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ORIGINAL ARTICLE

In vitro activity of favipiravir and neuraminidase inhibitor combinations against oseltamivir-sensitive and oseltamivir-resistant pandemic influenza A (H1N1) virus

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Abstract Few anti-influenza drugs are licensed in the United States for the prevention and therapy of influenza A and B virus infections. This shortage, coupled with continuously emerging drug resistance, as detected through a global surveillance network, seriously limits our anti-influenza armamentarium. Combination therapy appears to offer several advantages over traditional monotherapy in not only delaying development of resistance but also potentially enhancing single antiviral activity. In the present study, we evaluated the antiviral drug susceptibilities of fourteen pandemic influenza A (H1N1) virus isolates in MDCK cells. In addition, we evaluated favipiravir (T-705), an investigational drug with a broad antiviral spectrum and a unique mode of action, alone and in dual combination with the neuraminidase inhibitors (NAIs) oseltamivir, peramivir, or zanamivir, against oseltamivir-sensitive pandemic influenza A/California/07/2009 (H1N1) and oseltamivir-resistant A/Hong Kong/2369/2009 (H1N1) virus. Mean inhibitory values showed that the tested virus isolates remained sensitive to commonly used antiviral drugs, with the exception of the Hong Kong virus isolate. Drug dose-response curves confirmed complete drug resistance to oseltamivir, partial sensitivity to peramivir, and retained susceptibility to zanamivir and favipiravir against the A/Hong Kong/2369/2009 virus. Three-dimensional analysis of drug interactions using the MacSynergy™ II program indicated an overall synergistic interaction when favipiravir was combined with the NAIs against the oseltamivir-sensitive influenza virus, and an additive effect against the oseltamivir-resistant virus. Although the clinical relevance of these drug combinations remains to be evaluated, results obtained from this study support the use of combination therapy with favipiravir and NAIs for treatment of human influenza virus infections.

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Introduction

Influenza is a highly contagious acute viral infection that has afflicted mankind for centuries [1]. It continues to be a substantial source of morbidity and mortality with an enormous financial and socioeconomic impact [2]. Vaccination strategies to reduce disease burden have been implemented but have to be updated regularly to account for antigenic changes of the viral glycoproteins. The influenza pandemic of 2009 also highlighted the challenges of timely vaccine production and availability at the global level [3]. Antivirals used for the treatment and chemoprophylaxis of influenza virus infections during seasonal epidemics and sporadic pandemics therefore fill an important gap.

Presently licensed anti-influenza drugs in the United States include the M2 ion channel inhibitors amantadine and rimantadine and the neuraminidase inhibitors oseltamivir and zanamivir [4]. The M2 ion channel inhibitors have been used widely since their approval, but limited effectiveness towards only influenza A viruses, adverse side effects [5], and emergence and widespread transmission of resistant strains have rendered the usefulness of this class of drugs questionable [6]. Accordingly, the U.S. Centers for Disease Control and Prevention currently only recommend the use of oseltamivir and zanamivir for the treatment and chemoprophylaxis of influenza virus infections [7].

Oseltamivir and zanamivir both target the influenza virus sialidase (also known as neuraminidase, NA), a viral glycoprotein involved in the release of nascent virions from the host cell and spread within the respiratory tract [8, 9]. These compounds represent a success story in the rational design of novel anti-influenza drugs, as they were modeled and synthesized to fit into the active site and to competitively inhibit the viral NA [10]. Clinical data confirmed that oseltamivir and zanamivir could reduce the duration of symptoms and the occurrence of complications in healthy adults when given early. In addition, both drugs are generally well tolerated [11–15]. As with the M2 ion channel inhibitors, the emergence of virus strains with reduced susceptibility, particularly to oseltamivir, has been a public health concern [16]. During the first three years of their use (1999-2002), only 0.33 % of more than 2,200 seasonal influenza A (H1N1) viruses tested showed a tenfold decrease in susceptibility to oseltamivir [17], which was attributed to a low level of naturally occurring resistant variants. However, oseltamivir-resistant seasonal influenza A (H1N1) viruses emerged on a large scale in Europe unexpectedly during the 2007-2008 season [18–20]. Genotyping identified a mutation in the NA gene (H274Y, H275Y in N1 numbering) that was previously associated with decreased susceptibility to oseltamivir [21]. Although the substitution of histidine by tyrosine at residue 274 of the NA protein was initially shown to lead to lower viral fitness [21], permissive mutations in the hemagglutinin are speculated to have facilitated the worldwide spread of variants carrying this mutation [22, 23]. Fortunately, pandemic influenza A (H1N1) 2009 viruses are still almost completely susceptible to the NA inhibitors [24, 25]. But again, oseltamivir-resistant variants have been detected. Most of the cases were sporadic, epidemiologically unlinked, and occurred during oseltamivir treatment or prophylaxis [26, 27], but resistant viruses were also isolated from patients without prior oseltamivir exposure [28]. A rise in the prevalence of infections with oseltamivir-resistant pandemic influenza A (H1N1) 2009 viruses in the US during the 2010-2011 season [29], coupled with reports of community clusters in Australia [30], Vietnam [31], and evidence of nosocomial transmission [32], are reason for concern and emphasize the constant need for new drug development.

New drug development is a long and challenging process. As an alternative, existent antivirals may be used in combination with the hope of greater potency and clinical efficacy as well as a delay in the development of antiviral resistance [33, 34]. Combination therapy is neither a novel idea nor is it limited to certain human pathogens. The use of multiple drugs with the ability to act on different viral targets has become a routine treatment plan for the management of human immunodeficiency virus infections [35]. In addition, pegylated interferon-α, when used in combination with ribavirin, has been shown to be effective and safe in the treatment of hepatitis C virus infections in children and adults [36]. Several *in vitro* and *in vivo* studies have also demonstrated the enhanced activity of anti-influenza drugs when used in combination [37–45], although the clinical relevance for most combinations remains to be determined.

Here, we first evaluated the antiviral susceptibilities of 14 pandemic influenza A (H1N1) 2009 viruses and then examined the potential benefit of combining the investigational anti-influenza drug favipiravir (T-705) – a viral RNA polymerase inhibitor [46] – with the NAIs oseltamivir, zanamivir, and peramivir. The viruses used for the combination treatments included the pandemic oseltamivir-sensitive influenza A/California/07/2009 (H1N1pdm) virus and the oseltamivir-resistant influenza A/Hong Kong/2369/2009 (H1N1pdm) virus. A/California/07/2009 (H1N1pdm)-like viruses have been included for the past three years in the trivalent influenza seasonal vaccine and are also recommended for use in the 2013-14 influenza season [47]. The Hong Kong variant is a naturally occurring pandemic virus carrying the NA H275Y marker and was isolated from a 16-year old girl without prior oseltamivir exposure [28]. Virus-infected MDCK cells were treated with dual combinations of antiviral drugs based on a checkerboard matrix involving permutations of serial dilutions for each of the drugs. Virus yield reduction data were then analyzed with the MacSynergy™ II software program [48] to describe and quantitate drug interactions and to determine any possible areas of synergy (or antagonism) between the drug combinations.

Materials and methods

Cells and media

Madin-Darby canine kidney (MDCK) cells, strain number CCL-34, were obtained from the American Type Culture Collection (Manassas, VA) and maintained at 37 °C and 5 % CO_2 in antibiotic-free cell culture medium (minimum essential medium with Earle's balanced salts and L-glutamine, MEM/EBSS) supplemented with 5 % fetal bovine serum (both from HyClone, Fisher Scientific, Logan, UT). The test medium consisted of MEM/EBSS supplemented with trypsin (10 U/ml), EDTA (1 μg/ml) and gentamicin (50 μg/ml).

Viruses

Each influenza (H1N1) virus was passaged one time in MDCK cells to create a viral stock and then titrated for

antiviral experiments. The A/Utah/475/2009, A/Utah/476/2009, and A/Utah/727/2009 viruses were isolated from pediatric patients at the University of Utah Medical Center, Salt Lake City, UT. The A/Auckland/3/2009 virus was provided by Dr. Angela Luttick, Biota Holdings Limited (Australia). The A/California/07/2009, A/New York/18/2009, A/Hong Kong/2369/2009 (oseltamivir-resistant), A/Mexico/4108/2009, and A/Texas/15/2009 viruses were obtained from the Centers for Disease Control and Prevention (CDC, Atlanta, GA). The A/Brownsville/34H/2009, A/Netherlands/134/2009 and A/Wisconsin/629-D02473/2009 viruses were ordered from BEI Resources (Manassas, VA). The mouse-adapted A/California/04/2009 virus was received from Dr. Daniel Perez, Department of Veterinary Medicine, University of Maryland, College Park, MD, and the non-adapted A/California/04/2009 was received from Dr. Elena Govorkova, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN.

Antiviral compounds

Oseltamivir carboxylate, the active form of oseltamivir, was provided by Dr. Jack Nguyen (Adamas Pharmaceuticals, Emeryville, CA). Peramivir was a gift from Dr. Y. S. Babu (BioCryst Pharmaceuticals, Inc., Birmingham, AL). Zanamivir was obtained from Haorui Pharma-Chem Inc. (Edison, NJ). Favipiravir was provided by Dr. Furuta (Toyama Chemical Co., Toyoma, Japan). Compounds were dissolved in cell culture medium for antiviral evaluation. Antiviral concentrations included 0.032, 0.1, 0.32, 1, 3.2, 10, 32 and 100 μM favipiravir, and 0.0032, 0.01, 0.032, 0.1, 0.32 and 1 μM oseltamivir, zanamivir, or peramivir.

Antiviral studies in cell culture

The antiviral activity of each drug or drug combination was determined in MDCK cells. Effective antiviral concentrations (EC_{50} values) were computed based on the inhibition of virus-induced cytopathic effects (CPE) coupled with a neutral red dye uptake method [49]. Ninety-six-well plates were seeded with approximately 10^4 MDCK cells/well. Following overnight incubation, cells were infected with approximately 50 times the 50 % cell culture infectious dose ($CCID_{50}$) of virus. Microtiter plates were visually examined 3 days postinfection (dpi) and then treated for 2 h at 37 °C with neutral red (0.011 %) to quantify CPE. Excess dye was rinsed from cells with phosphate-buffered saline. The absorbed dye was eluted by addition of 0.1 ml of 50 % Sorensen's citrate buffer/50 % ethanol to each well. Optical density (OD) measurements were taken using a computerized microplate reader (SPECTRAmax® Plus^{384}, Molecular Devices Corporation, Sunnyvale, CA) with absorbance measurements at 560 nm. Optical density readings were converted to percent of uninfected control using an Excel spreadsheet by plotting CPE values versus $\log_{10}$ of drug concentration. Ninety percent virus-inhibitory concentrations (EC_{90} values) were determined based on virus yield reduction (VYR). MDCK cells were grown and infected as outlined above. Supernatants were collected 3 dpi when untreated control wells exhibited 100 % CPE, pooled from replicate wells, and stored at −80 °C until further processing. To quantify the virus produced in the wells, supernatants were thawed, centrifuged for 10 min at 4,000 rpm to pellet cell debris, and then titrated in new 96-well plates of confluent MDCK cells by tenfold serial dilution. Four wells were used per dilution. Plates were incubated for 3 days and then visually examined for appearance of virus-induced CPE. Wells were identified as plus or minus virus, and virus titers were calculated by the endpoint dilution method [49, 50]. EC_{90} values were computed by plotting $\log_{10}$ of viral titers versus $\log_{10}$ of drug concentration. The VYR assays completed in our laboratory have been designed to show peak CPE at 72 hours. Thus, minor antiviral effects remain undetectable by this method and may be underestimated when calculating the sums of effects for individual drugs. In addition, it is possible that the CPE observed at a single drug concentration could indicate a virus titer that is either rising to, or falling from the peak value. However, when considering CPE in VYR assays from studies involving multiple drug concentrations, we normally observe dose responsive effects that correlate with drug concentration. This is especially true when the VYR assays are completed in triplicate, which is essential for these types of cell-based assays. The limit of detection for the virus titration assays was ≤ 0.5 $\log_{10}$ $CCID_{50}$/0.1 ml.

Drug combination studies

Drug combination effects were determined by two methods. The first method identified possible regions of synergy in the VYR data based upon a standard definition of synergy as an antiviral effect of two drugs that is greater than the sum of the effects of the two drugs used independently; an additive effect is defined as a consistent decrease in virus titer demonstrated in replicate tests; antagonism is defined as a one $\log_{10}$ (or greater) increase in virus titer; an inhibitory effect is defined as a consistent increase in virus titer; and indifference is defined as no clear synergy or antagonism. In addition, three replicates were considered the minimum number of VYR tests required to characterize the effects of drug combination interactions. The second method determined the effects of drug combinations by the three-dimensional analysis (MacSynergy™ II) of Prichard and Shipman [48]. This software was kindly provided by Mark N. Prichard, University of Alabama, Birmingham,

AL. Briefly, data obtained from VYR assays were transformed to calculate interactions, assuming that each drug acts independently of the other (Bliss Independence [51]). The doses of two antiviral drugs used in a combination drug study represent independent variables. The effect that results from the drug combination is the dependent variable. Therefore, it is possible to represent the relationship for this combined antiviral activity as a three-dimensional (3-D) dose response surface. Analysis of the shape of the dose response surface can help in understanding any underlying drug interactions. The MacSynergy™ software was designed to identify regions of synergy or antagonism in the dose response surfaces determined by antiviral drug studies (52). The predicted additive interactions from a combination drug study are calculated from the dose response curves for each drug individually. This calculated dose response surface is then subtracted from the experimentally determined dose response surface of the drug combination to reveal regions of "non-additive activity". The resulting dose response surface would appear as a horizontal plane at 0 % change from the expected value if the interactions were only additive (52). In addition, the MacSynergy™ II program automatically quantifies the volumes of synergy (or antagonism) produced, expressed in μM^2 %, and statistically evaluates the interaction for significance. A broad classification system was proposed by Pritchard et al. in which values between 25 and 50 μM^2 % (at 95 % confidence) are considered to be minor but significant amounts of synergy, values of between 50 and 100 μM^2 % indicate moderate synergy, and values over 100 μM^2 % represent strong synergy [52]. Although this classification system has since been applied to *in vivo* data obtained from influenza virus infection studies in mice [44, 53], it was originally intended for *in vitro* data from virus yield reduction assays [52].

Statistical analysis

Mean EC_{50} values for the antiviral drugs tested against the influenza A (H1N1) viruses were analyzed by one-way (within group) or two-way (between groups) ANOVA followed by Bonferroni's multiple comparison tests using Prism 5.0f (GraphPad Software Inc., La Jolla, CA).

Results

Antiviral activity of favipiravir and neuraminidase inhibitors against pandemic influenza A (H1N1) viruses

Before testing antiviral drugs in combination, we evaluated the antiviral activity of each drug alone. Mean effective concentrations (EC_{50} and EC_{90} values) for the NAIs oseltamivir, peramivir and zanamivir, and the polymerase inhibitor favipiravir were determined in MDCK cells. Fourteen pandemic influenza A (H1N1pdm) viruses were tested, including the oseltamivir-sensitive influenza A/California/07/2009 (H1N1pdm) virus and the oseltamivir-resistant influenza A/Hong Kong/2369/2009 (H1N1pdm) virus with the NA H275Y point mutation. Results shown in Table 1 indicated a broad range of drug sensitivities. The mean EC_{50} values ranged from 0.2 to 1.1 μM for oseltamivir, from 0.004 to 0.13 μM for peramivir, and from 0.02 to 0.54 μM for zanamivir. Mean EC_{50} values for favipiravir (T-705) had a much smaller range, from 1.9 to 7.8 μM for all viruses. The values reported here fall into the range seen in the literature [49, 53, 54]. Notable exceptions in EC_{50} values were observed against the oseltamivir-resistant Hong Kong virus. EC_{50} and EC_{90} values of > 10 μM for oseltamivir indicated considerably reduced drug susceptibility compared to influenza viruses without the NA H275Y mutation. Peramivir was also not as effective against the Hong Kong virus (EC_{50} >10 μM, EC_{90} 4.48 μM), whereas drug susceptibility to zanamivir and favipiravir was unchanged (1.4 and 2.1 μM zanamivir, 7.8 and 10.8 μM favipiravir). As expected, none of the pandemic H1N1 viruses tested showed any susceptibility towards the M2 ion channel inhibitors amantadine and rimantadine (data not shown). It is important to note that MDCK cells did not exhibit any signs of cytotoxicity with any of these drugs (data not shown). These results were mirrored by the drug dose-response curves as presented in Fig. 1. The oseltamivir dose-response curve demonstrated a major shift in drug susceptibility for the drug-resistant Hong Kong virus compared to the drug-sensitive California virus, with an increase in EC_{90} from 0.32 to 14 μM (43.8-fold increase). The EC_{90} values for peramivir increased moderately from 0.48 to 4.5 μM (9.3-fold increase) against the drug-resistant virus, while zanamivir showed only a minor increase from 0.4 to 2.11 μM (5.3-fold increase). No significant differences in EC_{90} values for the two viruses were found for favipiravir.

Drug combination studies

To investigate drug potentiation effects on the inhibition of influenza virus replication in MDCK cells, serial dilutions of favipiravir were combined with serial dilutions of oseltamivir, peramivir, or zanamivir and tested against a representative oseltamivir-sensitive and an oseltamivir-resistant pandemic influenza A (H1N1) 2009 virus. Virus titers determined three days after drug exposure were used to characterize drug interactions by two methods, both assuming Bliss independence [51]: analysis of virus yield reduction (VYR) data, presented in Tables 2, 3, 4, 5, 6, 7, and three-dimensional analysis by MacSynergy™ II [48], shown in Fig. 2.

Table 1 *In vitro* antiviral efficacy of oseltamivir, peramivir, zanamivir, and favipiravir against 14 pandemic influenza A (H1N1) viruses

Virus	Oseltamivir		Peramivir		Zanamivir		Favipiravir	
	EC_{50}	EC_{90}	EC_{50}	EC_{90}	EC_{50}	EC_{90}	EC_{50}	EC_{90}
Auckland/3/2009 (Southern Hemisphere)	1.10 ± 0.74	3.90 ± 0.23	0.03 ± 0.01	0.13 ± 0.60	0.54 ± 0.45	0.73 ± 0.18	7.8 ± 1.9	9.30 ± 1.8
Brownsville/34H/2009	0.16 ± 0.02	0.18 ± 0.13	0.05 ± 0.04	0.06 ± 0.01	0.07 ± 0.01	0.11 ± 0.05	4.8 ± 2.8	4.10 ± 0.3
California/04/2009	0.12 ± 0.09	0.07 ± 0.00	0.04 ± 0.01	0.01 ± 0.00	0.02 ± 0.007	0.07 ± 0.01	2.7 ± 2.6	3.70 ± 1.4
California/04/2009 (Mouse-adapted)	0.30 ± 0.09	1.40 ± 0.50	0.11 ± 0.02	0.83 ± 0.25	0.30 ± 0.09	0.93 ± 0.11	6.8 ± 4.2	9.20 ± 0.6
California/07/2009 [b] (Oseltamivir-sensitive)	0.28 ± 0.12	0.32 ± 0.08	0.028 ± 0.04	0.48 ± 0.25	0.08 ± 0.004	0.40 ± 0.10	3.9 ± 0.1	4.10 ± 0.5
Hong Kong/2369/2009 [b] (Oseltamivir-resistant)	>10 [c]	>10	>10	4.48 ± 2.40	1.40 ± 1.20	2.10 ± 1.40	7.8 ± 0.0	10.8 ± 4.6
Netherlands/134/2009	0.07 ± 0.02	0.14 ± 0.01	0.02 ± 0.007	0.05 ± 0.01	0.06 ± 0.05	0.06 ± 0.01	3.5 ± 1.6	2.60 ± 0.5
New York/18/2009	0.76 ± 0.16	1.80 ± 0.30	0.06 ± 0.00	0.06 ± 0.06	0.09 ± 0.03	0.21 ± 0.16	4.3 ± 1.6	5.10 ± 0.6
Mexico/4108/2009	0.06 ± 0.06	0.14 ± 0.03	0.03 ± 0.004	0.06 ± 0.02	0.09 ± 0.09	0.86 ± 0.01	3.6 ± 1.1	0.81 ± 0.2
Utah/475/2009	0.20 ± 0.03	0.40 ± 0.05	0.024 ± 0.00	0.11 ± 0.09	0.12 ± 0.06	0.35 ± 0.08	1.9 ± 0.0	1.60 ± 0.7
Utah/476/2009	0.99 ± 0.45	1.24 ± 0.20	0.054 ± 0.04	0.20 ± 0.10	0.54 ± 0.23	0.73 ± 0.09	5.2 ± 3.2	6.10 ± 1.1
Utah/727/2009	0.68 ± 0.25	2.30 ± 0.90	0.013 ± 0.004	1.30 ± 0.23	0.12 ± 0.05	0.25 ± 0.07	4.3 ± 2.9	5.50 ± 4.1
Texas/15/2009	0.14 ± 0.02	0.22 ± 0.12	0.17 ± 0.04	0.09 ± 0.04	0.10 ± 0.02	0.14 ± 0.07	2.6 ± 1.9	1.20 ± 0.2
Wisconsin/629-D02473/2009	0.07 ± 0.02	0.18 ± 0.03	0.02 ± 0.01	0.06 ± 0.01	0.04 ± 0.04	0.17 ± 0.13	3.1 ± 2.2	4.30 ± 2.8

[a] Values are mean ± standard deviation from three independent experiments and are presented as 50 % and 90 % effective concentration (EC_{50} and EC_{90} in μM) for inhibition of virus-induced cytopathic effects in MDCK cells. EC_{50} and EC_{90} values were obtained by different assays

[b] These viruses were used for subsequent dual-combination drug studies

[c] Indicates the highest dose tested

When favipiravir was combined with oseltamivir, analysis of VYR data indicated a possible region of synergy – here defined as a one $\log_{10}$ or greater decrease in virus titer when compared to both individual drugs alone – ranging over four concentrations for both favipiravir (0.32-10 μM) and oseltamivir (0.0032-1 μM) against A/California/07/2009, and three concentrations for both favipiravir (1-10 μM) and oseltamivir (3.2-32 μM) against A/Hong Kong/2369/2009 (Tables 2, 5). The combination of favipiravir with peramivir produced a possible region of synergy ranging over four concentrations for favipiravir (0.32-10 μM) and even over five concentrations for peramivir (0.1-32 μM) against A/California/07/2009. When tested against the oseltamivir-resistant virus strain, however, possible regions of synergy covered only three concentrations for T-705 (1-10 μM) and two concentrations for peramivir (3.2-10 μM) (Tables 3, 6). VYR results for favipiravir plus zanamivir resembled those for favipiravir plus peramivir (Tables 4, 7). A possible region of synergy over three concentrations was observed for favipiravir (0.32-3.2 μM) and over six concentrations for zanamivir (0.0032-1 μM) and against the oseltamivir-sensitive virus strain. When cells were infected with the oseltamivir-resistant strain and treated with a combination of favipiravir and zanamivir, regions of synergy were 1-10 μM for favipiravir and 1-32 μM for zanamivir. No area of antagonism was observed with any of the combinations.

In addition, the virus titer datasets were evaluated for drug interactions by MacSynergy™ II [48]. Results are presented as three-dimensional bar graphs with bars above the null reference plane indicative of synergy and bars below indicative of antagonism, respectively. Surface volumes calculated from the combined volume of bar graphs above or below the reference plane (at 95 % confidence), respectively, are given to statistically evaluate the net effect of the drug combinations. Combinations of the polymerase inhibitor favipiravir with any of the three NAIs tested were found to be mostly synergistic against the oseltamivir-sensitive pandemic influenza A virus (Fig. 2a–c), as demonstrated by the bars above the null reference line with a volume of synergy between 28.6 and 35.1 μM^2 % and no statistically significant volume of antagonism (Table 8). Drug interactions against the oseltamivir-resistant pandemic virus, on the other hand, were generally characterized by a decrease in synergy in high concentration ranges and possibly minor antagonism in low concentration ranges, as indicated by the bars below the null reference line (Fig. 2d–f). This was reflected by lower

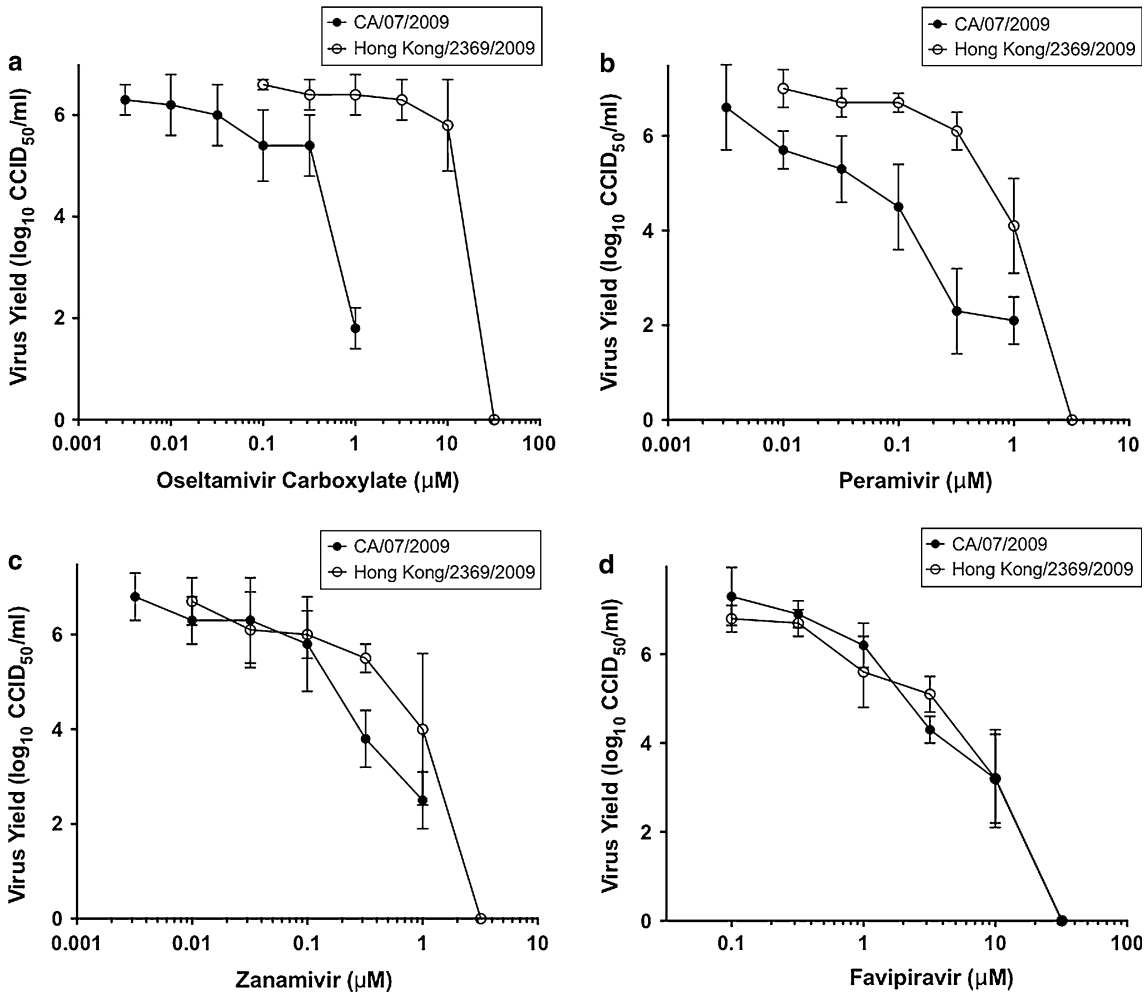

Fig. 1 Reduction in virus yield (dose-response curves) by neuraminidase inhibitors and favipiravir against pandemic influenza A viruses. MDCK cells were infected with oseltamivir-sensitive influenza A/CA/07/2009 virus or oseltamivir-resistant A/HK/2369/2009 virus and treated with serial dilutions of each antiviral drug. Virus yields were determined by neutral red dye uptake and expressed as $\log_{10}$ $CCID_{50}$/ml. Results are mean values ± standard deviation from three independent experiments. **a** Oseltamivir carboxylate, **b** peramivir, **c** zanamivir, **d** T-705

synergy volumes ranging from 11.6 to 12.8 μM^2 % (Table 8). Based on the classification system provided by Prichard and co-workers [52], the overall combination effect against the oseltamivir-resistant strain was additive, although certain drug combinations still resulted in a reduction in virus titer that was greater than tenfold higher than expected (Fig. 2d–f). Favipiravir concentrations above 32 μM did not increase the antiviral effect against either the oseltamivir-sensitive or oseltamivir-resistant virus strain. Results of both virus titer reduction analysis and interaction analysis by MacSynergy™ II are summarized in Table 8.

Discussion

Natural and drug-induced influenza virus variants with reduced susceptibility to currently licensed anti-influenza agents pose a great risk to the successful treatment of human influenza infections. Surveillance programs, such as the Global Neuraminidase Inhibitor Network, have been established to monitor these occurrences [17]. Our evaluation of 14 pandemic influenza A (H1N1) virus isolates from different regions of the world demonstrated that these isolates remained sensitive to commonly used antiviral drugs, with the exception of the Hong Kong isolate, which

Table 2 Antiviral activities of favipiravir-oseltamivir combinations against oseltamivir-sensitive influenza A/CA/07/2009 virus

Oseltamivir carboxylate (μM)[b]	Virus titer[a]								
	Favipiravir (μM)[c]								
	100	32	10	3.2	1	0.32	0.1	0.032	0
1	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
0.32	≤0.5	≤0.5	≤0.5	≤0.5	1.7±1.6	3.2±0.4	3.9±0.5	4.5	4.4±0.5
0.1	≤0.5	≤0.5	≤0.5	1.6±1.8	3.0±1.1	3.8±0.4	5.2±0.6	4.7	4.7±0.6
0.032	≤0.5	≤0.5	≤0.5	2.2±1.3	3.6±0.8	5.1±0.6	5.4±0.3	6.1±0.4	5.6±0.7
0.01	≤0.5	≤0.5	≤0.5	2.3±1.5	4.2±0.6	5.3±0.7	5.6±0.1	5.6±0.9	5.8±0.5
0.0032	≤0.5	≤0.5	1.3±1.3	2.7±1.0	4.3±0.7	5.9±0.6	6.1±0.3	5.9±0.6	6.0±0.5
0	≤0.5	≤0.5	2.2±1.1	3.3±0.3	5.2±0.5	5.9±0.4	6.3±0.7	6.0±0.4	6.2±0.1

Shaded area indicates possible range of synergy

[a] Values are mean $\log_{10}$ $CCID_{50}$/0.1 ml ± SD from three independent experiments, assay limit of detection ≤0.5 $\log_{10}$ $CCID_{50}$/0.1 ml

[b] Oseltamivir carboxylate EC_{90} = 0.17 μM

[c] Favipiravir EC_{90} = 1.0 μM

Table 3 Antiviral activities of favipiravir-peramivir combinations against oseltamivir-sensitive influenza A/CA/07/2009 virus

Peramivir (μM)[b]	Virus titer[a]								
	Favipiravir (μM)[c]								
	100	32	10	3.2	1	0.32	0.1	0.032	0
1	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	1.4±2.5	1.3±2.2	1.3±2.2	1.2±2.1
0.32	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	1.2±2.0	1.4±2.5	1.5±2.6	1.3±2.3
0.1	≤0.5	≤0.5	≤0.5	0.6±1.0	0.4±0.7	2.3±1.1	3.3±3.1	2.9±2.6	3.5±2.0
0.032	≤0.5	≤0.5	≤0.5	0.3±0.4	1.5±1.3	3.1±1.0	4.3±1.8	3.9±1.3	4.3±0.7
0.01	≤0.5	≤0.5	0.3±0.4	0.9±1.6	2.2±0.6	3.3±0.9	4.7±1.1	4.9±0.3	4.7±0.4
0.0032	≤0.5	≤0.5	≤0.5	1.7±1.5	2.5±1.1	4.1±0.6	5.1±1.3	5.4±0.9	5.6±0.9
0	≤0.5	≤0.5	1.2±1.3	2.6±1.0	5.2±0.3	5.3±0.6	5.7±0.5	5.8±0.3	5.8±0.6

Shaded area indicates possible range of synergy

[a] Values are mean $\log_{10}$ $CCID_{50}$/0.1 ml ± SD from three independent experiments, assay limit of detection ≤0.5 $\log_{10}$ $CCID_{50}$/0.1 ml

[b] Peramivir EC_{90} = 0.009 μM

[c] Favipiravir EC_{90} = 1.2 μM

Table 4 Antiviral activities of favipiravir-zanamivir combinations against oseltamivir-sensitive influenza A/CA/07/2009 virus

Zanamivir (μM)[b]	Virus titer[a]								
	Favipiravir (μM)[c]								
	100	32	10	3.2	1	0.32	0.1	0.032	0
1	≤0.5	≤0.5	0.3±0.4	≤0.5	0.8±1.3	1.1±1.9	1.2±2.0	1.3±2.2	1.5±2.6
0.32	≤0.5	≤0.5	0.3±0.4	≤0.5	1.4±1.8	2.0±1.8	2.8±2.5	1.2±2.0	2.8±2.6
0.1	≤0.5	≤0.5	≤0.5	0.6±1.0	3.4±0.8	3.8±1.6	5.0±1.4	3.8±0.6	4.8±1.0
0.032	≤0.5	≤0.5	0.3±0.4	0.4±0.7	3.8±1.3	4.3±0.7	5.3±1.4	3.8±0.4	5.3±0.9
0.01	≤0.5	≤0.5	0.3±0.4	1.7±0.1	4.0±0.7	4.5±0.9	5.2±0.9	4.4±0.3	5.3±0.5
0.0032	≤0.5	≤0.5	0.7±0.6	1.9±0.7	3.8±0.8	4.9±0.7	5.3±0.5	5.3	5.8±0.5
0	≤0.5	≤0.5	0.5±0.5	3.3±0.7	4.7±1.0	5.3±0.6	5.9±0.6	5.3±0.6	5.9±0.7

Shaded area indicates possible range of synergy

[a] Values are mean $\log_{10}$ $CCID_{50}$/0.1 ml ± SD from three independent experiments, assay limit of detection ≤0.5 $\log_{10}$ $CCID_{50}$/0.1 ml

[b] Zanamivir EC_{90} = 0.067 μM

[c] Favipiravir EC_{90} = 0.61 μM

Table 5 Antiviral activities of favipiravir-oseltamivir combinations against oseltamivir-resistant influenza A/HK/2639/2009 virus

Oseltamivir carboxylate (μM)[b]	Virus titer[a]								
	Favipiravir (μM)[c]								
	100	32	10	3.2	1	0.32	0.1	0.032	0
32	≤0.5	≤0.5	≤0.5	0.9±1.6	1.2±2.0	1.8±3.2	2.1±3.6	1.5±2.6	1.8±3.0
10	≤0.5	≤0.5	≤0.5	1.8±1.5	2.9±2.5	4.4±1.0	4.8±1.0	4.9±1.2	4.8±0.9
3.2	≤0.5	≤0.5	1.2±2.0	2.5±1.5	3.8±1.3	5.3±1.1	5.2±0.4	5.0±0.7	5.3±0.4
1	≤0.5	≤0.5	1.1±1.9	3.5±1.0	4.3±1.4	5.5±1.0	5.5±1.0	5.5±1.0	5.4±0.4
0.32	≤0.5	≤0.5	1.3±2.2	3.7±1.0	4.4±0.9	5.7±0.5	5.8±0.6	6.1±0.3	5.4±0.3
0.1	≤0.5	≤0.5	1.1±1.9	3.8±0.9	4.8±0.5	5.5±0.0	5.8±0.6	5.8±0.4	5.6±0.1
0	≤0.5	≤0.5	1.1±2.0	3.9±0.9	4.8±0.4	5.7±0.4	5.6±0.4	5.8±0.3	5.7±0.3

Shaded area indicates possible range of synergy

[a] Values are mean $\log_{10}$ $CCID_{50}$/0.1 ml ± SD from three independent experiments, assay limit of detection ≤0.5 $\log_{10}$ $CCID_{50}$/0.1 ml

[b] Oseltamivir carboxylate EC_{90} = 11.0 μM

[c] Favipiravir EC_{90} = 1.2 μM

Table 6 Antiviral activities of favipiravir-peramivir combinations against oseltamivir-resistant influenza A/HK/2639/2009 virus

Peramivir (μM)[b]	Virus titer[a]								
	Favipiravir (μM)[c]								
	100	32	10	3.2	1	0.32	0.1	0.032	0
32	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
10	≤0.5	≤0.5	≤0.5	0.9±1.6	2.0±1.8	3.4±0.6	4.1±0.5	4.4±0.6	4.1±1.0
3.2	≤0.5	≤0.5	≤0.5	2.5±0.2	4.2±0.4	4.9±0.5	4.8±0.0	5.0±0.4	5.1±0.4
1	≤0.5	≤0.5	0.8±1.4	3.3±0.5	4.5±0.3	5.4±0.1	5.5±0.3	5.4±0.3	5.7±0.0
0.32	≤0.5	≤0.5	1.1±1.9	3.4±0.3	4.2±0.4	5.2±0.6	5.7±0.1	5.8±0.4	5.7±0.3
0.1	≤0.5	≤0.5	1.3±2.2	3.5±0.3	4.8±0.4	5.6±0.6	6.1±0.6	5.8±0.4	6.0±0.4
0	≤0.5	≤0.5	1.2±2.1	3.6±0.9	4.8±0.4	5.4±0.4	5.8±0.7	5.7±0.1	6.0±0.3

Shaded area indicates possible range of synergy

[a] Values are mean $\log_{10}$ $CCID_{50}$/0.1 ml ± SD from three independent experiments, assay limit of detection ≤0.5 $\log_{10}$ $CCID_{50}$/0.1 ml

[b] Peramivir EC_{90} = 0.35 μM

[c] Favipiravir EC_{90} = 0.65 μM

Table 7 Antiviral activities of favipiravir-zanamivir combinations against oseltamivir-resistant influenza A/HK/2639/2009 virus

Zanamivir (μM)[b]	Virus titer[a]								
	Favipiravir (μM)[c]								
	100	32	10	3.2	1	0.32	0.1	0.032	0
32	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
10	≤0.5	≤0.5	≤0.5	0.8±1.3	1.8±1.6	2.7±2.4	2.9±2.5	2.8±2.4	3.0±2.6
3.2	≤0.5	≤0.5	≤0.5	2.9±0.6	3.5±0.3	4.5±0.8	4.9±0.5	4.8±0.4	4.5±0.3
1	≤0.5	≤0.5	0.9±1.6	2.9±0.5	3.8±0.0	4.9±0.5	5.1±0.3	5.5±0.3	5.0±0.5
0.32	≤0.5	≤0.5	1.1±1.9	3.3±0.4	4.3±0.0	5.3±0.5	5.7±0.1	5.9±0.3	5.1±0.8
0.1	≤0.5	≤0.5	1.2±2.0	3.6±0.8	4.5±0.8	5.6±0.3	5.8±0.0	5.6±0.1	5.7±0.5
0	≤0.5	≤0.5	1.2±2.0	4.1±0.4	4.6±0.8	5.7±0.3	5.8±0.3	6.0±0.4	5.7±0.6

Shaded area indicates possible range of synergy

[a] Values are mean $\log_{10}$ $CCID_{50}$/0.1 ml ± SD from three independent experiments, assay limit of detection ≤0.5 $\log_{10}$ $CCID_{50}$/0.1 ml

[b] Zanamivir EC_{90} = 0.22 μM

[c] Favipiravir EC_{90} = 0.9 μM

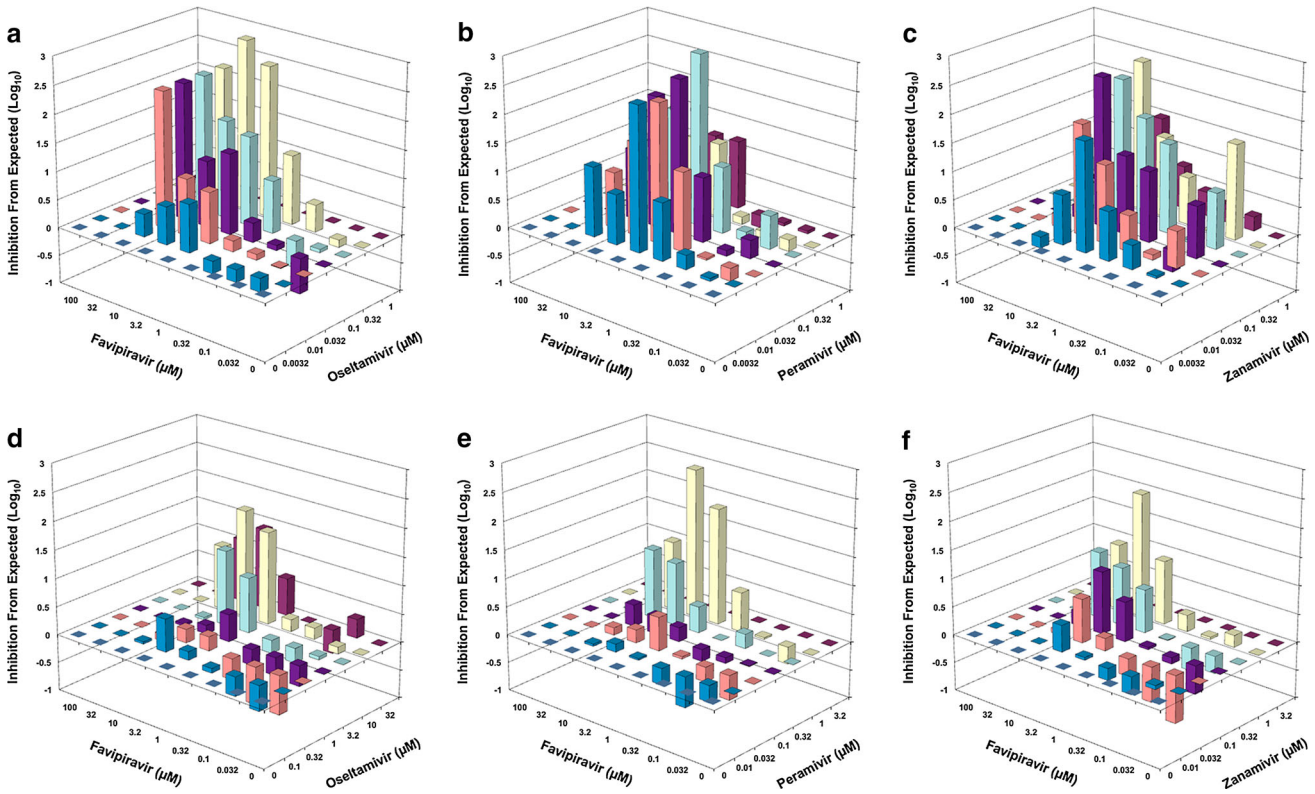

Fig. 2 Drug-drug interactions defined by three-dimensional dose-response surfaces (MacSynergy plots) for favipiravir combined with oseltamivir, peramivir, or zanamivir. MDCK cells were infected with oseltamivir-sensitive influenza A/CA/07/2009 virus (**a**–**c**) or with oseltamivir-resistant A/HK/2369/2009 virus (**d**–**f**) and treated with combinations of drugs. Virus yield reduction data from three independent experiments were used for the analysis. The x- and y-axes are the concentrations of favipiravir with oseltamivir, peramivir, and zanamivir, respectively. The z-axis is the calculated drug-drug interaction based on Bliss independence, expressed as reduction in virus titer relative to the expected value. The null reference plane represents additive interactions, whereas bars above the null reference plane represent synergistic interactions, and bars below the null reference plane represent antagonistic interactions

carries the established NAI resistance marker H275Y. Drug dose-response curves for the Hong Kong strain indicated reduced susceptibility to oseltamivir and peramivir – and to a minor extent to zanamivir – but no change in susceptibility to favipiravir. Although favipiravir generally inhibited virus replication at higher concentrations than the other three tested antivirals, it is not possible to say whether this translates into lower relative potency, since a direct comparison between agents with different modes of action is not recommended for data obtained from cell-based assays [55].

We also evaluated the effects of combination treatment with favipiravir (T-705) plus a neuraminidase inhibitor (oseltamivir, peramivir, or zanamivir) against an oseltamivir-resistant pandemic influenza virus infection in MDCK cells. Antiviral drugs have been tested and used in combination to delay the emergence and spread of drug resistance [56, 57]. In addition, combination therapy offers the benefit of potentiating drug activity and theoretically lowering individual drug dosage requirements [33]. Combinations of, for example, rimantadine or amantadine with ribavirin, a nucleoside analogue inhibiting RNA polymerases, have shown additive and synergistic effects in tissue culture and experimental influenza A virus infections in mice [37, 42, 58, 59]. Synergism was also observed between different NAIs (oseltamivir, peramivir, and zanamivir) and rimantadine in MDCK cells infected with influenza A virus subtypes H1N1 and H3N2 [39]. Moreover, mice that were lethally challenged with highly pathogenic avian influenza A/Vietnam/1203/04 (H5N1) virus and treated with combined doses of amantadine and oseltamivir [43] or ribavirin and oseltamivir [44] were significantly more protected than with either agent alone.

Results from our *in vitro* combination studies also indicated synergistic interactions between favipiravir and the NAIs oseltamivir, peramivir, and zanamivir. However, the degree of synergy depended on the virus strain tested and was generally lower against the oseltamivir-resistant influenza A/Hong Kong/2369/2009 (H1N1pdm) virus. Although the overall treatment effect was additive rather than synergistic against this particular virus strain, certain dual combinations still showed greater than tenfold higher

Table 8 Summary of effects of favipiravir in combination with different neuraminidase inhibitors on oseltamivir-sensitive influenza A/CA/07/2009 and oseltamivir-resistant A/HK/2369/2009 viruses

Virus strain	Drug[a] combination	Synergy dose range (μM)[b]	MacSynergy plot volume[c] (μM^2 %) and 95 % CI		Net combination effect
			Synergy	Antagonism	
California	Favi + Osel	Favi: 0.32-10	28.6	−1.97	Synergistic (minor)
		Osel: 0.01-0.32	(44-13)	(0 to −4)	
	Favi + Pera	Favi: 0.32-10	35.1	−0.62	Synergistic (minor)
		Pera: 0.0032-1	(55-16)	(N.S.) [d]	
	Favi + Zana	Favi: 0.32-10	32.9	−1.01	Synergistic (minor)
		Zana: 0.0032-1	(59-6)	(N.S.)	
Hong Kong	Favi + Osel	Favi: 1-10	12.8	−4.8	Additive
		Osel: 3.2-32	(23-2)	(N.S.)	
	Favi + Pera	Favi: 1-10	11.7	−2.5	Additive
		Pera: 3.2-10	(20-3)	(N.S.)	
	Favi + Zana	Favi: 1-10	11.6	3.7	Additive
		Zana: 1-32	(20-4)	(−1 to −6)	

[a] Favi = Favipiravir; Osel = Oseltamivir; Pera = Peramivir; Zana = Zanamivir

[b] Possible ranges of synergy as shown in Tables 2-7

[c] Synergy volumes at 95 % confidence interval (CI) of < 25, 25-50, 50-100 and > 100 μM^2 % indicate insignificant synergy, slight, moderate and strong synergy, respectively. Antagonism volumes of > −25, −50 to −25 and < −100 indicate insignificant antagonism, slight, moderate and strong antagonism, respectively. Classification adopted from Prichard et al. [52]

[d] N.S. = 'Not significant' as referred to by MacSynergy™ II - User Manual [52]

inhibition of virus replication than would be expected if the interaction had simply been additive. Notable here were the combined effects of favipiravir and peramivir at a concentration of 3.2 μM and 1 μM, respectively. These and other combinations might therefore be advisable for the treatment of oseltamivir-resistant pandemic influenza A (H1N1) virus infections in mice. A strongly synergistic interaction was demonstrated previously for such a combination in mice infected with the influenza A/California/04/2009 (H1N1pdm) virus. However, it should be noted that the pandemic strain tested in theses studies was fully susceptible to oseltamivir [41].

Of interest are the observed effects of certain low-dose combinations of favipiravir with the NAIs against the oseltamivir-resistant pandemic influenza virus strain. These small clusters of combinations were seen to negatively deviate from the null reference plane in the MacSynergy plots (Fig. 2d–f). According to the definitions given for synergy and antagonism by Prichard and colleagues [52], any bar below the null reference plane represents an antagonistic drug interaction. However, results have to be analyzed carefully and put in appropriate biological context. After thorough examination of the raw data (Tables 2, 3, 4, 5, 6, 7), it appeared that the observed antagonism was a result of variance in the virus yield reduction assay. It is not uncommon to find a half-$\log_{10}$ variance between virus titers in this assay. Thus, values in that range do not constitute either synergy or antagonism. However, when the data were analyzed in MacSynergy™ II at the 95 %, 99 %, and 99.9 % confidence intervals, no statistically significant area of antagonism was observed (data not shown). The areas of synergy remained comparable between the two analysis methods used in these studies, indicating the biological relevance of these combinations. One possible mechanism for the synergy observed between favipiravir and the neuraminidase inhibitors may involve a decrease in the virus replication rate following exposure to one drug, which provides more time for interaction with the second drug, leading to the enhanced effect. It was beyond the focus of these studies to evaluate the kinetics of viral replication for each drug and drug combination, but it is possible that the kinetics of virus replication would show a greater decrease (greater inhibition) following exposure to the two drug combination than would be expected for the additive effect from each individual drug. Future studies will be required to confirm this hypothesis.

Since zanamivir remains effective against influenza viruses carrying the NA H275Y point mutation associated with reduced susceptibility to oseltamivir and peramivir [27], and since the results from our *in vitro* tests also demonstrated that the Hong Kong isolate was susceptible to zanamivir when used as a monotherapy, we speculated that a combination of favipiravir and zanamivir would be the most synergistic against this strain. However, the results of our *in vitro* combination studies did not confirm this assumption. Based on the synergistic volumes

calculated with the MacSynergy™ II program, this particular combination was not superior to the other combinations tested. The reasons for these unexpected findings are not clear, but the results illustrate the fact that drug interactions can be unpredictable and therefore require empirical testing and evaluation over a broad concentration range and against a variety of virus subtypes and strains. However, our results do support the use of combination drug therapy, although its clinical relevance remains to be evaluated. This is particularly important in light of a recently published clinical trial on the efficacy of oseltamivir-zanamivir combinations for seasonal influenza in France during the pre-pandemic winter of 2009 [60]. The double-blinded clinical trial included 541 outpatients presenting with influenza-like illness for less than 36 hours and having tested positive for influenza A virus. Results from that study indicated that the oseltamivir-zanamivir combination was less effective than oseltamivir monotherapy, and a clinical antagonism between oseltamivir and zanamivir was suggested [60]. It is possible that the unfavorable drug interaction observed was due to the structural and functional similarity between oseltamivir and zanamivir. As much as synergistic drug interactions are desirable, it is equally important to point out combinations leading to antagonistic interactions, particularly in the clinical setting. In this regard, our results overall are encouraging, and favipiravir, with its broad antiviral spectrum and unique mode of action as a viral RNA polymerase inhibitor with low cytotoxicity [61], appears to be a promising new candidate for dual drug combinations with neuraminidase inhibitors in the therapy and management of human influenza virus infections.

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