Characterization of an influenza A (H3N2) virus resistant to the cyclopentane neuraminidase inhibitor RWJ-270201

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Abstract

The novel influenza virus neuraminidase (NA) inhibitor, (1S,2S,3R,4R)-3-[(1S)-(acetylamino)-2-ethylbutyl]-4-[(aminoiminomethyl)amino]-2-hydroxy-cyclopentanecarboxylic acid (RWJ-270201, BCX-1812), is a potent inhibitor of influenza A and B viruses in cell culture and in infected mice. A mouse-adapted strain of influenza A/Shangdong/09/93 (H3N2) virus was serially passaged in the presence of 1 μM compound. After the fourth passage, breakthrough of resistant virus occurred. By the tenth passage, a twice plaque purified isolate was obtained which could replicate in 10 μM inhibitor. The 50% effective concentration (EC$_{50}$) values for RWJ-270201 against wild-type and resistant viruses, determined by using a cytopathic effect inhibition assay, were 0.007 and 23 μM, respectively. Cross-resistance to zanamivir and oseltamivir carboxylate was observed. The hemagglutinin (HA) and NA genes of the virus were sequenced to determine the mutation(s) which conferred drug resistance. No differences were found between the resistant and wild-type viruses in the NA gene. However, a point mutation resulting in a single amino acid change (Lys189Glu) was found in the resistant viral HA. The wild-type and resistant viruses were compared for virulence in BALB/c mice. The resistant virus was approximately tenfold less virulent than the wild-type virus based upon virus challenge dose. Mice infected with a lethal dose of the resistant virus could still be effectively treated with RWJ-270201. Thus, the HA mutation may allow for the spread of the virus in cell culture in the presence of the NA inhibitor, but not in mice. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Antiviral; Neuraminidase inhibitor; Influenza virus; Drug resistance

1. Introduction

A novel cyclopentane compound, (1S,2S,3R,4R)-3-[(1S)-(acetylamino)-2-ethylbutyl]-4-[(aminoiminomethyl)amino]-2-hydroxy-cyclopentanecarboxylic acid (RWJ-270201, BCX-1812), was re-

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cently reported to be a potent inhibitor of influenza virus neuraminidase (NA) (Babu et al., 2000). This and related structures were highly active against influenza A (H1N1, H3N2, and H5N1) and influenza B viruses in MDCK cells (Smee et al., 2001), and in mice infected with influenza A (H1N1 and H3N2) and B viruses (Bantia et al., 2001; Sidwell et al., 2001). The potency of RWJ-270201 in these systems was comparable to or greater than the two licensed drugs, zanamivir and oseltamivir. RWJ-270201 has been used in experimental human challenge studies with influenza A and B virus infections (Hayden et al., 2000), where it is reported to be well tolerated and to have antiviral activity.

Viral resistance to NA inhibitors has been the topic of thorough and ongoing investigation, as recently reviewed (McKimm-Breschkin, 2000). Viruses resistant to zanamivir and oseltamivir have been particularly well studied, since the compounds have been available for several years. The majority of mutants isolated from cell culture in the presence of NA inhibitors have had mutations in the hemagglutinin (HA) gene with no mutations in the NA gene. Resistant viruses isolated in cell culture possessing a NA mutation almost always contain a HA mutation (with rare exceptions; Blick et al., 1995), with the HA mutation occurring first. Many but not all of the mutant viruses prepared in the cell culture are less virulent to mice. Some but not all of these viruses can still be effectively treated with zanamivir and/or oseltamivir in vivo (McKimm-Breschkin, 2000).

Isolation of drug-resistant viruses from humans has been infrequent. A double (HA and NA) mutant influenza B virus resistant to zanamivir was isolated from an immunosuppressed patient (Gubareva et al., 1998). Two different NA mutant viruses were isolated from patients treated with oseltamivir (Covington et al., 1999). Undoubtedly more drug-resistant virus isolates from humans will be isolated and characterized in the future.

It is important to understand the impact and frequency of emergence of drug-resistant viruses, in particular as mutations relate to new antiviral agents. For this reason we began the passage of a mouse-adapted influenza A/Shangdong/09/93 virus in cell culture in the presence of RWJ-270201. This particular virus strain was chosen because the wild-type virus is lethal to mice and has been used in chemotherapy studies with RWJ-270201 (Sidwell et al., 2001). A resistant virus that was obtained from this study has been genetically characterized and evaluated in cell culture and mouse chemotherapy experiments.

2. Materials and methods

2.1. Antiviral compounds

RWJ-270201 was synthesized by The R.W. Johnson Pharmaceutical Research Institute. Zanamivir, and oseltamivir carboxylate (previously referred to as GS4071; oseltamivir is the orally active prodrug of GS4071) were synthesized at BioCryst Pharmaceuticals (Birmingham, AL). The compounds were dissolved in cell culture medium for in vitro chemotherapy studies. Saline was the diluent for mouse experiments.

2.2. Cells and media

Madin Darby canine kidney (MDCK) cells were grown in antibiotic-free minimum essential medium (MEM) with non-essential amino acids (Gibco, Long Island, NY) containing 5% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT) and 0.1% $NaHCO_3$. Test medium consisted of MEM with 0.18% $NaHCO_3$, 10 units/ml trypsin, 1 μg of ethylenediaminetetraacetate (EDTA) per ml, and 50 μg gentamicin/ml. MDCK cells were used for all cell culture studies with virus.

2.3. Viruses

Influenza A/Shangdong/09/93 (H3N2) was provided by Dr Helen Regnery of the Influenza Branch of the Centers for Disease Control and Prevention (Atlanta, GA). The virus was passaged seven times in mice to increase its virulence. The virus was then divided into two pools. For isolation of resistant virus, one pool was serially passaged in the presence of RWJ-270201 starting at 1 μM. Little or no virus-induced cytopathic effect

(CPE) was noted until the fourth passage when virus breakthrough occurred. The virus was then passaged once in the absence of drug to increase the titer, followed by continued passage in the presence of 1 μM RWJ-270201. For passages 8 and 9, the inhibitor concentration was increased to 10 μM. At this point the virus was diluted to extinction in 96-well plates and individual virus foci isolated. Several of these isolates were grown individually. Drug susceptibility tests were conducted to select the most drug-resistant isolates. The final pool of virus selected was passaged (passage 10) in the absence of the inhibitor. The newly derived virus resistant to RWJ-270201 was termed 'RWJ-R'.

For control wild-type (WT) virus, the other pool from mice was passaged ten times without drug in parallel with the virus propagated in the presence of RWJ-270201. For passages 8 and 9, individual virus foci were isolated and tested for drug sensitivity as described above for the resistant virus. In the case of the WT virus, isolates most sensitive to inhibition by RWJ-270201 were kept and amplified. Virus isolates varied from three- to tenfold from each other in sensitivity to the inhibitor, possibly the result of assay variability rather than true differences.

2.4. Antiviral drug susceptibility testing

Inhibition of virus-induced CPE, as determined by visual (microscopic) examination of the cells, was used to assess antiviral activity as described previously (Sidwell and Huffman, 1971; Smee et al., 2001). Seven half-$\log_{10}$ concentrations of test compound (0.1–100 μM for viruses less sensitive to inhibition by the agent; 0.001–1 μM for more sensitive viruses) were evaluated in quadruplicate wells against each virus in 96-well microplates. The compounds were added 5–10 min prior to virus, and the extent of CPE was determined after incubation at 37 °C for 72 h. The EC_{50} values (effective concentrations for 50% inhibition of virus-induced CPE) were determined graphically.

Virus yield reduction assays were performed by a method which separated extracellular (supernatant) from cell-associated virus prior to quantitation (Smee et al., 2001). Virus was first replicated 72 h in 24-well plates in the presence of varying concentrations of drug. Extracellular virus was removed from cell monolayers and centrifuged at 3200g for 5 min. The supernatant was frozen at −80 °C. The cell pellets were recombined with cells remaining in the wells (which had fresh medium added to them), representing cell-associated virus. These plates were frozen until the day of titration. Cells from thawed plates were sonicated 30 s each, then were serially diluted in 96-well plates for virus titer determinations. Supernatant virus samples were similarly titrated. Four replicate samples were assayed separately in order to derive standard deviations. Virus titers were calculated by a 50% end point dilution method (Reed and Muench, 1938). The EC_{90} values (effective concentrations for 90% inhibition of virus yield) were determined graphically.

2.5. Genetic characterization of viruses

Viral RNA was prepared from infected tissue culture fluid using a QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA). Viral complementary deoxyribonucleic acid (cDNA) was synthesized using a Superscript Preamplification System (Gibco BRL, Rockville, MD). The primer for transcription was designed from the common nucleotide sequence in the 3′ non-coding region of viral RNA segments (U12: 5′AGCAAAAGCAGG3′) (Lamb and Choppin, 1983). HA1 and 2 overlapping fragments of NA were then amplified by polymerase chain reaction (PCR) from cDNA using gene specific primers designed from published sequence (HA1, Accession number Z46417; NA, Accession number U43419). PfuTurbo (Stratagene, La Jolla, CA) was used for PCR amplification to minimize nucleotide changes due to polymerase error. PCR products were purified (Wizard PCR Preps, Promega, Madison, WI) and sequenced by a PRISM Dye Deoxy Terminator Cycle Sequencing system (Applied Biosystems, Foster City, CA). Sequences were analyzed using Sequencher 3.1 (Gene Codes Corp., Ann Arbor, MI) and CLUSTAL W (Thompson et al., 1994).

2.6. Neuraminidase assay

Standard fluorometric endpoint assays were used to monitor NA activity and inhibition (Mendel et al., 1998). The substrate, 2′-(4-methylumbelliferyl)-D-N-acetylneuraminic acid (MU-NANA, Sigma Chemical Co., St. Louis, MO), is cleaved by NA to yield a fluorescent product, 4-methylumbelliferone, that can be quantified. NA activity was assayed in 50 µl reactions in Dynatech MicroFLUOR 96-well plates containing 30 µM MUNANA, 32.5 mM MES (2-[N-morpholino]ethanesulfonic acid) buffer pH 6.0, and 4 mM calcium chloride. Preliminary titrations of virus were performed to ensure that NA activity was in the linear range. An HA immunoassay was done to verify that total virus particle numbers (i.e. infectious and non-infectious virus) correlated directly with plaque forming units of virus. Final concentrations of $2–3 \times 10^6$ plaque forming units/ml of the WT and RWJ-R viruses were used for the determination of IC_{50} values (concentration of RWJ-270201 that inhibited 50% of NA activity). Similar numbers of infectious virus particles from the WT and RWJ-R viruses resulted in similar amounts of NA activity. Virus and RWJ-270201 were pre-incubated in buffer for 10 min at 37 °C, followed by addition of substrate and continued incubation for 20 min. Reactions were terminated by the addition of 75 µl 0.2 M glycine–NaOH, pH 10.2, and 4-methylumbelliferone was detected using a SpectraMax Gemini XS fluorometer (Molecular Devices, excitation wavelength 355 nm, emission wavelength 460 nm).

2.7. Immunoassay for viral standardization

WT and RWJ-R viruses were serially diluted in phosphate buffered saline (PBS), and 5 µl samples were spotted on a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) and blocked for 1 h in PBS–0.1% Tween 20 containing 1% blocking grade non-fat dry milk (BioRad). The membrane was then incubated with a monoclonal antibody to influenza A H3 antigen (1:200; Chemicon International, Temecula, CA). After washing with PBS–Tween 20, the membrane was incubated with horseradish peroxidase-conjugated

goat anti-mouse serum (1:5000, BioRad). The dot blot was developed using an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ) before exposure to film.

2.8. Binding assay for influenza virus

A binding assay which monitors the adherence of viral HA to immobilized fetuin (Bethell et al., 2000) was used with modification. ELISA plates (Corning, Corning, NY) were coated overnight with fetuin (20 µg/ml; Sigma Chemical Co.) in coating buffer (3 mM NaN_3, 5 mM $MgCl_2$, 15 mM Na_2CO_3, 35 mM $NaHCO_3$) and blocked with PBS–Tween 20. Virus was serially diluted with PBS and 50 µl of each dilution incubated overnight at 4 °C in fetuin coated wells. After washing with PBS–Tween 20, plates were incubated with goat anti-influenza A antibody [1:1000 (Chemicon) in PBS–Tween 20 containing 1% non-fat dry milk], washed and incubated with horseradish-peroxidase conjugated rabbit anti-goat Ig (1:5000; Chemicon). Bound virus was detected with OPD (Sigma Fast o-phenylenediamine dihydrochloride; Sigma Chemical Co.) substrate, and read using a microplate reader (450 nm; Molecular Devices, Sunnyvale, CA).

WT and RWJ-R virus isolates were equilibrated by immunoassay, and the binding assay was used to select a test dilution that was in the linear range of absorbance. Aliquots of virus at this dilution were prepared in triplicate using PBS and incubated at 0, 37, 43, 58, and 62 °C for 1 h. Viral samples were then tested for binding to fetuin as described above.

2.9. Animal studies

Specific pathogen-free BALB/c mice were obtained from B&K Universal (Fremont, CA). They were infected with virus intranasally in a volume of 90 µl, while under anesthesia (ketamine, 100 mg/kg, by intraperitoneal injection). In the initial study, different challenge doses of WT and resistant viruses were compared for virulence in 18–21 g mice to determine the approximate 50% lethal dose (LD_{50}) of virus. In a second experiment examining the antiviral activity of RWJ-270201, a

greater degree of lethality was required than was achieved using the 18–21 g mice. Thus, 8–10 g weanling mice were used and infected with 10^7 cell culture infectious doses ($CCID_{50}$) of the resistant virus per mouse. In this experiment, the animals were treated orally twice daily with RWJ-270201 or placebo for 5 days starting 4 h pre-virus exposure. Animals were held 21 days for death. Arterial oxygen saturation (SaO_2) levels were monitored daily for 10 days by pulse oximetry as described previously (Sidwell et al., 1992), with the day 6 data reported here for brevity. Statistical evaluations of increases in the number of survivors were made using the chi square with Yates' correction test. Differences in mean day of death and in mean arterial oxygen saturation levels were made using the Students *t* test. All statistical analyses were two-tailed.

3. Results

3.1. Sensitivities of WT and RWJ-R viruses to NA inhibitors in cell culture

The sensitivity of the RWJ-R virus was assayed in parallel with the WT virus against three NA inhibitors in cell culture by CPE assay (Table 1). The mutant virus was > 3000-fold more resistant to RWJ-270201 than WT virus based on EC_{50} values. Cross-resistance was also observed against two other NA inhibitors, zanamivir and os-

Table 1
Sensitivities of wild-type (WT) and RWJ-270201-resistant (RWJ-R) forms of influenzaA/Shangdong/09/93 (H3N2) virus to inhibition by RWJ-270201, zanamivir, and oseltamivir carboxylate in cell culture

Virus	50% Effective concentration[a] (µM)		
	RWJ-270201	Zanamivir	Oseltamivir carboxylate
Wild-type	0.007 ± 0.003	0.043 ± 0.02	0.012 ± 0.005
RWJ-R	23 ± 15	80 ± 30	>100

[a] Determined by cytopathic effect inhibition assays. Data represent means ± standard deviations (three independent assays).
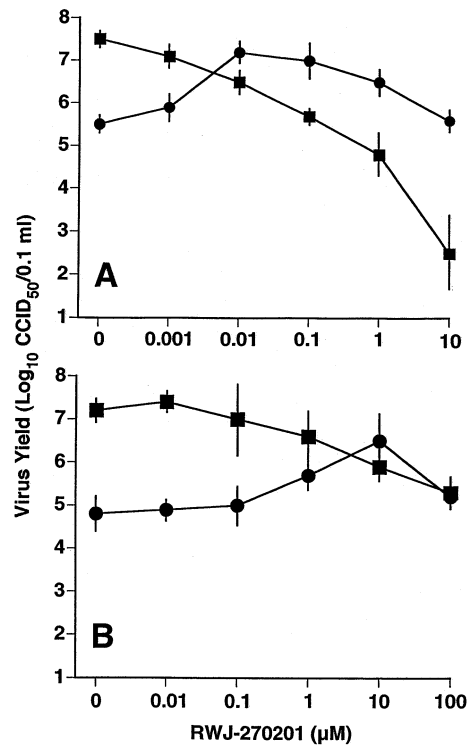

Fig. 1. Cell-associated (●) and extracellular (■) influenza A/Shangdong/09/93 (H3N2) WT (panel A) and RWJ-R (panel B) virus yields produced in the presence of RWJ-270201. Data represent mean titers ± standard deviations (four replicate samples).

eltamivir carboxylate. To determine whether the decrease in drug sensitivity of the RWJ-R virus resulted in increased virus production, virus yield reduction assays were performed, analyzing for both cell-associated and extracellular virus. As observed in Fig. 1A, RWJ-270201 inhibited extracellular but not cell-associated WT virus production in a concentration dependent manner, similar to its effects against other strains of influenza virus (Smee et al., 2001). For the RWJ-R virus, decreases in extracellular virus production only occurred at high RWJ-270201 concentrations (≥ 10 µM) (Fig. 1B). The 90% inhibitory concentration values for the compound against WT and resistant virus were 0.01 and 3 µM, respectively. These values approximated the 50% CPE inhibition data for RWJ-270201 in Table 1.

3.2. Characterization of drug resistance

The WT and RWJ-R viruses were characterized by sequence analysis of their HA and NA genes. No differences between these viruses were seen in their NA genes. A single point mutation was present in the HA gene of the RWJ-R virus compared to the WT virus. This resulted in an amino acid change in the HA protein from lysine to glutamic acid at position 189 (Lys189Glu). The WT mouse-adapted virus differed from the published (GenBank) influenza A/Shangdong/09/93 (H3N2) virus sequence in 42 amino acid positions (data not shown), probably as a result of mouse adaptation. The RWJ-R virus possessed these amino acid changes as well. Thus, the only observed difference between the RWJ-R and the WT viruses was the Lys189Glu amino acid change in HA.

To elucidate whether there were any biochemical differences between the WT and RWJ-R NAs, assays were conducted using enzymatic activity obtained from purified whole (infectious and non-infectious) virions. Similar amounts of NA activity were present in WT and RWJ-R whole virus preparations which had been equalized by determining HA content by immunoassay; equalization by HA immunoassay was in agreement with quantitation of the preparations by determination of plaque forming units (data not shown). The RWJ-270201 IC_{50} mean values (from two independent assays) of WT and RWJ-R viruses were 0.47 ± 0.11 and 0.45 ± 0.08 nM, respectively. The dose–response curves of inhibition of WT and RWJ-R NAs were nearly the same (Fig. 2), indicating that the NAs of RWJ-R and WT viruses had the same sensitivity to RWJ-270201.

A fetuin-binding assay was used to compare HA binding of WT and RWJ-R viruses following pre-incubation temperatures (for 1 h) of 0, 37, 45, 58, and 62 °C. The RWJ-R virus binding to fetuin was approximately $60 \pm 6\%$ that of the WT virus regardless of the pre-incubation condition.

3.3. Animal infection studies

Because drug-resistant mutations often lead to viruses with reduced virulence in animals (McKimm-Breschkin, 2000), we investigated the infectivities of RWJ-R and WT viruses in mice. Table 2 shows comparative lethality titrations of the two viruses. Based on LD_{50} values, the RWJ-R virus proved to be about tenfold less virulent than the WT virus in these animals. Mice infected with the WT virus died much more rapidly than did animals infected with the resistant virus in this particular experiment. Higher virus titers were recovered from the lungs of mice infected with the WT virus (data not shown), correlating with greater disease severity.

In a follow-up study, weanling mice were infected with the RWJ-R virus and treated with RWJ-270201 to determine if the compound would show protective activity (Table 3). Doses of 100, 10, and 1 mg/kg/day caused statistically significant increases in numbers of survivors. The decline in SaO_2 values in the three treated groups was significantly less compared to those of the placebo control on days 4–10 of the infection (data shown only for day 6). Complete protection from mortality was afforded by the 100 mg/kg/day dose. Thus, RWJ-270201 was able to provide substantial protection to mice from a lethal infection caused by this virus which is resistant to the drug in tissue culture. We previously reported that infections with the WT virus A/Shangdong/09/93 (H3N2) virus could be effectively treated with RWJ-270201 (Sidwell et al., 2001).
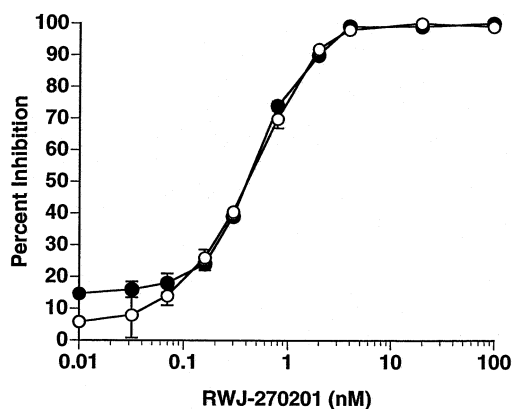

Fig. 2. Inhibition by RWJ-270201 of influenza A/Shangdong/09/93 (H3N2) neuraminidase of WT (●) and RWJ-R (○) viruses. Data represent mean values ± standard deviations (three replicate samples).

Table 2
Comparison of virulence of a wild-type (WT) and an RWJ-270201-resistant (RWJ-R) influenza A/Shangdong/09/93 (H3N2) viruses in 18–21 g BALB/c mice

Virus description	$\log_{10}$ Infecting virus dose (CCID$_{50}$)/mouse	Survivors/total	MDD[a] $\pm$ SD	LD$_{50}$ ($\log_{10}$ CCID$_{50}$/mouse)
WT	7.0	0/10	3.9 ± 0.6	
	6.5	0/10	4.1 ± 1.3	
	6.0	1/10	6.2 ± 2.0	5.5
	5.5	5/10	10.2 ± 2.1	
	5.0	6/10	9.5 ± 1.9	
	4.5	7/10	10.7 ± 1.9	
RWJ-R	7.0	2/10	8.3 ± 3.1***	
	6.5	4/10	11.7 ± 3.0***	
	6.0	8/10**	10.0 ± 0.0***	6.4
	5.5	10/10*	$>21.0 \pm 0.0$	
	5.0	10/10	$>21.0 \pm 0.0$	
	4.5	10/10	$>21.0 \pm 0.0$	

*$P<0.05$; **$P<0.01$; ***$P<0.001$. Statistical comparison made between respective WT and RWJ-R virus infection groups.
[a] Mean day of death of mice dying prior to day 21.

4. Discussion

The influenza A Shangdong/09/93 (H3N2) virus that was derived by cell culture passage in the presence of RWJ-270201 was found to be highly resistant to the compound and to the two other NA inhibitors, zanamivir and oseltamivir carboxylate. Virus yield reduction assays confirmed that RWJ-270201 had a reduced ability to inhibit extracellular virus production of the resistant compared to the WT virus. As was reported previously for the WT virus (Smee et al., 2001), cell associated virus titers tended to increase as extracellular virus release was inhibited by high concentrations of the neuraminidase inhibitor. This effect applied to both WT and RWJ-R viruses. Thus, RWJ-270201 and other neuraminidase inhibitors do not appear to inhibit overall virus production, but only affect whether viruses remain cell-associated or are released from the cell surface.

The RWJ-R virus contains a single point mutation in the HA gene (Lys189Glu), and no mutation in the NA gene compared to the WT virus. Position 189 has been identified as being proximal to the sialic acid binding site of HA (Weis et al., 1988). We hypothesize that, in tissue culture, the lysine to glutamic acid change weakens binding of HA to sialic acid, thus reducing the necessity for the viral NA to cleave sialic acid residues to release nascent virus from the surface of the infected cells. In support of this, the RWJ-R virus exhibited reduced binding to fetuin compared to WT virus. Although the reduction in binding to fetuin was moderate (about 40%), the binding of these same viruses to MDCK cells could not be studied since virus would be internalized. Other investigators have used an erythrocyte hemagglutination assay to show that certain HA mutations led to HA instability (McKimm-Breschkin et al., 1996), whereas the HA of the RWJ-R virus was as heat-stable as the HA of the WT virus.

The NA activity of the resistant virus was nearly identical to that of the wild-type virus. This further implicates the HA as the protein responsible for resistance to RWJ-270201. We cannot rule out the possibility that other mutations occurred elsewhere in the virus that may have contributed to drug resistance, but focusing on the NA and HA was a

Table 3
Effect of oral treatment with RWJ-270201 on an infection in 8–10 g BALB/c mice induced by a mutant influenza A/Shangdong/09/93 (H3N2) virus (RWJ-R) displaying in vitro resistance to RWJ-270201

Compound	Dose[a] (mg/kg/day)	Infected, treated Mice		
		Survivors/total	MDD[b] ± SD	Mean SaO$_2$[c] (% ± SD)
RWJ-270201	100	9/9***	>21.0 ± 0.0	86 + 1***
	10	7/10***	4.6 ± 0.6	82 ± 5**
	1	6/10**	7.3 ± 4.4	83 ± 5***
Saline	–	1/19	5.9 ± 3.3	77 ± 4
Uninfected controls	–	5/5	>21.0 ± 0.0	87 ± 2

*$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to saline controls.
[a] Twice daily for 5 days starting 4 h pre-virus exposure.
[b] Mean day of death of mice dying prior to day 21.
[c] Arterial oxygen saturation level, determined on day 6 of the infection.

reasonable approach. The most definitive way to prove that a particular mutation is responsible for drug resistance would be to insert the particular mutation into a WT virus and demonstrate resistance. We are not aware of such studies with HA mutations which were identified by resistance to NA inhibitors.

Because the resistant virus isolated from these studies was found to possess an HA mutation but no NA mutation, this does not mean that NA mutations cannot be derived by cell culture selection methods with this compound. Indeed, we have been working on other mutant viruses, some having both HA and NA mutations (P.C. Wagaman, unpublished). Longer periods of time of viral exposure to RWJ-270201 in cell culture appear to be necessary to derive these NA mutations.

The RWJ-R virus was about tenfold less virulent than the wild-type virus in mice. Thus, it is one of a number of influenza virus mutants that exhibits reduced virulence in vivo (McKimm-Breschkin, 2000). The mutation in HA may reduce the ability of the virus to infect cells of the mouse pulmonary tract, resulting in a less robust infection.

Mice infected with the RWJ-R virus can still be effectively treated with RWJ-270201. This indicates that in mouse lungs, the NA is important for the release of virus from cells, whereas NA is not as important for the spread of virus in cell culture if a compensatory mutation (such as the purported HA Lys189Glu mutation) is present. The HA

receptor in mice differs from that of MDCK cells (McKimm-Breschkin, 2000), suggesting that HA binding in vivo is tighter than it is in cell culture, thus requiring the activity of NA for cleavage of sialic acid and subsequent release of nascent virions from the infected cells. Thus, the HA mutation described here may be important to confer resistance to NA inhibitors in cell culture but does not lead to reduced drug efficacy in mice.

It has been difficult to isolate viruses resistant to NA inhibitors in humans (Gubareva et al., 1998; Covington et al., 1999). The recovered viruses have always been shown to be altered in their NA protein. No drug-resistant viruses have been isolated to date from in vivo sources that contained solely a HA mutation. Efforts to isolate RWJ-270201 resistant virus from mice, and to ascertain whether resistance arises in humans, are ongoing.

Laboratory Animal Resources, National Research Council (National Institutes of Health Publication No. 86-23, revised 1985), and used facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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