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In Vitro Antiviral Activity of Favipiravir (T-705) against Drug-Resistant Influenza and 2009 A(H1N1) Viruses[▽]

Katrina Sleeman,[1] Vasiliy P. Mishin,[1] Varough M. Deyde,[1] Yousuke Furuta,[2] Alexander I. Klimov,[1] and Larisa V. Gubareva[1]*

Virus Surveillance and Diagnosis Branch, Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia,[1] and Toyama Chemical Co., Ltd., Tokyo, Japan[2]

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Favipiravir (T-705) has previously been shown to have a potent antiviral effect against influenza virus and some other RNA viruses in both cell culture and in animal models. Currently, favipiravir is undergoing clinical evaluation for the treatment of influenza A and B virus infections. In this study, favipiravir was evaluated *in vitro* for its ability to inhibit the replication of a representative panel of seasonal influenza viruses, the 2009 A(H1N1) strains, and animal viruses with pandemic (pdm) potential (swine triple reassortants, H2N2, H4N2, avian H7N2, and avian H5N1), including viruses which are resistant to the currently licensed anti-influenza drugs. All viruses were tested in a plaque reduction assay with MDCK cells, and a subset was also tested in both yield reduction and focus inhibition (FI) assays. For the majority of viruses tested, favipiravir significantly inhibited plaque formation at 3.2 μM (0.5 μg/ml) (50% effective concentrations [EC_{50}s] of 0.19 to 22.48 μM and 0.03 to 3.53 μg/ml), and for all viruses, with the exception of a single dually resistant 2009 A(H1N1) virus, complete inhibition of plaque formation was seen at 3.2 μM (0.5 μg/ml). Due to the 2009 pandemic and increased drug resistance in circulating seasonal influenza viruses, there is an urgent need for new drugs which target influenza. This study demonstrates that favipiravir inhibits *in vitro* replication of a wide range of influenza viruses, including those resistant to currently available drugs.

In the United States alone, seasonal influenza is responsible annually for infecting between 5 and 20% of the American population, resulting in more than 200,000 hospitalizations and 36,000 deaths (8). Globally, seasonal influenza causes between 250,000 and 500,000 deaths every year (60). Influenza is not only a disease of great medical importance but also of economic importance. Despite available vaccines, a recent study predicted that in the United States influenza results in direct medical costs of the order of $10.4 billion each year, with the total economic burden for the United States being projected at $87.1 billion each year (44). It is widely accepted that vaccination remains the most effective approach for the prevention of viral infections (48). Although there is a safe and effective annual trivalent influenza vaccine, a large proportion of the global population does not receive the yearly influenza vaccine. This can be due to a variety of reasons, including the lack of access to adequate health care, unavailability of vaccine supply, allergies, and adverse reactions. During the 2009 pandemic (pdm), in addition to the vaccination and epidemiological control measures being exerted by health care officials, antivirals targeting influenza offer an essential tool in treating infected patients, in addition to protecting those at high risk of infection, such as the young, elderly, and health care workers.

Currently, there are two classes of anti-influenza drugs licensed in the United States for use in the treatment and management of influenza infections in humans: M2 ion channel blockers (also known as adamantanes) and neuraminidase (NA) inhibitors (NAIs) (30). Influenza antivirals are highly effective in the treatment of influenza infections if used promptly following the onset of symptoms or following exposure (45, 46). Both the M2 blockers amantadine and rimantadine are taken by the patient orally (45). However, of the two available NAIs, only oseltamivir is available as an oral formulation (zanamivir has to be inhaled [14, 53]), although other routes of administration have been investigated (31). The use of the M2 blockers amantadine and rimantadine is limited due to the rapid emergence of transmissible drug-resistant mutant viruses and the fact that they offer protection only against influenza A virus infections (32). The high prevalence of adamantane resistance in seasonal A(H3N2) viruses and oseltamivir resistance in seasonal A(H1N1) viruses is reflected in the CDC recommendations for the use of influenza antivirals (6).

The majority of adamantane-resistant A(H3N2) and A(H1N1) viruses circulating globally in recent years share the same mutation, S31N, in the M2 protein (20), although other resistance-conferring mutations have been detected also (including A30T, L26F, and V27A) (20, 49). The globally spread oseltamivir-resistant seasonal A(H1N1) viruses share the same mutation, H275Y (H274Y in N2 subtype amino acid numbering), in the drug-targeted enzyme neuraminidase, although other mutations are known to cause reduced susceptibility *in vitro* (19, 47, 50).

Seasonal A(H1N1) viruses resistant to both the adamantanes and the NAI oseltamivir have previously been reported, without an apparent link to treatment (12, 50). Currently, zanamivir is the only drug effective against both adamantane-resistant and/or oseltamivir-resistant influenza viruses, but due to the fact that it has to be inhaled, it is less suitable for use

* Corresponding author. Mailing address: Virus Surveillance and Diagnosis Branch, Influenza Division, National Center of Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, MS G16, 1600 Clifton Road, Atlanta, GA 30329-4018. Phone: (404) 639-3204. Fax: (404) 639-0080. E-mail: LGubareva@cdc.gov.

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with several high-risk groups, including the severely ill (41), infants (33), and the elderly (22). Furthermore, zanamivir may decrease pulmonary function, so it is not recommended for the treatment of infections in individuals with chronic underlying lung and heart disease conditions (23).

Since 1997, there have been several outbreaks of highly pathogenic avian influenza A(H5N1) infections in poultry, with a substantial number of infections occurring in humans (1). The overall case fatality of A(H5N1) infections in humans is over 60% and, unlike seasonal influenza, is most deadly in the young and healthy (ages 10 to 19 years) (59). Oseltamivir is the medication of choice for treating individuals infected with A(H5N1) (17). However, resistance in A(H5N1) viruses has been detected following the treatment of patients with oseltamivir (18, 38). In addition, naturally occurring reduced susceptibility to oseltamivir (35, 40) and possibly to zanamivir (29) has been documented for circulating A(H5N1) viruses, including novel mutations in the NA (29, 35). Adamantane resistance is widely spread among A(H5N1) viruses that carry mutations at amino acid residues 26, 27, and 31 in the M2 protein (13, 35) and among swine viruses circulating in Eurasia (27).

In April 2009, a novel reassortant A(H1N1) virus was first identified as circulating in humans in both Mexico and the United States (7, 9). Since April, the virus has continued to transmit among humans, and on 11 June 2009 the World Health Organization classified the outbreak as the first influenza pandemic of the 21st century (58). The 2009 A(H1N1) pandemic viruses consist of a unique combination of gene segments, including those of the North American (triple reassortants) and Eurasian swine lineages (27, 54). The 2009 A(H1N1) pandemic viruses are resistant to the adamantanes and sensitive to the NAIs (3, 16). Yet, concerns exist about the possibility of acquisition of resistance to the NAI oseltamivir, since the majority of A(H1N1) viruses which have been circulating predominantly worldwide during the 2008–2009 influenza season are oseltamivir resistant due to the resistance-conferring H275Y mutation in the NA. Such an acquisition of resistance by the 2009 A(H1N1) pandemic viruses would be a major setback and would further limit the already sparse therapeutic options (15, 57). There have been laboratory-confirmed cases of oseltamivir-resistant 2009 A(H1N1) pandemic viruses (each carrying the H275Y resistance-conferring mutation in the NA) in the United States (5).

Collectively, these recent findings emphasize not only the need for new effective antivirals to control and treat influenza infections but also the need to identify new molecular targets (47).

One such compound which is currently being investigated and undergoing clinical trials for the treatment of influenza infections is favipiravir (T-705), a pyrazine derivative (2, 26, 31). Favipiravir targets the RNA-dependent RNA polymerase (RdRp), a component of influenza virus different from that of currently licensed influenza antivirals (24, 25). It was shown that favipiravir can inhibit the viral replication of influenza type A, B, and C viruses (24, 25, 55). Favipiravir reduces influenza virus replication by selectively inhibiting the viral RdRp, since it does not affect the synthesis of host cellular DNA and RNA (25). Favipiravir has also shown great potential to act as a broad-spectrum antiviral against many RNA viruses, as reviewed by Furuta and coworkers (26).

The purpose of this study was to evaluate the ability, *in vitro*, of favipiravir to inhibit the viral replication of contemporary influenza viruses as well as viruses with pandemic potential, including viruses resistant to the currently available and licensed anti-influenza drugs. In this report we demonstrate that favipiravir is a potent inhibitor of seasonal influenza A and B virus replication, including that of drug-resistant and drug-sensitive viruses. In addition, favipiravir was shown to effectively inhibit influenza A viruses of other antigenic subtypes, including A(H2N2), viruses of avian origin [A(H4N2), A(H7N2), and A(H5N1)], and viruses of swine origin [A(H1N1) and A(H1N2)], as well as the 2009 A(H1N1) pandemic viruses.

MATERIALS AND METHODS

Cells and viruses. MDCK cells from two sources (ATCC and Mill Hill, United Kingdom; a kind gift of Alan Hay) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (DMEM-S10), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco).

Influenza viruses were submitted to the World Health Organization Collaborating Center for Surveillance, Epidemiology, and Control of Influenza at the Centers for Disease Control and Prevention (CDC), Atlanta, GA, for antigenic and antiviral resistance surveillance. All viruses were propagated in MDCK cells prior to use in this study. A panel of previously characterized drug-resistant viruses and their drug-sensitive counterparts were also grown in MDCK cells and used as controls for a corresponding type and subtype of the NA (43). Following propagation in cell culture, the drug susceptibility profile of each virus was confirmed using both the NA-Star chemiluminescent neuraminidase inhibition assay (to determine the susceptibility of the viruses to zanamivir and oseltamivir) (50) and by using the pyrosequencing method to detect the presence of markers of drug resistance to either the neuraminidase inhibitors (19) and/or the adamantanes (20).

The A(H2N2), A(H4N2), and A(H7N2) influenza viruses used in this study were obtained from the influenza strain depository at the Influenza Division at the CDC in Atlanta, GA. H5N1 viruses used in this study, with the exception of the A/Vietnam/HN30408/2005 virus pair, were also submitted to the World Health Organization Collaborating Center for Surveillance, Epidemiology, and Control of Influenza at the CDC, Atlanta, GA, for virus surveillance. The A/Vietnam/HN30408/2005 virus, kindly provided by Q. M. Le (National Institute of Hygiene and Epidemiology, Hanoi, Vietnam) (38), was plaque purified at the CDC to separate the two clones, H274Y and N294S (N2 numbering), in the NA.

The viruses used in this study were selected to include reference virus strains resistant to FDA-approved drugs (43), a pool of community isolates of wild-type influenza viruses which were in circulation during the 2007–2008 (4) and 2008–2009 influenza seasons, and a set of oseltamivir-resistant A(H1N1) viruses also isolated during the past two influenza seasons. To address the emergent and reemergent zoonotic influenza viruses, A(H5N1) viruses isolated from both humans and birds, including drug-resistant variants (38), were incorporated into the study. Furthermore, A(H1N1) and A(H1N2) viruses of swine origin (triple reassortants) which were isolated from humans during 2007 and 2008 (51) were also tested against favipiravir in this study. Pandemic 2009 A(H1N1) viruses isolated in the United States and Mexico were also included.

Seasonal influenza viruses and avirulent avian viruses were tested in a biosafety level 2 (BSL2) laboratory. Experiments using A(H1N1) and A(H1N2) triple swine reassortant influenza viruses, in addition to the 2009 pandemic viruses, were performed in a BSL2-enhanced laboratory. All experiments using A(H2N2), A(H7N2), and highly virulent A(H5N1) influenza viruses were carried out in the biosafety level 3-enhanced laboratory.

Antiviral compounds. Favipiravir was kindly provided by Toyama Chemical Company, Ltd., Tokyo, Japan. Ribavirin was purchased from Sigma Chemical Co. Both compounds were dissolved in DMEM (Gibco) at the time of each experiment.

Plaque reduction assay. MDCK-ATCC cells were seeded in 6-well plates to form a confluent monolayer and infected with pretitrated dilutions of influenza virus (influenza virus stocks were previously titrated by plaque assay to determine the dilution of virus required to generate 15 to 45 plaques per well). Virus adsorption was carried out in the absence of either favipiravir or ribavirin (Sigma) for a period of 1 h at 37°C in an incubator with 5% CO_2. Following the 1-h incubation period, the viral inoculum was removed from the cell monolayer, and the cells were washed once with phosphate-buffered saline (PBS; Gibco), pH

7.2. A 2-ml 0.8% SeaKem LE agarose (Lonza) overlay in modified Eagle medium (MEM) containing the relevant concentration of favipiravir or ribavirin was then added to each well. Tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin was added at a concentration of 3 μg/ml to media to support the replication of seasonal viruses, A(H2N2), A(H4N2), and A(H7N2) viruses and at a concentration of 2 μg/ml to media to support the replication of 2009 pandemic A(H1N1) viruses. One set of wells received neither the favipiravir compound nor ribavirin. Plates were incubated for 3 days at 37°C in an incubator with 5% CO_2. Agar overlays were then removed from each well, and the cells were fixed with 70% ethanol for a period of 30 min at room temperature. Following fixation, the 70% ethanol was removed from the cells, and 1 ml of Gram crystal violet stain (BD) was added to each well for a period of 5 min at room temperature. The crystal violet stain was then removed from the wells, and cells were washed twice with PBS. Plaques were then visualized and counted. GraphPad Prism (San Diego, CA) version 5.0 was used for curve fitting and 50% effective concentration (EC_{50}) determination.

Focus inhibition (FI) assay. MDCK cells were seeded in flat-bottomed 96-well plates to form a confluent monolayer. Serial 10-fold dilutions of influenza virus were made starting with a 10^{-1} dilution of virus in DMEM (Gibco) supplemented with bovine albumin fraction V solution (Gibco), HEPES buffer solution (Gibco), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco). Thirty microliters of each virus dilution assayed (multiplicity of infection [MOI] of ~0.001, ~0.01, and ~0.1) was added to cells, and following virus adsorption for 1 h at 4°C, the unbound virus was removed. Cells were washed twice with PBS, and 100 μl of DMEM containing 2 μg/ml TPCK-treated trypsin (Sigma) and favipiravir at the desired concentration was added. After incubation for 24 h at 37°C, supernatants were harvested from each well and titrated on MDCK cells (see "Determination of virus titer in cell culture supernatant by focus formation assay (immunofluorescence)").

The monolayers were fixed with 50 μl per well of ice cold fixative (95% methanol and 5% glacial acetic acid) for 30 min at −20°C. Cells were then rinsed twice with PBS and incubated with 100 μl of blocking solution (PBS containing 1% bovine serum albumin [BSA]) for 15 min at room temperature. Blocking buffer was then removed, and the fixed cells were incubated overnight at 4°C with 30 μl per well of a combination of six murine monoclonal antibodies (MAbs) targeting the influenza A nucleoprotein (kind gift of Robert Webster, St. Jude Children's Research Hospital, Memphis, TN). Next, the MAbs were removed, the cells were rinsed twice with PBS, and 30 μl of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse secondary antibody (Sigma) was added to the cells and incubated for 1 h at room temperature. The secondary antibody was then removed, and cells were rinsed twice with PBS. To each well, 50 μl of blocking solution was added, the number of immunostained foci was counted in each well using an Axiovert 200 fluorescent microscope (Zeiss), and viral titers were calculated. As an alternative for the visualization of cells expressing influenza virus nucleoprotein antigen, following incubation with primary antibodies, cells were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Millipore) and then incubated for 30 min with precipitate-forming peroxidase substrate (True Blue; KPL).

Determination of virus titer in cell culture supernatant by focus formation assay (immunofluorescence). MDCK cells were seeded in 96-well flat-bottom plates to form a confluent monolayer. Serial 10-fold dilutions of supernatants containing influenza virus were made, starting with a 10^{-1} dilution of virus in DMEM (Gibco) supplemented with bovine albumin fraction V solution (Gibco), HEPES buffer solution (Gibco), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco). Cell monolayers were washed twice with PBS, and the virus was added for adsorption of 1 h at 4°C. Unbound virus was then removed from the cell monolayer. Cells were washed twice with PBS, and 100 μl of DMEM containing 2 μg/ml TPCK-treated trypsin (Sigma) was added. Plates were incubated at 37°C in an incubator with 5% CO_2 for 24 h, at which point the supernatants were discarded and the cells were fixed with 50 μl per well of ice cold fixative (95% methanol [Fisher Scientific] and 5% glacial acetic acid [Fisher Scientific]) for 30 min at −20°C. Fixed cell monolayers were then immunostained (as described in "Focus inhibition [FI] assay").

RESULTS

Assessment of the anti-influenza activity of favipiravir in a plaque reduction assay. The ability of favipiravir to inhibit the replication of influenza viruses was determined using a plaque reduction assay on MDCK-ATCC cells. A representative set of viruses ($n = 53$), including seasonal A(H3N2) and A(H1N1) influenza viruses and nonseasonal viruses, including A(H2N2), A(H4N2), A(H7N2), highly virulent avian A(H5N1), swine triple-reassortant viruses, and the 2009 A(H1N1) pandemic (pdm) strains were tested. Some of the tested viruses were previously reported as resistant to either M2 blockers and/or an NAI(s) (38, 43, 50), and some expressed reduced drug susceptibility in the NA inhibition assays (Tables 1, 2, and 3). Tables 1, 2, and 3 contain information on the molecular markers in M2 and NA for the viruses used in this study, which have previously been associated with resistance or reduced susceptibility to adamantanes, oseltamivir, and/or zanamivir. The levels of viral resistance to licensed anti-influenza drugs listed in Tables 1, 2, and 3 were assigned based on surveillance criteria and on our previously published 50% inhibitory concentrations (IC_{50}s) (50). In the plaque reduction assay experiments, the antiviral drug ribavirin was used as a positive control against a subset of the seasonal influenza viruses used in this study. Experiments were performed in duplicate three times on three separate occasions using fresh preparations of cells. EC_{50}s provided in the tables are averages obtained from three independently executed experiments performed in duplicate and in each table, standard deviations are reported.

Inhibition of the replication of seasonal influenza A and B viruses. A panel of 15 A(H1N1) viruses, including six carrying the most common oseltamivir resistance-conferring H275Y mutation (corresponds to H274Y in N2 numbering), were tested. Favipiravir completely inhibited plaque formation at concentrations of 15.9 μM (2.5 μg/ml), with the EC_{50}s ranging from 0.19 μM (0.03 μg/ml) to 5.03 μM (0.79 μg/ml) (Table 1). A dually resistant virus, A/Luhansk/18/2008, was shown to be sensitive to favipiravir, with an EC_{50} of 2.93 μM (0.46 μg/ml) (Table 1).

The EC_{50}s for the nine A(H3N2) viruses tested ranged from 0.45 μM (0.07 μg/ml) to 5.99 μM (0.94 μg/ml) (Table 1). The A/Bethesda/956/2006 virus, resistant to both oseltamivir and zanamivir, exhibited susceptibility to favipiravir, with an EC_{50} of 1.21 μM (0.19 μg/ml) (Table 1).

Eight seasonal influenza B viruses tested showed EC_{50}s ranging from 0.57 μM (0.09 μg/ml) to 5.3 μM (0.83 μg/ml) (Table 1). This pool of B viruses consisted of four viruses which are sensitive to an NAI(s) and four which are either resistant to an NAI(s) or which showed reduced drug susceptibility (R152K, D198N, E119A, and H274Y in NA; N2 numbering). As a control, a subset of the seasonal influenza viruses ($n = 7$) was tested in parallel using ribavirin and was shown to have EC_{50}s in the range of 4.92 to 46.7 μM (1.15 μg/ml to 11.39 μg/ml) (Table 2).

Inhibition of the replication of A(H5N1) viruses. Favipiravir was tested against highly virulent A(H5N1) viruses ($n = 6$) which were isolated from either humans or birds and which are representative of two different genetic clades, 1 and 2.3.4. All six viruses, including the isolate dually resistant to oseltamivir and the adamantanes, the A/Vietnam/HN30408/2005 H274Y variant, were susceptible to favipiravir, with EC_{50}s ranging from 1.27 μM (0.2 μg/ml) to 5.22 μM (0.82 μg/ml) (Table 3).

Inhibition of the replication of subtype H2N2, H4N2, and H7N2 influenza A viruses. In addition to seasonal and highly virulent A(H5N1) viruses, the susceptibility of influenza A viruses of other antigenic subtypes was also assessed. The A/Ann Arbor/6/1960 [A(H2N2)], the internal genes of which

TABLE 1. Testing of virus susceptibility to favipiravir in plaque reduction assay with MDCK cells[b]

Strain designation	Subtype	Adamantane phenotype	M2 mutation	Oseltamivir phenotype	Zanamivir phenotype	NA mutation[a]	Favipiravir EC_{50} ± SD (μM)
A/Georgia/17/2006	H1N1	S		S	S		1.46 ± 0.25
A/Georgia/20/2006	H1N1	S		R	S	**H274Y**	2.55 ± 0.06
A/California/27/2007	H1N1	S		S	S		3.63 ± 0.06
A/New Jersey/15/2007	H1N1	S		R	S	**H274Y**	4.90 ± 0.06
A/Ecuador/5179/2008	H1N1	S		S	S		2.48 ± 0.12
A/Santiago/5248/2008	H1N1	S		R	R	D198E	4.70 ± 0.06
A/Brazil/1067/2008	H1N1	S		S	S		1.85 ± 0.06
A/Brazil/1633/2008	H1N1	S		R	R	Q136K	0.83 ± 0.06
A/Luhansk/18/2008	H1N1	R	**G34E**	R	S	**H274Y**	2.93 ± 0.06
A/New York/34/2008	H1N1	S	L26I	S	S		0.19 ± 0.06
A/Washington/10/2008	H1N1	R	**S31N**	S	S		3.25 ± 0.19
A/Florida/21/2008	H1N1	S		R	S	**H274Y**	1.59 ± 0.06
A/Wisconsin/16/2008	H1N1	S		R	S	**H274Y**	2.87 ± 0.06
A/North Carolina/02/2009	H1N1	R	**S31N**	S	S		5.03 ± 0.06
A/Idaho/01/2009	H1N1	S		R	S	**H274Y**	2.93 ± 0.06
A/Wuhan/395/1995-like	H3N2	S		S	S		5.99 ± 0.06
A/Wuhan/395/1995-like	H3N2	S		R	S	**E119V**	5.41 ± 0.12
A/Bethesda/956/2006	H3N2	R	**S31N**	R	R	**R292K**	1.21 ± 0.06
A/Washington/01/2007	H3N2	R	**S31N**	S	S		4.46 ± 0.06
A/Texas/12/2007 (clone)	H3N2	R	**S31N**	R	S	E119I	3.95 ± 0.19
A/Texas/12/2007 (clone)	H3N2	R	**S31N**	R	S	**E119V**	5.22 ± 0.06
A/Florida/01/2009	H3N2	R	**S31N**	S	S		0.45 ± 0.06
A/New Hampshire/01/2009	H3N2	R	**S31N**	S	S		4.08 ± 0.06
A/Massachusetts/03/2009	H3N2	R	**S31N**	S	S		4.20 ± 0.06
B/Memphis/20/1996		R	N/A	S	S		1.21 ± 0.06
B/Memphis/20/1996		R	N/A	R	R	**R152K**	0.57 ± 0.06
B/Rochester/01/2001		R	N/A	S	S		1.40 ± 0.06
B/Rochester/01/2001		R	N/A	R	S	D198N	1.72 ± 0.06
B/New York/22/2008		R	N/A	S	S		5.30 ± 0.06
B/Illinois/03/2008		R	N/A	R	R	E119A	3.00 ± 0.19
B/Illinois/47/2005		R	N/A	S	S		4.01 ± 0.06
B/Michigan/20/2005		R	N/A	R	S	H274Y	5.03 ± 0.06

[a] N2 amino acid numbering.
[b] S, sensitive; R, resistant, based on surveillance criteria (50). Values reported represent means of experiments performed in duplicate on three separate occasions ± standard deviations. Boldface type, established resistance-conferring mutation.

are present in some live influenza vaccines, was shown to be highly susceptible to favipiravir (Table 3). A pair of the NAI-sensitive and NAI-resistant variants of A/turkey/MN/833/1980 [A(H4N2)] were shown to have EC_{50}s of 0.96 μM (0.15 μg/ml) and 0.89 μM (0.14 μg/ml), respectively (Table 3). In addition, two A(H7N2) virus isolates, from a bird and a human, were also susceptible, with EC_{50}s ranging from 1.53 μM (0.24 μg/ml) to 10.2 μM (1.6 μg/ml), respectively (Table 3).

Inhibition of the replication of swine triple-reassortant viruses and 2009 A(H1N1) pandemic viruses. Three triple-reassortant swine viruses isolated from humans in the United States during 2007 and 2008 showed EC_{50}s ranging from 0.83 μM (0.13 μg/ml) to 4.52 μM (0.71 μg/ml) (Table 3). These viruses were also sensitive to all four licensed anti-influenza drugs. In addition to these three triple-reassortant viruses, five 2009 A(H1N1) pandemic viruses isolated from infected people in the United States and Mexico from March to April of 2009 were tested and showed EC_{50}s ranging from 0.83 μM (0.13 μg/ml) to 1.97 μM (0.31 μg/ml) (Table 3). All five 2009 A(H1N1) pdm viruses tested are resistant to the adamantanes and sensitive to NAIs (16). Two 2009 A(H1N1) pdm viruses recently isolated from infected patients in the United States

TABLE 2. Comparison of virus susceptibilities to favipiravir and ribavirin in plaque reduction assays with MDCK cells[b]

Strain designation	Subtype	Adamantane phenotype	M2 mutation	Oseltamivir phenotype	Zanamivir phenotype	NA mutation[a]	Favipiravir EC_{50} ± SD (μM)	Ribavirin EC_{50} ± SD (μM)
A/Georgia/17/2006	H1N1	S		S	S		1.46 ± 0.02	4.92 ± 0.25
A/Georgia/20/2006	H1N1	S		R	S	**H274Y**	2.55 ± 0.02	19.67 ± 0.06
A/Washington/01/2007	H3N2	R	**S31N**	S	S		4.46 ± 0.01	35.6 ± 0.12
A/Texas/12/2007 (clone)	H3N2	R	**S31N**	R	S	**E119V**	5.22 ± 0.01	46.7 ± 0.06
A/Florida/01/2009	H3N2	R	**S31N**	S	S		0.45 ± 0.01	20.92 ± 0.06
B/Memphis/20/1996		R	N/A	S	S		1.21 ± 0.01	13.93 ± 0.06
B/Memphis/20/1996		R	N/A	R	R	**R152K**	0.57 ± 0.01	25.00 ± 0.12

[a] N2 amino acid numbering.
[b] S, sensitive; R, resistant, based on surveillance criteria (50). Values reported represent means of experiments performed in duplicate on three separate occasions ± standard deviations. Boldface type, established resistance-conferring mutation.

TABLE 3. Testing of 2009 A(H1N1) and animal virus susceptibility to favipiravir in plaque reduction assay with MDCK cells[b]

Strain designation	Subtype	Adamantane phenotype	M2 mutations	Oseltamivir phenotype	Zanamivir phenotype	NA mutation[a]	Favipiravir EC_{50} ± SD (μM)
A/South Dakota/03/2008	swH1N1	S		S	S		0.83 ± 0.06
A/Texas/14/2008	swH1N1	S	V27T, V28D	S	S		4.52 ± 0.06
A/Michigan/09/2007	swH1N2	S	V27I, V28D	S	S		2.23 ± 0.12
A/Mexico/4604/2009	H1N1pdm	R	V28I, **S31N**	S	S		1.21 ± 0.06
A/California/04/2009	H1N1pdm	R	V28I, **S31N**	S	S		1.97 ± 0.06
A/California/05/2009	H1N1pdm	R	V28I, **S31N**	S	S		0.83 ± 0.06
A/California/07/2009	H1N1pdm	R	V28I, **S31N**	S	S		1.40 ± 0.06
A/New York/18/2009	H1N1pdm	R	V28I, **S31N**	S	S		0.89 ± 0.06
A/Illinois/10/2009	H1N1pdm	R	V28I, **S31N**	R	S	**H274Y**	22.48 ± 0.06
A/Washington/29/2009	H1N1pdm	R	V28I, **S31N**	R	S	**H274Y**	6.62 ± 0.12
A/Ann Arbor/6/1960	H2N2	S		S	S		0.38 ± 0.12
A/turkey/Minnesota/833/1980	H4N2	S		S	S		0.96 ± 0.06
A/turkey/Minnesota/833/1980	H4N2	S		S	R	**E119G**	0.89 ± 0.06
A/duck/Vietnam/NCVD93/2007 clade 2.3.4	H5N1	S		S	S		1.59 ± 0.06
A/duck/Vietnam/NCVD94/2007 clade 2.3.4	H5N1	S		R	R	I117V	3.38 ± 0.06
A/chicken/Vietnam/NCVD103/2007 clade 2.3.4	H5N1	S		S	S	I222T	1.27 ± 0.12
A/Vietnam/1203/2004 clade 1	H5N1	R	L26I, **S31N**	S	S		5.22 ± 0.06
A/Vietnam/HN30408/2005 H274Y clade 1	H5N1	R	L26I, **S31N**	R	S	**H274Y**	4.14 ± 0.06
A/Vietnam/HN30408/2005 N294S clade 1	H5N1	R	L26I, **S31N**	R	R	N294S	1.341 ± 0.06
A/turkey/VA/4529/2002	H7N2	S		S	S		1.53 ± 0.12
A/New York/107/2003	H7N2	R	V28A, **S31N**	S	S		10.2 ± 0.44

[a] N2 amino acid numbering.
[b] S, sensitive; R, resistant, based on surveillance criteria (50). Values reported represent means of experiments performed in duplicate on three separate occasions ± standard deviations. Boldface type, established resistance-conferring mutation.

following treatment with oseltamivir were also tested in this assay. These two viruses are resistant to both the adamantanes and the NAI oseltamivir. Each of these two viruses remained susceptible to treatment with favipiravir, although the EC_{50}s were slightly elevated in this assay (6.62 and 22.48 μM [1.04 and 3.53 μg/ml]) compared to the oseltamivir-sensitive 2009 A(H1N1) pdm viruses which were tested in the same manner (Table 3).

Assessment of the anti-influenza activity of favipiravir in FI and viral yield reduction assays. To further assess the ability of favipiravir to inhibit viral replication, the focus inhibition (FI) assay was used. It allows the detection of virus spread by immunostaining the influenza virus NP antigen in infected cells. MDCK cells were infected with two 2009 pandemic viruses, isolated in March and April of 2009 in Mexico and the United States, which are resistant to the adamantanes but sensitive to both oseltamivir and zanamivir. In addition, two 2009 pandemic viruses recently isolated from patients in the United States following treatment with oseltamivir were also tested. These two viruses are resistant to both the adamantanes and the NAI oseltamivir. Favipiravir at 128.0 μM (20 μg/ml) was shown to completely inhibit focus formation for all four viruses tested, at all three MOI assayed (data not shown). At an MOI of 0.001, favipiravir at concentrations as low as 1.6 μM (0.25 μg/ml) greatly inhibited focus formation. With a higher MOI, a greater concentration of favipiravir was required to inhibit virus replication (data not shown). Thus, a dose-dependent response was observed between the amount of favipiravir used to treat the cells during the course of infection and the amount of viral input.

Favipiravir was also assessed for its ability to inhibit the yield of infectious virus in cell culture supernatants. These experiments were performed on two separate occasions in quadruplicate. For this purpose, MDCK cells from two different sources were used, and virus yields were determined following treatment with favipiravir for a period of 24 h. The experiments described here were performed mainly using a low-passage MDCK-ATCC cell line. However, it has been shown that results for antiviral testing on different MDCK cell lines with different receptor repertoires can result in a substantial difference of any observed inhibitory effects against the virus (39). To determine if such an effect was observed using the compound favipiravir, a subset of the newly isolated 2009 pandemic viruses from Mexico and the United States were tested using MDCK-Mill Hill cells (courtesy of Alan Hay, United Kingdom) in parallel with the MDCK-ATCC cells. Favipiravir was shown to have a more potent inhibitory effect when screened using MDCK-ATCC cells compared to MDCK-Mill Hill cells, for all viruses tested, with the exception of A/Illinois/10/2009 (Table 4). Against the A/California/04/2009 oseltamivir-sensitive virus using MDCK-ATCC cells, favipiravir was shown to reduce the viral titer by more than one $\log_{10}$ at a concentration as low as 1.6 μM (0.25 μg/ml), and at a concentration of 14.2 μM (2.22 μg/ml), virus yield was undetectable (Table 4). Against the same virus, but using MDCK-Mill Hill cells, favipiravir reduced viral output to undetectable levels following treatment at a 3-fold higher concentration, 42.7 μM (6.67 μg/ml). Similarly, favipiravir was shown to have a more potent effect when used in the MDCK-ATCC cell line (Table 4) with the second oseltamivir-sensitive pandemic virus A/Mexico/4604/2009. The A/California/04/2009 virus was slightly more susceptible to favipiravir in both cell lines compared to the A/Mexico/4604/2009. The two H275Y oseltamivir-resistant H1N1 pdm viruses were slightly less susceptible to favipiravir compared to the oseltamivir-sensitive viruses (Table 4).

Against three of the four viruses and in both cell lines tested, favipiravir was shown to completely inhibit virus growth at 128.0 μM (20 μg/ml). For the A/Washington/29/2009 H275Y

TABLE 4. Reduction of infectious virus yield in MDCK cells infected with the pandemic 2009 A(H1N1) influenza viruses in the presence of favipiravir[a]

2009 H1N1 virus	Strain or NA sequence[b]	MDCK source	Viral yield (log_{10} FFU/well) in the presence of favipiravir (μM)[c]						Mean EC_{90} ± SD (μM)
			0.0	1.6	4.7	14.2	42.7	128.0	
A/California/04/2009	WT	ATCC	4.3 ± 0.2	2.7 ± 0.3	1.8 ± 0.8	<1*	<1	<1	0.48 ± 0.06
A/Mexico/4604/2009	WT	ATCC	6.2 ± 0.1	4.4 ± 0.1	3.5 ± 0.2	2.4 ± 0.3	1.8 ± 0.4	<1	0.35 ± 0.06
A/Washington/29/2009	H275Y	ATCC	5.7 ± 0.3	4.2 ± 0.1	3.9 ± 0.1	2.2 ± 0.4	1.8 ± 0.5	1**	0.78 ± 0.13
A/Illinois/10/2009	H275Y	ATCC	5.3 ± 0.4	4.9 ± 0.8	4.4 ± 0.6	3.1 ± 0.2	2.1 ± 0.3	<1	4.21 ± 0.38
A/California/04/2009	WT	Mill Hill	4.5 ± 0.7	4.1 ± 0.1	3.6 ± 0.2	2.3 ± 0.1	<1	<1	3.75 ± 0.26
A/Mexico/4604/2009	WT	Mill Hill	6.4 ± 0.1	6.2 ± 0.1	6.2 ± 0.2	4.6 ± 0.2	2.1 ± 0.2	<1	36.16 ± 1.02
A/Washington/29/2009	H275Y	Mill Hill	6.0 ± 0.3	5.4 ± 0.4	4.7 ± 0.5	3.1 ± 0.2	1.5 ± 0.4	<1	6.32 ± 0.58
A/Illinois/10/2009	H275Y	Mill Hill	4.6 ± 0.5	4.5 ± 0.3	3.8 ± 0.2	1.8 ± 0.2	1.7**	<1	5.03 ± 0.70

[a] MDCK cells from two sources (ATCC and Mill Hill, United Kingdom) were infected with either A/California/04/2009, A/Mexico/4604/2009, A/Washington/29/2009, or A/Illinois/10/2009 A(H1N1) 2009 pandemic viruses at an MOI of ~0.01. At 24 h postinfection, the cell culture supernatants (media above the cell monolayers) were collected from individual wells and titrated using the focus inhibition assay (see Materials and Methods). The infectious viral titers were expressed as log_{10} focus forming units (FFU) per well (100 μl).

[b] WT, wild type; H275Y, a mutation known to confer resistance to oseltamivir.

[c] Values represent mean of quadruplicate experiments ± standard deviation. *, below the limit of detection (<10 FFU per 100 μl of supernatant); **, virus was detected in one well of four.

oseltamivir-resistant virus, a single well was found to be positive for virus replication in the presence of favipiravir at 128.0 μM (20 μg/ml) using the MDCK-ATCC cells; when using the MDCK-Mill Hill cells, no detectable virus was observed in the presence of favipiravir at 128.0 μM (20 μg/ml).

DISCUSSION

The ongoing influenza pandemic necessitates the development of new measures to reduce the impact on human health of the 2009 A(H1N1) pdm strain. While these circulating pandemic viruses are resistant to the adamantanes, they remain susceptible to the NAI class of influenza antivirals (3, 16). However, it is plausible that the pandemic viruses could acquire resistance to NAIs in the future. Rare cases of oseltamivir resistance have been reported in patients infected with the 2009 A(H1N1) pdm strain following exposure to oseltamivir (10, 11, 61).

In the present study we tested the susceptibility of both oseltamivir-sensitive and oseltamivir-resistant 2009 pandemic viruses, which were isolated from patients in the United States and Mexico, to favipiravir. These viruses were tested using a combination of plaque reduction, focus inhibition, and viral yield reduction assays with MDCK cells and were shown to be potently inhibited by favipiravir. It is interesting to note that the potency of favipiravir varied, depending upon which of the two MDCK cell lines were used in this assay. For each of the 2009 pandemic viruses tested, with the exception of A/Illinois/10/2009, favipiravir was found to inhibit viral infection more effectively when used to treat infections in MDCK-ATCC cells. The A/Illinois/10/2009 virus has a passage history different than that of the other pandemic viruses used in this study and was passaged an additional two times in MDCK-ATCC cells in order to gain a virus of high hemagglutination (HA) titer. Therefore, cell selection could play a role in the elevated EC_{50} seen with this virus compared to the other 2009 A(H1N1) pandemic viruses. The observation that favipiravir appears to be more efficacious in MDCK-ATCC cells compared to MDCK-Mill Hill cells could be due to a difference in the rates of virus replication and viral spread, although at this time, other factors cannot be ruled out. It is possible that the two cell lines differ in their receptor repertoires or receptor densities, and this may potentially account for this observation. The varying efficacy of favipiravir in the two cell lines could also be due to differing ratios of conversion of the inactive form of favipiravir into its active ribofuranosyltriphosphate form by the cellular enzymes of each of the two cell lines (26, 52). It could be useful to investigate the antiviral potency of favipiravir against the pandemic viruses using human primary respiratory epithelium culture.

Since 2005 there have been sporadic cases of human infections with swine A(H1N1) and A(H1N2) viruses in the United States (51), and human infection with swine influenza has become a nationally notifiable disease (51). Favipiravir targets the influenza virus polymerase complex (PB1, PB2, and PA proteins). Phylogenetic analyses have revealed that the PB2 and PA genes of the 2009 H1N1 pdm viruses, as well as those of the swine triple-reassortant viruses recovered from humans prior to 2009, originated from avian viruses that crossed the species barrier and entered the swine population around 1998. In contrast, the PB1 gene originated from human A(H3N2) influenza viruses and entered swine at approximately the same time (27, 54). In our study, favipiravir potently inhibited the plaque formation of the viruses sharing this distinctive constellation of the polymerase genes. Our data are in accord with the published results on *in vitro* susceptibility of the 2009 pandemic viruses (36).

In this study, in addition to showing the potent activity of favipiravir against viruses of swine origin and novel pandemic viruses, we also demonstrate that favipiravir is highly effective among multiple strains of drug-sensitive and drug-resistant seasonal influenza isolates as well as A(H5N1) viruses of avian origin and oseltamivir-resistant pandemic viruses. Using plaque reduction assays with MDCK-ATCC cells, favipiravir was shown to potently inhibit this panel of contemporary influenza viruses, with EC_{50}s in the μg/ml range (expressed as μM in Tables 1, 2, and 3). Some of the oseltamivir-resistant viruses tested were also resistant to adamantanes. Although it would be ideal to provide a direct comparison of the potency of favipiravir in cell culture relative to that of the neuraminidase inhibitors, this is not possible, since cell culture-based assays

are not recommended for the assessment of drug susceptibility for this class of drugs (56). The *in vitro* favipiravir susceptibility of viruses resistant to currently licensed anti-influenza drugs is particularly encouraging at a time when the incidence of seasonal influenza viruses resistant to currently licensed influenza antiviral drugs is high (12, 21, 28, 34, 37, 42, 47, 50). These recent developments in antiviral resistance have made treatment decisions for health care workers and physicians challenging (6). To date, either oseltamivir or zanamivir is recommended for the treatment of 2009 pandemic virus infections.

Our *in vitro* study further validates favipiravir as a highly effective broad spectrum antiviral with a mechanism of action different from that of the currently available anti-influenza drugs which target either the neuraminidase or M2 protein.

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