of distilled water (cotton rat 1) or JNJ 2408068 (cotton rats 2–10; aerosol delivery reservoir concentration = 5 mg/ml). The animals administered the distilled water and three of the nine drug-treated animals were sacrificed on Day 1 at which time their lungs were lavaged. On each of the next two days, three more cotton rats were sacrificed and similarly treated. The resulting lung lavage fluids were added undiluted to the first wells of an assay plate and then serially diluted in duplicate up the plate. Respiratory syncytial virus (RSV) then was added to each well in the plate. The assay plates were placed in a 5% CO_2 incubator. Final readings were made seven days later. A "0" indicates no evident inhibition of virus CPE compared to the CPE in control wells in which virus and medium but no lung fluids were added. All other numbers indicate the number of wells in each column that evident reduction in virus-induced syncytium was present. (Slant bars separate results seen in the duplicate columns.)

[b] Other abbreviations: no.: number; Rx: treatment; LF: lung fluids; $TCID_{50}$: median tissue culture infectious doses.

[c] The amount of virus added was determined by back titration.

and/or the presence of inflammatory cells (IC; i.e. polymorphonuclear neutrophils, macrophages, lymphocytes and eosinophils), in the alveoli or in or around the bronchi and bronchioles. Ten consecutive microscopic fields of each section were observed using the 100× objective of an Olympus CK2 light microscope. The fields were selected utilizing an up, over, and down search pattern. Because no edema or evident changes in pulmonary architecture were seen in any of the stained sections observed, scoring consisted primarily of counting the number of IC present in each field of observation. The mean number of IC per section of lung were determined for each group (test and control) and compared as discussed below. The BCM Center of Comparative Medicine Chemistry Laboratory determined the levels of ALT, AST, creatine, and BUN in the serum of each treated and control cotton rat using a COBAS Integra 400 plus chemistry analyzer (Roche Diagnostics, Indianapolis, IN). Means for each of these was determined and compared as described in the Section 2.11 below.

2.9. Preparation of tissues for pharmacokinetic studies

In order to document the exposure and the pharmacokinetic characteristics of JNJ 2408068 in cotton rats after inhalation, three small replicate experiments were performed using conditions similar to those utilized in the in vivo efficacy studies. However, none of the test animals received virus. Eleven animals were included in these experiments. In each, three cotton rats were harvested at time 0, immediately after stopping drug delivery, and then again 4 and 96 h later. One animal in each experiment was also sacrificed 1 and 24 h after the end of the inhalation regimen. Blood was collected from these animals by cardiac puncture using sodium ethylenediaminetetraacetic acid (Na-EDTA) as an anticoagulant. The plasma from these samples was separated by centrifugation and frozen until quantification of the drug levels present in each were performed. Tissues (muscle-femur, liver and lung) and lung fluid were also collected from each cotton rat, always taking care to avoid contaminating the tissues with any JNJ 2408068 that might be on the fur of the test animals. The latter was obtained by transpleurally lavaging each set of lungs with 2 ml of a 5% bovine serum albumin (BSA, Sigma Chemical Company, cat. no. A-7284) solution in PBS. The addition of 5% BSA to the PBS solution was necessary to prevent the adsorption of JNJ 2408068 to the syringes or other containers. Subsequently, the trachea was removed from the lungs and all tissue samples were frozen as wet tissue until analysis.

2.10. Bioanalysis of JNJ 2408068 in plasma, tissue, and lung lavage fluid samples

Determination of JNJ 2408068 concentrations in plasma and tissue samples was done using a liquid chromatography-mass spectrometry (LC-MS/MS) method. Extra precautions and adequate sample preparation were required since JNJ 2408068 has unfavorable physicochemical characteristics towards bioanalysis including adsorption to glassware or other recipients and poor reproducibility of the extraction from biological matrices at low concentrations. Moreover, the compound exhibits poor chromatographic properties, especially in the lower nanogram range. To get around the extraction, adsorption, and chromatographic problems, a bioanalytical method was developed using in situ derivatiza-

tion (Husek, 1998). This was accomplished by adding butyl chloroformate (Aldrich Chemical Company Inc., Milwaukee, WI; cat. no. 18,446-2) to the plasma or homogenated tissue, followed by a liquid/liquid extraction of the obtained chemical derivative of JNJ 2408068. This derivatization procedure drastically changes the physicochemical properties of JNJ 2408068, but results in a reproducible bioanalytical method for plasma and tissue homogenates. For LF, the method still proved to be inaccurate. As a consequence, absolute concentrations for the latter samples were unreliable.

2.11. Statistics

Student's *t*-test (two-tailed) was used to compare mean changes in body weight, the average number of IC present in lung sections and the mean levels of AST, ALT, creatine, and BUN present in the various test groups. Comparison of the mean geometric RSV titers obtained for the different groups in each experiment were generally made using the Kruskal–Wallis non-parametric analysis of variance (ANOVA) program. However, in experiments in which there were only two test groups, the Mann–Whitney non-parametric test was utilized to compare mean levels of virus. Regardless of the test, all comparisons of mean virus titers were made to the mean pulmonary virus titer obtained for the group given placebo. For statistical evaluation, LF with undetectable virus titers were assigned a value of 2.3 (the minimal detection limit being 2.8) $\log_{10}$/g lung. Instat, a statistical program designed for IBM compatible computers (version 3, Graphpad Software, Inc., San Diego, CA) was used to perform this testing, as well as to obtain all of the descriptive statistics (e.g. means, geometric mean virus titers, and standard deviations).

3. Results

Because the antiviral efficacy and toxicity of the two forms of JNJ 2408068 were equivalent in these studies and to reduce redundancy, only the results of experiments utilizing the salt form are shown. For the same reasons, although most of the experiments presented in this report were also performed utilizing RSV A2 (another RSV subtype A strain) and RSV 18537 (a RSV subtype B strain), as well as RSV Long, only data from experiments using RSV Long are displayed.

3.1. Antiviral activity of JNJ 2408068 in cotton rats following therapeutic administration

In the therapeutic studies displayed in Table 2, the test compound was always administered just once, for 15 min, starting 1 h after the animals were inoculated with virus on Day 0, or once on Days 1, 2, or 3 post-infection. All of the aerosols of JNJ 2408068 were generated from delivery reservoirs containing 5 mg of active compound per milliliter. In these experiments, placebo (dH_2O) was delivered similarly to control animals once daily on every day that drug was given (e.g. once daily on Day 0 through Day 3 in Experiment 1).

As the bolded values displayed in the last column of Table 2 for each Group 2 indicate, statistically significant reductions in pulmonary virus levels ranging from at least 2 to 3.6 $\log_{10}$/g lung were seen in all groups of cotton rats administered JNJ 2408068 starting 1 h after virus inoculation compared to the mean pulmonary virus titer determined for the respective group in each experiment given placebo (Group 1 in each experiment). In three of the four experiments shown, statistically significant reductions in mean pulmonary virus titers were also observed in the groups of animals exposed to sda of JNJ 2408068 for 15 min once, 24 h after virus inoculation (Group 3 in each experiment). These reductions in mean pulmonary virus titer ranged from 1.9 to 3.7 $\log_{10}$/g of lung. In contrast to these results, in none of the four experiments shown was a significant reduction in mean pulmonary RSV titers seen in the groups of cotton rats treated for 15 min with JNJ 2408068 approximately 48 h after virus replication, compared to the mean RSV titers seen in the placebo control groups. Significant reductions in virus titers were seen in the groups of cotton rats treated with test compound once on Day 3 after virus inoculation in both of the experiments shown in which this treatment schedule was done. This seemingly paradoxical antiviral activity on Day +3 after virus inoculation and not on Day +2 was most likely due to an artifact resulting from "carry over" of drug in LF at the time of their collection into the in vitro assays that were performed to measure pulmonary virus levels (discussed in Section 2). In contrast to the inhibition of RSV seen when JNJ 2408068 was used, ribavirin (delivery reservoir concentration = 60 mg/ml) administered for 15 min one day before or after virus inoculation had little effect on mean pulmonary RSV titers (e.g. in Experiment 3 there was less than a $\leq$0.4 $\log_{10}$/g lung reduction in both of the groups administered ribavirin compared to the mean virus titer in the group given placebo).

3.2. Antiviral activity of JNJ 2408068 in cotton rats following prophylactic administration

As can be seen from the mean pulmonary virus titers presented in the last column in Table 3, with only one exception (middle experiment, Group 3), $\geq$2 $\log_{10}$ reductions in mean virus titer were detected in all groups of cotton rats exposed to just one aerosol of JNJ 2408068 for 15 min (drug reservoir concentrations = 5 mg active drug per milliliter) 1–48 h prior to virus inoculation. However, using the Kruskall–Wallis non-parametric ANOVA to compare the mean pulmonary virus titers obtained in the treated groups to the mean pulmonary virus titers seen in the placebo control group, statistically significant reductions only occurred when the test compound was administered one day before or the same day (1 h prior to virus) as the virus.

Table 2
Comparison of mean pulmonary virus titers in cotton rats experimentally infected with respiratory syncytial virus and treated by small droplet aerosol of JNJ 2408068, ribavirin or placebo on different days post-virus infection[a]

Expt. no.[b]	Group no.	Material aerosolized	Reservoir concentration (mg/ml)	Day(s) of treatment	Mean pulmonary RSV titer (log_{10}/g lung ± S.D.)
1	1	dH_2O	0	Days 0–3	4.4 ± 0.3
	2	JNJ 2408068	5	Day 0 only	**1.4 ± 1.6**[c*]
	3	JNJ 2408068	5	Day 1 only	**0.4 ± 1.4***
	4	JNJ 2408068	5	Day 2 only	3.9 ± 0.6
	5	JNJ 2408068	5	Day 3 only	**0 (≤2.3)****
2	1	dH_2O	0	Days 0–3	4.8 ± 0.4
	2	JNJ 2408068	5	Day 0 only	**0 (≤2.3)****
	3	JNJ 2408068	5	Day 1 only	**0.5 ± 1.0***
	4	JNJ 2408068	5	Day 2 only	1.8 ± 2.5
	5	JNJ 2408068	5	Day 3 only	**1.7 ± 0.5****
3	1	dH_2O	0	Days 0–2	4.8 ± 0.4
	2	JNJ 2408068	5	Day 0 only	**1.2 ± 1.3***
	3	JNJ 2408068	5	Day 1 only	2.9 ± 0.3
	4	JNJ 2408068	5	Day 2 only	4.2 ± 0.3
	5	Ribavirin	60	Day 1 only	4.4 ± 0.3
	6	Ribavirin	60	Day −1 only	4.5 ± 0.3
4	1	dH_2O	0	Days 0–2	4.3 ± 0.4
	2	JNJ 2408068	5	Day 0 only	**0 (≤2.3)****
	3	JNJ 2408068	5	Day 1 only	**0.6 ± 1.6***
	4	JNJ 2408068	5	Day 2 only	3.8 ± 0.4

[a] Each cotton rat was inoculated intranasally with respiratory syncytial virus (Long strain) on Day 0. Placebo (dH_2O), ribavirin, or JNJ 2408068 was delivered by small droplet aerosol on the day or days indicated, always for only 15 min. On Day 0, aerosolization was started 1 h after virus inoculation.

[b] Other abbreviations: expt.: experiment; no.: number; S.D.: standard deviation; RSV: respiratory syncytial virus.

[c] A bolded mean indicates that it is statistically significant compared to the mean obtained for the placebo control group using the Kurskall–Wallis non-parametric analysis of variance test. One asterisk (*) indicates that the *P* value of this mean was ≤0.05 while two asterisks (**) signify that it was ≤0.01. A mean titer of "0" indicates that no virus was detected in the lungs of any animal in this group (minimum detection 2.8 log_{10} $TCID_{50}$/g lung). The number of cotton rats per group = 4 or 5.

Table 3
Comparison of mean pulmonary virus titers in cotton rats exposed for 15 min to a small droplet aerosol of JNJ 2408068 once on different days prior to being experimentally infected with respiratory syncytial virus[a]

Group no.	Material aerosolized	Drug reservoir concentration (mg/ml)[b]	Day and duration of aerosol	Mean pulmonary of RSV titer on Day +4 (log_{10}/g lung ± S.D.)
1	dH_2O	0	Day 0 only	4.3 ± 0.4
2	JNJ 2408068	5	Day −4 only	2.8 ± 1.8
3	JNJ 2408068	5	Day −2 only	1.7 ± 1.5
4	JNJ 2408068	5	Day −1 only	**1.1 ± 1.5**[c*]
5	JNJ 2408068	5	Day 0 only	**0.6 ± 1.3***
1	dH_2O	0	Day 0 only	4.6 ± 0.3
2	JNJ 2408068	5	Day −4 only	4.4 ± 0.3
3	JNJ 2408068	5	Day −2 only	3.3 ± 0.4
4	JNJ 2408068	5	Day −1 only	**0 (≤2.3)****
1	dH_2O	0	Day 0 only	4.6 ± 0.3
2	JNJ 2408068	5	Day −4 only	3.7 ± 0.8
3	JNJ 2408068	5	Day −2 only	1.7 ± 1.9
4	JNJ 2408068	5	Day −1 only	**0 (≤2.3)****

[a] Placebo (dH_2O) or JNJ 2408068 was delivered for 15 min by small droplet aerosol on the day indicated. On Day 0, aerosolization of these materials was started 1 h after inoculation of the cotton rats with respiratory syncytial virus (RSV) Long.

[b] Other abbreviations: S.D.: standard deviation.

[c] A bolded mean indicates that it is statistically significant compared to the mean obtained for the placebo control group using the Kurskall–Wallis non-parametric analysis of variance test. One asterisk (*) indicates that the *P* value of this mean was ≤0.05 while two asterisks (**) signify that it was ≤0.01. A mean titer of "0" indicates that no virus was detected in the lungs of any animal in this group (minimum detection 2.8 log_{10} $TCID_{50}$/g lung). The number of cotton rats per group = 4 or 5.

Table 4
Determination of the minimum protective dose for JNJ 2408068 when administered to cotton rats experimentally infected with respiratory syncytial virus by continuous small droplet aerosol[a]

Expt. no.[b]	Group no.	Material aerosolized	Reservoir concentration (mg/ml)	Day and duration of treatment	Mean pulmonary of RSV titer on Day +4 (log_{10}/g lung ± S.D.)
1	1	dH_2O	0	Day −1	4.6 ± 0.4
	2	JNJ 2408068	1.25	Day −1	3.9 ± 0.5
	3	JNJ 2408068	2.5	Day −1	**0.8 ± 1.7**[c]*
	4	JNJ 2408068	5.0	Day −1	**1.3 ± 1.5***
2	1	dH_2O	0	Day −1	4.3 ± 0.4
	2	JNJ 2408068	1.25	Day −1	3.4 ± 0.8
	3	JNJ 2408068	2.5	Day −1	1.4 ± 1.6
	4	JNJ 2408068	5.0	Day −1	**0 (≤2.3)****

[a] Placebo (dH_2O) or JNJ 2408068 was delivered for 15 min by small droplet aerosol (sda) on Day −1 relative to inoculation of the animals with respiratory syncytial virus (RSV) Long. Animals were sacrificed and their lungs tested for pulmonary virus levels on Day +4 after virus inoculation.
[b] Other abbreviations: S.D.: standard deviation; expt.: experiment.
[c] A bolded mean indicates that it is statistically significant compared to the mean obtained for the placebo control group using the Kurskall–Wallis non-parametric analysis of variance test. One asterisk (*) indicates that the P value of this mean was ≤0.05 while two asterisks (**) signify that it was ≤0.01. A mean titer of "0" indicates that no virus was detected in the lungs of any animal in this group (minimum detection 2.8 log_{10} $TCID_{50}$/g lung). The number of cotton rats per group = 4 or 5.

These results were confirmed in experiments performed to determine the minimal dose of JNJ 2408068 that provided protection against pulmonary RSV infection in cotton rats. As seen from the representative data displayed in Table 4, cotton rats exposed for 15 min to a single sda of the test compound generated from delivery reservoirs containing 5 mg active drug per milliliter one day before virus challenge again had significant ($P \leq 0.05$ using the Kruskall–Wallis non-parametric ANOVA) and ≥2 log_{10} reductions in mean pulmonary RSV titers compared to animals that received placebo. A statistically significant reduction in mean pulmonary RSV titer also was seen in one of the two experiments shown in groups of animals exposed to 15 min aerosols generated from ADR containing 2.5 mg of active compound. However, in neither of the experiments displayed was a statistically significant reduction in virus titer seen in groups of cotton rats exposed to aerosols of JNJ 2408068 generated from delivery reservoirs containing 1.25 mg of test compound per milliliter. The estimated dose given the latter animals was 0.1 mg/kg. Thus, under the conditions utilized, 0.2 mg/kg was the median protective dose of JNJ 2408068 and 0.39 mg/kg the minimal protective dose.

3.3. Toxicity testing of JNJ 2408068 in cotton rats

Experiments were performed to gauge the toxicity of JNJ 2408068 in cotton rats. In these experiments, the animals were exposed 15 min daily to an estimated 3.9 mg of test compound per kilogram per day for four consecutive days. Thus, these animals got approximately 40 times more test compound than the cotton rats used in the antiviral studies. Despite this increased dosage and exposure, no morbidity, mortality, or overt untoward activity was seen in any of the test animals during the five-day test period (Table 5, middle section). Neither was there any significant effect on body weight (top portion of Table 5) or levels of ALT, AST, BUN, or creatine in the sera (bottom portion of this table) of the test animals. All of the groups had an equivalent increase in weight during the course of this experiment and all had comparable mean levels of the selected serum markers when tested. In addition, there were no overt changes in pulmonary architecture or significant differences in the number of IC seen in the sections of lung taken from animals dosed with JNJ 2408068 compared to what was observed in similarly prepared and stained sections of lung from cotton rats that were not infected with virus or exposed either of the test compounds (data not shown).

3.4. Pharmacokinetic studies

Plasma and tissue concentration-time profiles were subjected to a non-compartmental pharmacokinetic analysis using WinNonlin Professional (v2.1 Pharsight Corporation, Cary, NC, 1998). As the data in Table 6 indicate, plasma concentrations declined rapidly following cessation of drug delivery and within 4 h, plasma levels were near the lower limit of quantification (LLOQ) of the LC-MS/MS assay (i.e. 0.5 ng/ml). The calculated mean half-life ($T_{1/2\,1–4\,h}$) of JNJ 2408068 in the plasma obtained in the three replicate experiments was 1.5 ± 0.5 h and the average area under the curve value ($AUC_{0–4\,h}$) for drug plasma concentrations was determined to be 25.6 ng h/ml. These results indicated that systemic exposure to the test compound was limited. Concentrations of JNJ 2408068 in the liver and femur muscle confirmed this. Levels of drug in these tissues were always low (5–13 ng/g tissue) or below the LLOQ of 5 ng/ml and even the former levels were only observed in a few animals at 0 or 0.5 h post-dosing.

As seen by the data presented in Table 7 and Fig. 2, much higher concentrations of JNJ 2408068 were seen in the lungs. These levels initially decreased rapidly (average $T_{1/2\,1–4\,h}$ = 3.1 h), but the rate of clearance slowed from 24 h after ap-

Table 5
Results of preliminary toxicity testing of JNJ 2408068 in cotton rats administered these compounds by continuous small droplet aerosol[a]

Material aerosolized	Estimated daily dose (mg/kg per day)	Number of administrations	Parameter evaluated	Observation after five days
Placebo	0	4 (1× pre day)	Body weight	+5.4 g mean wt gain
JNJ 2408068	3.9	4 (1× pre day)	Body weight	+4.7 g mean wt gain[b]
JNJ 2408068	3.9	4 (1× pre day)	Body weight	+5.0 g mean wt gain[b]
Placebo	0	4 (1× pre day)	Mort./morb./UA	None observed
JNJ 2408068	3.9	4 (1× pre day)	Mort./morb./UA	None observed
JNJ 2408068	3.9	4 (1× pre day)	Mort./morb./UA	None observed
Placebo	0	4 (1× pre day)	ALT/AST/BUN/creatine	125/239/22.2/0.55[c]
JNJ 2408068	3.9	4 (1× pre day)	ALT/AST/BUN/creatine	143/282/21.4/0.48[b,c]
JNJ 2408068	3.9	4 (1× pre day)	ALT/AST/BUN/creatine	138/276/20.9/0.53[b,c]

[a] Test animals were exposed once daily to continuous small droplet aerosols of placebo (distilled water) or JNJ 2408068 for four continuous days, 15 min per day. In each instance, the concentration of drug in the delivery reservoirs used to generate the aerosols was 50 mg drug per milliliter of water, so that all of the cotton rats exposed to these compounds received an estimated 3.9 mg/kg per day of test compound. Mortality (mort.), morbidity (morb.), or untoward activity (UA) seen in each test group was looked for daily. All animals were weighed at the beginning and at the end of the five-day observation period. In addition, levels of ALT, AST, creatine, and BUN in the serum of the placebo and treated cotton rats were compared.

[b] These values are not significantly different ($P > 0.05$) from the mean obtained for the placebo control group using Student's unpaired t test (two-tailed; number of animals per group = 4).

[c] Slash bars separate the mean values obtained for ALT (units/l), AST (units/l), creatine (mg/dl), and BUN (mg/dl).

plication onwards (average $T_{1/2\,24–96\,h} = 52.3$ h) and even at 96 h after dosing, average lung levels in the lungs ranged from 119 to 460 ng/g. Most telling of the high average exposure of the lungs to the test compound was the $AUC_{0–\infty}$ value obtained, i.e. 91,027 ng h/ml. Unfortunately, the compound could not be completely extracted from LF. For this reason, the levels shown represent "at least" values and for these samples are not completely accurate. Nevertheless, as seen in Fig. 2, the data suggest that after an initial faster decline of JNJ 2408068 concentrations in LF, both lung and LF concentrations declined at a similar rate. One possible complicating factor is that the lavaging process may damage lung tissue to an extent that the LF are contaminated with traces of lung tissue, adding to the apparent level of test compound in them. Regardless, the levels of JNJ 2408068 measured in LF at all times exceeded the EC_{50} determined for this compound in in vitro studies (i.e. 1 ± 1 ng/ml; see previous article in this journal).

Table 6
Mean plasma concentrations (ng/ml) of JNJ 2408068 in cotton rats after a single 15-min dose inhalation of an aqueous solution of this compound based on an average estimated dosage of 0.39 mg base-eqv./kg[a]

	Mean plasma level JNJ 2408068 (ng/ml)			Average mean plasma level (ng/ml ± S.D.)[c]
	Expt. 1	Expt. 2	Expt. 3	
Time (h) after ceasing aerosol[b]				
0 ($n = 3$)[d]	20.7	23.6	36.4	26.9 ± 8.4
0.5 ($n = 1$)	14.8	11.9	14.4	13.7 ± 1.6
1 ($n = 1$)	2.6	7.8	11.3	7.2 ± 4.4
4 ($n = 3$)	0.9	1.4	2.0	1.4 ± 0.5
24 ($n = 3$)	<0.5	<0.5	<0.5	<0.5 ± 0
96 ($n = 1$)	<0.5	<0.5	<0.5	<0.5 ± 0
Pharmacokinetic characteristics				
C_{max} (ng/ml)	20.7	23.6	36.4	26.9 ± 8.3
T_{max} (h)	0	0	0	0
$t_{1/2,\,1–4\,h}$ (h)	2.0	1.2	1.2	1.5 ± 0.5
$AUC_{0–4\,h}$ (ng h/ml)	17.1	24.5	34.3	25.6 ± 8.6
$AUC_{0–\infty}$ (ng h/ml)	19.8	26.9	37.7	28.2 ± 9.0

[a] Blood was taken by cardiac puncture using sodium ethylenediaminetetraacetic acid as an anticoagulant. The plasma from these samples was separated by centrifugation and frozen until quantification of the drug levels present in each were performed.

[b] Abbreviations: h: hour; n: number of animals in each group; C: concentration; max: maximum; T: time; $t_{1/2}$: half-life; AUC: area under the curve; S.D.: standard deviation, expt.: experiment.

[c] Shown are the means and standard deviation obtained for the three replicate experiments. The number of animals per group are indicated in parentheses in column 1.

[d] End of 15 min inhalation.

Table 7
Mean concentrations (ng/g) of JNJ 2408068 in lung tissue of cotton rats after a single 15-min dose inhalation of an aqueous solution of test drug based on an average estimated dosage at 0.39 mg/kg[a]

	Mean plasma level JNJ 2408068 (ng/ml)			Average mean level (ng/ml ± S.D.)[c]
	Expt. 1	Expt. 2	Expt. 3	
Time (h) after ceasing aerosol[b]				
0 ($n = 3$)[d]	5270	5425	6998	5898 ± 956
0.5 ($n = 1$)	5690	4792	3994	4826 ± 849
1 ($n = 1$)	2013	4665	4318	3665 ± 1441
4 ($n = 3$)	1788	1767	2092	1882 ± 182
24 ($n = 3$)	436	584	1130	717 ± 366
96 ($n = 1$)	249	119	460	276 ± 172
Pharmacokinetic characteristics				
C_{max} (ng/ml)	5690	5425	6998	5898 ± 842
T_{max} (h)	0.5	0	0	0 ± 0.29
$t_{1/2,\,1–4\,h}$ (h)	17.5	2.1	2.9	3.1 ± 8.67
$t_{1/2,\,24–96\,h}$ (h)	89.1	31.4	55.5	52.3 ± 29.0
$AUC_{0–96\,h}$ (ng h/ml)	53400	56287	98884	70211 ± 25468
$AUC_{0–\infty}$ (ng h/ml)	85403	61673	35735	91027 ± 37819

[a] Lung fluids were obtained from each set of lungs by transpleurally lavaging each set utilizing 2 ml of a 5% bovine serum albumin solution in PBS. Subsequently, the trachea was removed from the lungs and all tissue samples were frozen as wet tissue until analysis.

[b] Abbreviations: h: hour; *n*: number of animals in each group; *C*: concentration; max: maximum; *T*: time; $t_{1/2}$: half-life; AUC: area under the curve; S.D.: standard deviation; expt.: experiment.

[c] Shown are the means and standard deviation (S.D.) obtained for the three sessions. Number of animals per group are indicated in parentheses in column 1.

[d] End of 15 min inhalation.

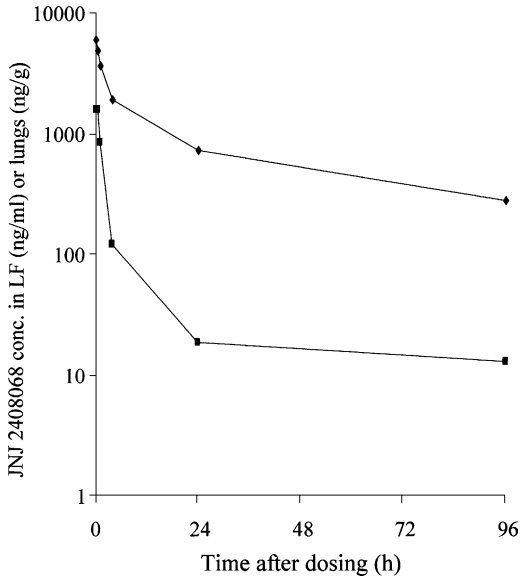

Fig. 2. Concentration of JNJ 2408068 in cotton rat lungs (ng/g lung; ◆) or lung lavage fluids (ng/ml; ■) with time after these animals were exposed to a single 15-min dose inhalation of an aqueous solution of the test drug. The concentrations are based on a theoretical delivery dose of 0.39 mg base-eqv./kg. Due to limitations of the bioanalytical method, the lung lavage fluid concentrations are only indicative.

4. Discussion

Throughout the present studies, consistent and marked reductions in mean pulmonary RSV titers occurred in groups of cotton rats administered JNJ 2408068, despite using only 15 min delivery times (see Tables 2–4). This activity appeared to support the reported in vitro findings (Andries et al., 2000; Andries et al., in press) that this compound is very active against RSV. However, because of the very high specific activity of JNJ 2408068, it was possible that test compound was being "carried over" in LF and inactivating virus ex vivo or during in vitro testing. Bolstering this possibility were results obtained in pharmacokinetic studies (Tables 6 and 7 and Fig. 2) that clearly showed that significant levels of the test compound were being successfully delivered to the lungs (average $AUC_{0–\infty}$ value = 91,027 ng h/ml) and that despite an initial rapid decrease in the levels of the test compound ($T_{1/2\,1–4\,h}$ = 3.1 h), there was still sufficient drug present at 96 h after dosing to inhibit virus (119–460 ng drug/g lung). Interpretation of the data was made still more difficult by the fact that the pharmacokinetic studies could not distinguish between "free and available" and "bound and unavailable" compound. Despite the problems, much evidence was obtained indicating that JNJ 2408068 is indeed quite active in vivo. First, it is unlikely that any compound present in the LF at the time of harvest was inactivating virus ex vivo since binding of JNJ 2408068 to the virus has been found to be fully reversible by dilution (Andries et al., in press). In addition, "carry over" experiments indicated that LF obtained from lungs taken from mice exposed to sda of the test compound ≥48 h after stopping aerosol delivery did not inhibit replication of exogenously added RSV (e.g. Table 1). More conclusive were the results obtained in prophylactic studies where significant reductions in mean pulmonary RSV titers occurred in animals given drug the same day (1 h prior to virus inoculation)

or one day prior to virus inoculation—up to 120 h prior to harvesting lungs for virus testing.

It is also noteworthy that mean pulmonary virus titers were generally inversely related to the interval in time between drug administration and virus inoculation. Thus, as shown in Table 3, maximal reductions in mean virus titers occurred when JNJ 2408068 was given 1–24 h prior to virus with reductions ranging from at least 2.3 to 3.7 $\log_{10}$ $TCID_{50}$/g lung. (The 2.3 $\log_{10}$ reductions represent minimal decreases because of the 2.8 $\log_{10}$/g lung minimum detection limit in our assay.) Generally lower (1.3–2.9 $\log_{10}$ $TCID_{50}$/g lung) reductions occurred when groups of cotton rats were administered test compound two days prior to virus and no significant reductions in mean pulmonary virus titers were obtained for any group of animals given the test compound four days prior to virus. As important, many of the means obtained for groups of animals given test material two days prior to virus had relatively large standard deviations (e.g. 1.5 ± 1.7 $\log_{10}$/g lung and 1.5 ± 1.9 $\log_{10}$/g lung in two of the experiments shown in Table 3) and were not statistically significant. This variability was not likely due to carryover of compound into the in vitro assays performed to test for levels of pulmonary virus since these were performed 144 h later. Instead, the variable pulmonary virus titers suggest that the levels of test drug in the lungs of the animals at the time that they were inoculated with virus (48 h after drug administration) may have been at or near the threshold required for antiviral efficacy, causing greater reduction in pulmonary virus in some animals and less in others.

The results obtained in the carryover and prophylactic studies also help explain the seemingly paradoxical results in which statistically significant reductions in mean pulmonary RSV titers were seen in cotton rats given test compound three days after virus administration, but not two days after the animals were inoculated with RSV (compare the mean virus titers and reductions in titer for Groups 4 and 5 in Experiment 1 and Groups 4 and 5 in Experiment 2 in Table 2). The reductions in virus titer observed in the groups of animals administered test compound on Day +3 were most likely due to the presence of sufficient levels of unbound drug still being present in the lungs of the test animals on Day 4 (only one day after drug delivery) when the LF were collected from these animals and tested for antiviral activity. It is probable that no similar reduction occurred when LF obtained from cotton rats given drug two days after inoculation of the animals with virus were used since by the time that these lungs were collected for testing 48 h had elapsed and the levels of available drug in them had likely decreased sufficiently so as not to significantly interfere with the in vitro testing. It should be kept in mind that the lungs were always lavaged with 3 ml of fluid, a procedure that inherently decreases the concentration of any compound in the LF.

Also arguing against the decreases in viral titer being a result of drug carryover was the finding in pharmacokinetic studies (Fig. 2) indicating that the levels of the test compound in LF remain virtually constant from 24 through 96 h after drug dosing. As discussed above, this finding did not correlate with the virus levels detected in the lungs of animals administered drug 24 h (Day +3) to 144 h (i.e. Day −2) prior to harvesting. These tended instead to vary inversely with the time elapsed between drug administration and virus inoculation. Together the results obtained in the biological studies belie those of the pharmacokinetic studies and suggest that although test compound is detectable in the lungs and LF for days, drug concentrations reach threshold levels between 24 and 48 h and are not available for virus inhibition thereafter.

The minimal protective dose of JNJ 2408068 for cotton rats was determined by exposing test animals to graded doses of this compound one day prior to virus; 0.39 mg active drug per kilogram (the estimated amount of test drug delivered to cotton rats from an aerosol delivery reservoirs containing 5 mg active drug per milliliter) was chosen as the maximum drug dose to be tested since animals given this quantity of test drug within a day of virus inoculation almost always had significantly reduced mean pulmonary virus titers compared to those seen in the placebo control groups (e.g. see the mean pulmonary virus titers and reductions in mean virus titer displayed in Tables 2 and 3). Similarly, administering the compounds one day prior to virus was picked because earlier studies had indicated that this timing was consistently effective. As discussed above, under these conditions, the minimum protective dose of JNJ 2408068 appeared to be approximately 0.39 mg/kg, a value at least 20 times lower than that obtained for ribavirin whose minimal protective dose is between 8 and 15 mg/kg (Gilbert et al., 1992). Indeed, in the present studies, when ribavirin was administered for just 15 min (e.g. see Table 2, Experiment 3) using 60 mg/ml aerosol reservoir concentrations, it did not have any significant effect on pulmonary RSV levels. These studies clearly indicate that JNJ 2408068 has markedly more specific activity than the nucleoside analog.

One of the most important findings obtained in these studies was the marked inhibition of virus that occurred in animals exposed for very short treatment intervals (i.e. 15 min) and relatively low doses of compound (estimated to be only 0.39 mg/kg). Although surprising, these results were not incongruous with the remarkable activity of these compounds in tissue culture assays. Most evocative, such short delivery times approach conditions amenable for outpatient care, perhaps even in a doctor's office.

Toxicity testing in cotton rats was limited. Regardless, none of the animals included in this testing exhibited any overt toxic effects (i.e. morbidity, mortality, weight loss, significant changes in selected serum markers, or other untoward responses; Table 5 and data not shown), although they received approximately 40 times the amount of drug shown to be consistently protective against RSV replication in cotton rats (i.e. 40×0.39 mg/kg). As important, there were no evident histopathological changes induced in the lungs of treated cotton rats. H&E stained sections of lung obtained from these animals appeared to be no different

than comparably stained sections prepared from lungs removed from untreated animals (data not shown). This said, it is clear that more comprehensive toxicity studies need to be performed. For example, it is not known what this compound does to rapidly proliferating tissues such as bone marrow or T- and B-lymphocytes, or what prolonged treatment with JNJ 2408068 would do to immunosuppressed individuals infected with RSV. Moreover, the limited extrapulmonary distribution of drug seen in these studies is contrary to results obtained in dogs and rats (data not shown). Nevertheless, based on the extraordinary selective antiviral activity seen in tissue culture and cotton rats, and the lack of overt toxicity in the latter, further testing of 2[[2-[[1-(2-aminoethyl)-4-piperidinyl]amino]-4-methyl-1H-benzimidazol-1-yl]methyl]-6-methyl-3-pyridinol and/or derivatives of this compound appears justified.

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