

# Resistance to Neuraminidase Inhibitors Conferred by an R292K Mutation in a Human Influenza Virus H7N9 Isolate Can Be Masked by a Mixed R/K Viral Population

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**ABSTRACT** We characterized the A/Shanghai/1/2013 virus isolated from the first confirmed human case of A/H7N9 disease in China. The A/Shanghai/1/2013 isolate contained a mixed population of R (65%; 15/23 clones) and K (35%; 8/23 clones) at neuraminidase (NA) residue 292, as determined by clonal sequencing. A/Shanghai/1/2013 with mixed R/K at residue 292 exhibited a phenotype that is sensitive to zanamivir and oseltamivir carboxylate by the enzyme-based NA inhibition assay. The plaque-purified A/Shanghai/1/2013 with dominant K292 (94%; 15/16 clones) showed sensitivity to zanamivir that had decreased by >30-fold and to oseltamivir carboxylate that had decreased by >100-fold compared to its plaque-purified wild-type counterpart possessing dominant R292 (93%, 14/15 clones). In Madin-Darby canine kidney (MDCK) cells, the plaque-purified A/Shanghai/1/2013-NA<sup>K292</sup> virus exhibited no reduction in viral titer under conditions of increasing concentrations of oseltamivir carboxylate (range, 0 to 1,000  $\mu$ M) whereas the replication of the plaque-purified A/Shanghai/1/2013-NA<sup>R292</sup> and the A/Shanghai/2/2013 viruses was completely inhibited at 250  $\mu$ M and 31.25  $\mu$ M of oseltamivir carboxylate, respectively. Although the plaque-purified A/Shanghai/1/2013-NA<sup>K292</sup> virus exhibited lower NA enzyme activity and a higher  $K_m$  for 2'-(4-methylumbelliferyl)- $\alpha$ -D-N-acetylneuraminic acid than the wild-type A/Shanghai/1/2013-NA<sup>R292</sup> virus, the A/Shanghai/1/2013-NA<sup>K292</sup> virus formed large plaques and replicated efficiently *in vitro*. Our results confirmed that the NA R292K mutation confers resistance to oseltamivir, peramivir, and zanamivir in the novel human H7N9 viruses. Importantly, detection of the resistance phenotype may be masked in the clinical samples containing a mixed population of R/K at NA residue 292 in the enzyme-based NA inhibition assay.

**IMPORTANCE** The neuraminidase (NA) inhibitors oseltamivir and zanamivir are currently the front-line therapeutic options against the novel H7N9 influenza viruses, which possess an S31N mutation that confers resistance to the M2 ion channel blockers. It is therefore important to evaluate the sensitivity of the clinical isolates to NA inhibitors and to monitor for the emergence of resistant variants. We characterized the A/Shanghai/1/2013 (H7N9) isolate which contained a mixed population of R/K at NA residue 292. While the clinical isolate exhibited a phenotype of sensitivity to NA inhibitors using the enzyme-based NA inhibition assay, the plaque-purified A/Shanghai/1/2013 virus with dominant K292 was resistant to zanamivir, peramivir, and oseltamivir. Resistance to NA inhibitors conferred by the R292K mutation in a human influenza virus H7N9 isolate can be masked by a mixed R/K viral population, and this should be taken into consideration while monitoring antiviral resistance in patients with H7N9 infection.

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Within 3 months of its emergence, the novel avian-origin H7N9 influenza virus had caused more than 130 confirmed human infections in eastern China. These infections led to variable clinical outcomes, with the majority of the patients presenting with pneumonia that progressed to acute respiratory distress syndrome and a fatal outcome in approximately 25% of cases so far (1–3). Only a few mild or asymptomatic infections have been reported so far (3, 4). Contact with live poultry, especially within live-poultry markets appears to be a

major source of human infection (5), although other sources of exposure may also contribute to human infection. H7N9 viruses that are genetically homologous to the human H7N9 viruses have been isolated at a low frequency in poultry and pigeons within live-poultry markets (1). The virus has so far not been detected in poultry farms, and the source of the virus that seeds the wholesale and retail live-poultry markets is still unclear. While the majority of the patients had exposure to live animals, clusters of human infection have occurred and, in

**TABLE 1** IC<sub>50</sub> values of the NA inhibitors for the human and avian H7N9 isolates in the MUNANA-based enzyme inhibition assay

Virus	IC <sub>50</sub> value (nM) [mean (95% CI)]	
	Zanamivir	Oseltamivir carboxylate
A/Shanghai/1/13	1.59 (1.01–2.50)	1.18 (0.85–1.66)
A/Shanghai/2/13	1.30 (0.49–3.49)	1.21 (1.00–1.47)
A/Dk/JX/3286/09	1.46 (1.09–1.96)	1.16 (0.95–1.42)

some of these instances, the possibility of limited human-to-human transmission cannot be excluded (3).

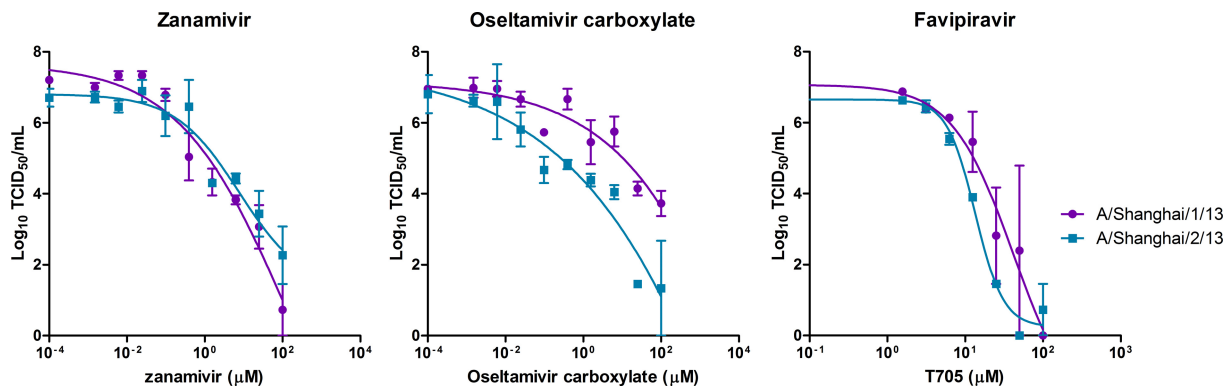
Neuraminidase (NA) inhibitors have been used as the front-line therapeutic option because the novel H7N9 viruses contain the S31N mutation in the M2 protein conferring resistance to the M2 ion channel blockers (2). It is therefore critical to evaluate the sensitivity of the clinical H7N9 isolates to NA inhibitors and to monitor for the emergence of resistant variants, especially in patients undergoing NA inhibitor treatment. We characterized the A/Shanghai/1/2013 virus isolated from the first confirmed human case of A/H7N9 disease in China at 2 days after commencement of oseltamivir therapy (2). The A/Shanghai/1/2013 isolate has been reported to possess an R292K NA mutation (N2 numbering; R294K for N9 numbering), which is R289K for the novel H7N9 viruses due to the five-amino-acid deletion in the NA stalk region (2). Recently, the emergence of the R292K mutation in 2 of 14 patients infected with the novel H7N9 influenza virus was reported to be associated with poor clinical outcome (6). This mutation has been previously associated with resistance to both zanamivir and oseltamivir carboxylate in seasonal H3N2 influenza viruses (7) and has been previously reported in an H1N9 influenza virus selected in the presence of a zanamivir derivative *in vitro* (8).

## RESULTS

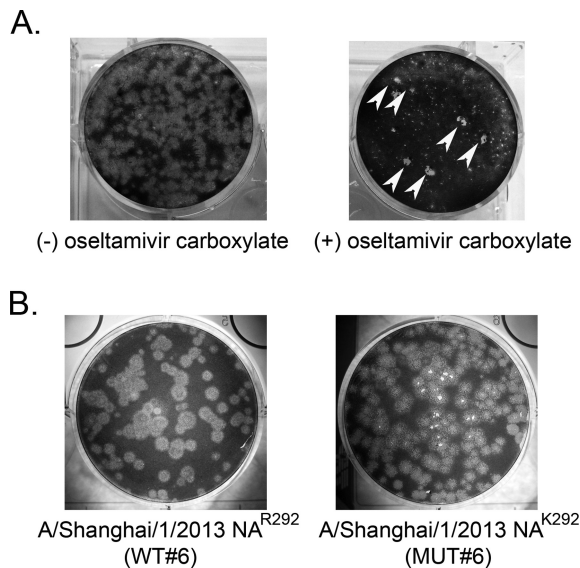
**The A/Shanghai/1/2013 isolate with a mixed R/K population at NA residue 292 exhibited a phenotype that is sensitive to NA inhibitors.** We first evaluated the sensitivity of two human H7N9 influenza viruses (A/Shanghai/1/2013 and A/Shanghai/2/2013) as well as a control avian H7N9 isolate (of a different genetic derivation from the novel human H7N9 viruses) to NA inhibitors using an enzyme-based NA inhibition assay. The original sample provided by China CDC has been passaged in eggs and Madin-Darby

canine kidney (MDCK) cells, and we determined the percentages of the wild-type (WT; R292) and the mutant (MUT; K292) populations using 454 sequencing. The results showed that the sample contained a mixed population of the wild type (R292) and mutant (K292) at 67% (31/46 reads) and 33% (15/46 reads), respectively. The received sample was further passaged once in MDCK cells to prepare stock virus in the laboratory. Clonal sequencing analysis for the NA gene of the stock virus showed that the wild type (R292) and the mutant (K292) were present at frequencies of 65% (15/23) and 35% (8/23), respectively. The results suggest that the percentages of the wild-type (R292) and mutant (K292) populations did not alter significantly after one passage in MDCK cells. The 50% inhibitory concentrations (IC<sub>50</sub>s) of zanamivir (1.59 nM; 95% confidence interval [CI], 1.01 to 2.50 nM) and oseltamivir carboxylate (1.18 nM; 95% CI, 0.85 to 1.66 nM) with respect to the A/Shanghai/1/2013 virus containing mixed R/K292 populations were comparable to those seen with the A/Shanghai/2/2013 and the A/Duck/Jiangxi/3286/2009 H7N9 influenza viruses (Table 1). In addition, we evaluated the sensitivity of the A/Shanghai/1/2013 and A/Shanghai/2/2013 viruses using increasing concentrations of zanamivir, oseltamivir carboxylate, or favipiravir (T-705) in MDCK cells (Fig. 1). The A/Shanghai/1/2013 virus was less sensitive than the A/Shanghai/2/2013 virus under conditions of increasing concentrations of oseltamivir carboxylate. The two human H7N9 isolates exhibited comparable dose-response curves under conditions of increasing concentrations of zanamivir or favipiravir. Overall, the results suggested that the resistance phenotype cannot be easily identified from the clinical H7N9 isolate with a mixed population of R/K at NA residue 292.

**Plaque-purified A/Shanghai/1/2013 virus with dominant K292 exhibited resistance to zanamivir, peramivir, and oseltamivir.** Since the clinical H7N9 isolate A/Shanghai/1/2013 contained a mixed R/K population at NA residue 292, we performed plaque purification in the presence and absence of 10 μM of oseltamivir carboxylate. Plaque purification (Fig. 2A) yielded three clones of wild-type populations containing R292 in the NA protein and four mutant populations containing K292 in the NA protein as confirmed by Sanger sequencing. To monitor if any compensatory mutation might have arisen together with the NA R292K mutation, full-length hemagglutinin (HA) and NA sequences of these plaque-purified viruses were verified by Sanger



**FIG 1** Human H7N9 influenza virus sensitivity to zanamivir, oseltamivir, and favipiravir (T-705) *in vitro*. MDCK cells were infected with A/Shanghai/1/13 (mixed R/K at NA residue 292) or A/Shanghai/2/13 virus at MOI = 0.001 TCID<sub>50</sub>/cell. Supernatants were collected at 48 h postinfection and were titrated in MDCK cells (log<sub>10</sub> TCID<sub>50</sub>/ml).



**FIG 2** Morphology of the plaque-purified A/Shanghai/1/2013 viruses. (A) A/Shanghai/1/2013 was grown in the absence or presence of 10  $\mu$ M oseltamivir carboxylate for plaque purification. The arrows indicated the hole left after plaque picking. (B) Morphology of the plaque-purified A/Shanghai/1/2013 NA<sup>R292</sup> (WT no. 6) and NA<sup>K292</sup> (MUT no. 6) viruses.

sequencing. HA mutations were found in 3 of 4 plaque-purified mutant viruses and 1 of 3 of the wild-type viruses at different residues (Table 2). Overall, we observed that the wild-type viruses bearing the R292 in NA protein replicated to approximately 10-fold-lower peak titers (range, 7.07 to 7.57  $\log_{10}$  50% tissue culture infective doses [TCID<sub>50</sub>]/ml) than the mutant viruses carrying the K292 in the NA protein (range, 8.21 to 8.96  $\log_{10}$  TCID<sub>50</sub>/ml) (Table 2). Plaque-purified WT variant no. 6 and MUT variant no. 6, which differed only by the NA R292K mutation in their HA and NA genes, were selected for further analysis. In addition to Sanger sequencing, we verified the ratio of R/K at residue 292 for both WT no. 6 and MUT no. 6 using clonal sequencing. It was observed that the WT no. 6 strain was dominated by R292 (93%, 14/15), with detection of one clone of G292 (1/15). The MUT no. 6 strain was dominated by K (94%, 15/16) with one clone of R292 (1/16) after two rounds of expansion under conditions of exposure to 10  $\mu$ M oseltamivir carboxylate. Both the plaque-purified WT no. 6 and MUT no. 6 strains formed large plaques in MDCK cells (Fig. 2B);

**TABLE 3** IC<sub>50</sub> values of pdm(H1N1) 09 viruses and plaque-purified A/Shanghai/1/13 viruses in the MUNANA-based enzyme inhibition assay

Virus	IC <sub>50</sub> value (nM) [mean (95% CI)]	
	Zanamivir	Oseltamivir carboxylate
A/Shanghai/1/13 NA <sup>R292</sup> (WT no. 6)	1.80 (1.34–2.41)	0.97 (0.73–1.30)
A/Shanghai/1/13 NA <sup>K292</sup> (MUT no. 6)	52.02 (15.39–175.8)	>100
RG-A/CA/04/09	0.52 (0.47–0.58)	1.09 (0.94–1.25)
RG-A/CA/04/09 NA <sup>H274Y</sup>	0.88 (0.79–0.98)	1,067 (878.1–1,297)

however, it was noted that the MUT no. 6 strain formed a more diffused plaque morphology than the WT no. 6 strain.

Applying the enzyme-based NA inhibition assay, we observed that the plaque-purified A/Shanghai/1/2013 NA<sup>K292</sup> (MUT no. 6) virus exhibited reduced sensitivity to zanamivir (52.02 nM; 95% CI, 15.39 to 175.8 nM) and oseltamivir carboxylate (>100 nM) compared to the plaque-purified wild-type A/Shanghai/1/2013 NA<sup>R292</sup> virus (WT no. 6) (Table 3). Interestingly, the dose-response curve for the plaque-purified A/Shanghai/1/2013 NA<sup>K292</sup> (MUT no. 6) strain with oseltamivir carboxylate showed a two-shoulder effect (Fig. 3A) and a poorly fitted IC<sub>50</sub> value (Table 3). This may have been due to the presence of the wild-type R292 population (6%; 1/16) in the A/Shanghai/1/2013 NA<sup>K292</sup> (MUT no. 6) virus that may have been a result of reversion or from residual wild type in the original population. We further evaluated the sensitivity of other plaque-purified clones using the enzyme-based NA inhibition assay (Fig. 3B). Two mutant clones (MUT no. 2 and MUT no. 3) that bear additional HA mutations showed better dose-response curves, and a more accurate IC<sub>50</sub> estimation conferred by the NA R292K mutation was able to be determined (Table 4). Using the IC<sub>50</sub> determined from the A/Shanghai/1/2013 NA<sup>K292</sup> (MUT no. 3) and A/Shanghai/1/2013 NA<sup>R292</sup> (WT no. 6) viruses (Table 4), it was observed that the R292K mutation confers resistance to zanamivir that is increased by 62-fold, to peramivir that is increased by 1,445-fold, and to oseltamivir carboxylate that is increased by 33,862-fold.

To determine the sensitivity of the plaque-purified virus *in vitro*, we evaluated the sensitivity of the plaque-purified virus in MDCK cells under conditions of increasing concentrations of oseltamivir carboxylate. The plaque-purified A/Shanghai/1/2013 NA<sup>K292</sup> (MUT no. 6) virus exhibited no reduction in viral titer

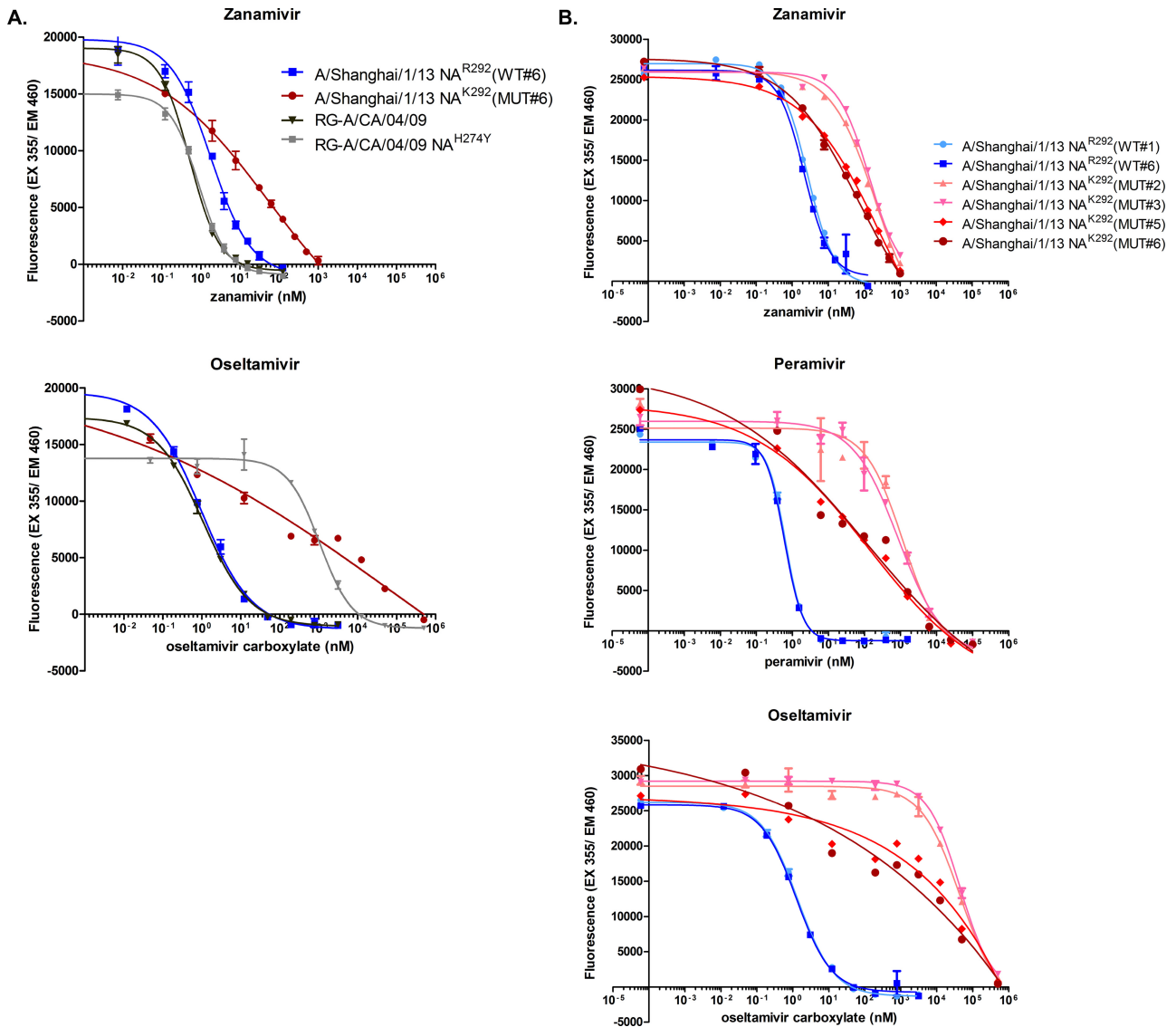
**TABLE 2** Amino acid changes identified in the HA and NA protein of the plaque-purified viruses of A/Shanghai/1/2013 by Sanger sequencing

Clone	HA titer <sup>a</sup>	Log <sub>10</sub> TCID <sub>50</sub> /ml <sup>b</sup>	Mutation(s) in the HA protein <sup>c</sup> (H3 numbering)	Mutation in the NA protein <sup>c</sup> (N2 numbering)
WT no. 1	64	7.07	None	R292
WT no. 2	64	7.18	E121G/A122S/D513N	R292
WT no. 6	128	7.57	None	R292
MUT no. 2	64	8.34	I118T	K292
MUT no. 3	256	8.76	G218E/A479T	K292
MUT no. 5	32	8.96	G65E/T547P	K292
MUT no. 6	32	8.21	None	K292

<sup>a</sup> Determined with 0.5% turkey red blood cells.

<sup>b</sup> Titer after one expansion in the presence of 10  $\mu$ M oseltamivir carboxylate followed by one expansion in the absence of oseltamivir carboxylate.

<sup>c</sup> Compared to the parental A/Shanghai/1/2013 sequence.



**FIG 3** Dose-response curve of the plaque-purified A/Shanghai/1/2013 and the A(H1N1) pdm09 viruses in the fluorescence-based neuraminidase inhibition assay. A/Shanghai/1/13 NA<sup>R292</sup> (WT no. 6), A/Shanghai/1/13 NA<sup>K292</sup> (MUT no. 6), RG-A/CA/04/09, and RG-A/CA/04/09 NA<sup>H274Y</sup> viruses (A) and A/Shanghai/1/13 NA<sup>R292</sup> (WT no. 1 and WT no. 6) and A/Shanghai/1/13 NA<sup>K292</sup> (MUT no. 2, MUT no. 3, MUT no. 5, and MUT no. 6) viruses (B) were preincubated with zanamivir, peramivir, or oseltamivir carboxylate for 45 min at 37°C before incubation with the fluorogenic substrate MUNANA at a final concentration of 167  $\mu$ M at 37°C for 30 min. The neuraminidase-cleaved product (4-methylumbelliferone) was detected using an FLUOstar OPTIMA microplate reader (BMG Labtech). EX, excitation; EM, emission (in nm).

under conditions of increasing concentrations of oseltamivir carboxylate (range, 0 to 1,000  $\mu$ M), whereas the replication of the plaque-purified A/Shanghai/1/2013-NA<sup>R292</sup> and the A/Shanghai/2/2013 viruses was completely inhibited at 250  $\mu$ M and 31.25  $\mu$ M of oseltamivir carboxylate, respectively (Fig. 4A). Similarly, the plaque-purified A/Shanghai/1/2013 NA<sup>K292</sup> (MUT no. 6) virus exhibited no reduction in viral titer under conditions of increasing concentrations of zanamivir (range, 0 to 500  $\mu$ M), whereas the replication of the A/Shanghai/2/2013 viruses was completely inhibited at 125  $\mu$ M zanamivir. The plaque-purified A/Shanghai/1/2013-NA<sup>R292</sup> (WT no. 6) virus was not completely inhibited at the highest concentration tested (125  $\mu$ M zanamivir) (Fig. 4B). The observation that the plaque-purified A/Shanghai/1/2013-NA<sup>R292</sup> (WT no. 6) virus exhibited reduced sensitivity to oseltamivir car-

boxylate or zanamivir compared to the A/Shanghai/2/2013 virus suggests that there may still be a residual NA<sup>K292</sup> population existing in the plaque-purified A/Shanghai/1/2013-NA<sup>R292</sup> (WT no. 6) virus. The plaque-purified A/Shanghai/1/2013-NA<sup>R292</sup> (WT no. 6) (90% effective concentration [EC<sub>90</sub>] = 10.41  $\mu$ M; 95% CI, 7.74 to 13.99  $\mu$ M) and A/Shanghai/1/2013 NA<sup>K292</sup> (MUT no. 6) (EC<sub>90</sub> = 4.84  $\mu$ M; 95% CI, 1.90 to 12.36  $\mu$ M) viruses exhibited dose-response curves comparable to that of the A/Shanghai/2/2013 virus (EC<sub>90</sub> = 1.43  $\mu$ M; 95% CI, 2.28 to 15.92  $\mu$ M) under conditions of increasing concentrations of favipiravir, indicating that alternative therapeutic agents such as favipiravir, which targets the influenza virus polymerase, would still be effective against the NA inhibitor-resistant variants.

To evaluate the effect of the R292K mutation on the NA en-

**TABLE 4** IC<sub>50</sub> values of plaque-purified A/Shanghai/1/13 viruses with R292 or K292 in the NA protein in the MUNANA-based enzyme inhibition assay

Virus	IC <sub>50</sub> value (nM) [mean (95% CI)]		
	Zanamivir	Peramivir	Oseltamivir carboxylate
A/Shanghai/1/13 NA <sup>R292</sup> (WT no. 1)	2.7 (2.2–3.3)	0.6 (0.6–0.7)	1.4 (1.2–1.5)
A/Shanghai/1/13 NA <sup>R292</sup> (WT no. 6)	2.2 (1.7–2.7)	0.6 (0.5–0.7)	1.3 (1.0–1.5)
A/Shanghai/1/13 NA <sup>K292</sup> (MUT no. 2)	195.1 (84.7–449.1)	1169 (610.1–2,241)	44,093 (26,168–74,299)
A/Shanghai/1/13 NA <sup>K292</sup> (MUT no. 3)	136.3 (82.5–225.4)	867.5 (558.3–1,348)	44,021 (37,315–51,933)
A/Shanghai/1/13 NA <sup>K292</sup> (MUT no. 5)	283.5 (51.2–1,570)	134.5 (22.1–818.9)	>100
A/Shanghai/1/13 NA <sup>K292</sup> (MUT no. 6)	65.7 (35.4–122.2)	116.1 (0.6–22,739)	>100

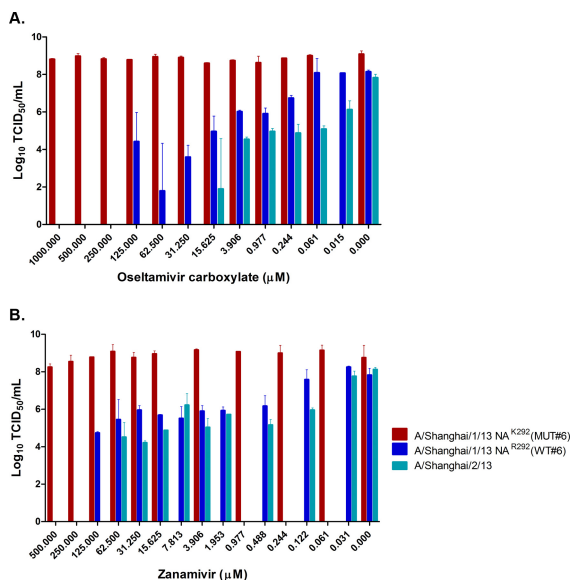
zyme activity, we performed the NA kinetics assay using the 2'-(4-methylumbelliferyl)- $\alpha$ -D-N-acetylneuraminic acid (MUNANA) substrate (Table 5). We observed that the  $V_{\max}$  of the plaque-purified wild-type A/Shanghai/1/2013 NA<sup>R292</sup> (WT no. 6) virus was 4-fold higher than that of the plaque-purified A/Shanghai/1/2013 NA<sup>K292</sup> (MUT no. 6) virus (Table 5). The R292K mutation also increased the  $K_m$  value, indicating a reduced binding affinity for the MUNANA substrate. In parallel, the A/Shanghai/2/13 NA showed higher NA activity but a  $K_m$  comparable to that of the plaque-purified wild-type A/Shanghai/1/2013 NA<sup>R292</sup> (WT no. 6) virus. Overall, our results showed that the R292K NA mutation

confers resistance to zanamivir, peramivir, and oseltamivir in the novel H7N9 influenza virus and that the mutation had impaired the NA enzyme function as previously reported for the H1N9 or seasonal H3N2 influenza viruses (8, 9).

**The plaque-purified viruses replicate efficiently in MDCK-SIAT1 cells.** To evaluate if the reduced NA activity conferred by the R292K mutation would compromise viral growth, we performed multicycle replication kinetics analyses for the plaque-purified A/Shanghai/1/2013 NA<sup>R292</sup> and A/Shanghai/1/2013 NA<sup>K292</sup> viruses in MDCK-SIAT1 cells that overexpress the alpha-2,6-linked terminal sialic acid (10). We observed that the mutant virus continuously replicated to 1-log<sub>10</sub>-higher titers than the wild-type virus from 8 h postinfection onward (Fig. 5). This observation suggests that the NA R292K mutation did not compromise the replication efficiency of the A/Shanghai/1/2013 virus in the MDCK-SIAT1 cells.

## DISCUSSION

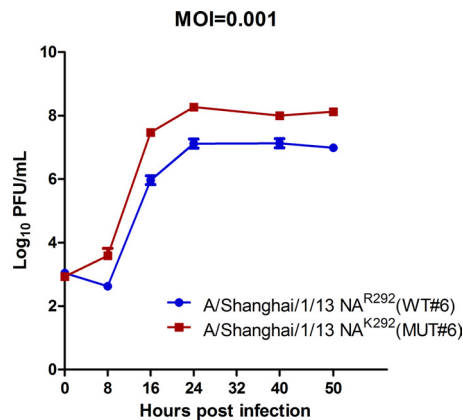
We report here that the R292K NA mutation confers resistance to zanamivir, peramivir, and oseltamivir carboxylate in the novel H7N9 virus. However, in the field isolate where the K292 mutation comprised 35% of the total population, it exhibited a sensitive phenotype by the NA enzyme-based assay. This could have been due to the low NA activity (25%) possessed by the R292K mutant virus relative to the A/Shanghai/1/2013 wild-type virus, comparable to the previously reported results that the R292K mutation reduced the enzyme activity in an H1N9 virus to only 20% of the wild-type level (8). Hence, when the K292 mutant is present at 35% of the total population, the activity and drug sensitivity of the wild-type R292 virus would predominate, as determined using the enzyme-based NA inhibition assay to assess the sensitivity of the viruses to NA inhibitors. Our results suggest that direct assess-



**FIG 4** Sensitivity of the plaque-purified A/Shanghai/1/2013 viruses to zanamivir and oseltamivir carboxylate *in vitro*. MDCK cells were preincubated with NA inhibitors for 2 h prior to infection with A/Shanghai/1/13 NA<sup>R292</sup> (WT no. 6), A/Shanghai/1/13 NA<sup>K292</sup> (MUT no. 6), or A/Shanghai/2/13 viruses at MOI = 0.001 TCID<sub>50</sub>/cell for 1 h and overlaid with media containing oseltamivir carboxylate (0 to 1,000  $\mu$ M for the A/Shanghai/1/13 NA<sup>K292</sup> MUT no. 6 virus and 0 to 500  $\mu$ M for the A/Shanghai/1/13 NA<sup>R292</sup> WT no. 6 and the A/Shanghai/2/2013 viruses) (A) or zanamivir (0 to 500  $\mu$ M for the A/Shanghai/1/13 NA<sup>K292</sup> MUT no. 6 virus and 0 to 125  $\mu$ M for the A/Shanghai/1/13 NA<sup>R292</sup> WT no. 6 and the A/Shanghai/2/2013 viruses) (B). Supernatants were collected at 48 h postinfection and were titrated in MDCK cells ( $\log_{10}$  TCID<sub>50</sub>/ml).

**TABLE 5** NA enzyme kinetics using MUNANA substrate

Virus	$V_{\max}$ [mean (95% CI)]	$K_m$ ( $\mu$ M) [mean (95% CI)]
A/Shanghai/1/13 NA <sup>R292</sup> (WT no. 6)	6.06 (5.03–7.09)	222.9 (141.1–304.6)
A/Shanghai/1/13 NA <sup>K292</sup> (MUT no. 6)	1.42 (0.61–2.23)	1086 (239.2–1,933)
A/Shanghai/2/13	20.78 (14.82–26.75)	221.1 (83.6–358.6)



**FIG 5** Replication kinetics of the plaque-purified A/Shanghai/1/2013 NA<sup>R292</sup> and NA<sup>K292</sup> viruses in MDCK-SIAT cells. Cells were infected with an MOI = 0.001 TCID<sub>50</sub>/cell. The supernatants were collected at the time points indicated and titrated in MDCK cells (log<sub>10</sub> PFU/ml).

ment of the cultured clinical samples with the enzyme-based neuraminidase inhibition assay may not accurately identify the presence of the resistant variants. It is crucial to monitor the emergence of the R292K mutation among H7N9 patients by genotypic methods.

Surveillance studies suggest that the emergence of NA mutations conferring resistance to NA inhibitors has reportedly been low, with the exception of the naturally emergent H274Y NA mutation in H1N1 seasonal influenza viruses during the influenza season of 2007 to 2008 (7, 11). The R292K mutation is one of the most commonly identified mutations among seasonal H3N2 isolates with significantly reduced sensitivity to oseltamivir carboxylate (7, 11), intermediate resistance to peramivir, and slightly reduced sensitivity to zanamivir (12, 13). This mutation was first selected *in vitro* using an avian H4N2 influenza virus under the selection pressure of zanamivir (14) and was subsequently isolated *in vitro* from a seasonal H3N2 influenza virus and a reassortant H1N9 influenza virus under the selection pressure of oseltamivir carboxylate and a zanamivir derivative, respectively (8, 15). Using the enzyme-based NA inhibition assay, it was shown that the R292K mutation in the H1N9 virus conferred reduced sensitivity to zanamivir (by 55-fold), peramivir (by 1,000-fold), and oseltamivir carboxylate (by 6,500-fold) (16), comparable to the results we observed with the novel H7N9 influenza viruses. Structurally, R292 is one of the three catalytic arginines that form a triad interacting with the carboxylate group of the sialic acid (17) or the NA inhibitors (zanamivir and oseltamivir carboxylate). Changes in hydrogen bonding in the R292K mutant lead to a reduced interaction between the inhibitor carboxylate and the protein. This resulted in reduced binding of all the NA inhibitors and additionally the sialic acid substrate, thus correlating with reduced enzyme activity. The mechanism by which the R292K confers higher resistance to oseltamivir is through preventing rotation of the E276 to form the salt link to R224, which is required for the formation of a hydrophobic pocket to accommodate the bulky pentyl ether group of the oseltamivir carboxylate (18).

The R292K mutation that confers resistance to both zanamivir and oseltamivir carboxylate in a reassortant H1N9 virus was selected *in vitro* after 8 passages in the presence of the 6-carboximide derivative of zanamivir (8). Subsequent passage yielded variants

with additional HA (G143E or N199S) in combination with the NA R292K mutation. It was reported that the R292K mutation reduced the NA activity to 20% of the wild-type virus activity and that the virus carrying this mutation alone formed plaques significantly smaller than those formed by the parent strain. The HA mutations were observed to increase the plaque size of the H1N9 virus carrying the R292K mutation. The R292K mutants were serially passaged in the absence of NA inhibitors 10 times, and no reversion in the HA or NA mutation was observed previously (8). We observed here that the plaque-purified A/Shanghai/1/2013 NA<sup>K292</sup> (MUT no. 6) virus, without an additional HA mutation as confirmed by Sanger sequencing, formed large plaques and replicated efficiently *in vitro* despite possessing significantly lower NA activity than was seen with the plaque-purified wild-type A/Shanghai/1/2013 NA<sup>R292</sup> virus. It is not known if the HA protein of A/Shanghai/1/13 possess a low binding affinity contributing to the lack of NA dependence. A reduced HA binding affinity would allow the virus to tolerate the low NA activity caused by the NA R292K mutation and allow the virus to form large plaques and to replicate efficiently *in vitro*. In addition, it is known that the viral sensitivity *in vitro* is dependent on both HA and NA, while the IC<sub>50</sub> determined by the NA inhibition assay is purely dependent on the NA property. This could contribute to the observed discrepancy between the enzyme-based NA inhibition assay and the *in vitro*-based assay, in which the K292 variant had an IC<sub>50</sub> value of 52 nM for zanamivir (Table 3) but failed to be inhibited at 500,000 nM *in vitro* (Fig. 4). It should be noted that the A/Shanghai/1/2013 sequence differed from the A/Shanghai/2/2013 sequence by 52 nucleotides (19), including 9 amino acid differences in the HA protein. One major molecular difference between the A/Shanghai/1/2013 and A/Shanghai/2/2013 viruses in the HA protein is the absence of the avian-to-human-adaptation Q226L change in the A/Shanghai/1/2013 virus. It remains to be investigated whether the R292K mutation would exhibit similar plaque morphology or replication characteristics in the other novel H7N9 viruses.

The stability of the R292K mutation in the novel H7N9 viruses also needs to be further characterized. This needs to be studied in different culture systems, including embryonic chicken eggs, conventional cell lines, differentiated human respiratory epithelium cells, and *in vivo* animal models, to evaluate the fitness and the public health risk posed by this mutation. It was observed previously that the seasonal H3N2 virus carrying the R292K mutation showed compromised replication efficiency in MDCK-SIAT1 cells and possessed inefficient transmissibility to naive direct-contact ferrets (9, 20). In contrast, we observed that the plaque-purified A/Shanghai/1/2013 NA<sup>K292</sup> virus replicated efficiently in the MDCK-SIAT1 cells, which could have been due to a different HA-NA functional balance *in vitro*. It is not clear if balanced HA-NA activity would be maintained *in vivo* during ferret transmission experiments, including the transmissibility of the resistant strain carrying the R292K mutation and the potential of a wild-type revertant to arise *in vivo* over time. In the present study, a wild-type NA<sup>R292</sup> population was still detectable at 6% (1/16) in the plaque-purified A/Shanghai/1/2013 NA<sup>K292</sup> virus after two passages in the presence of 10 μM oseltamivir carboxylate. It is not clear if the wild-type NA<sup>R292</sup> population detected that emerged during the one subsequent passage in the absence of the NA inhibitor was revertant or if it consisted of residual wild-type virus from the original mixture.

In summary, we confirm that the R292K mutation in the novel H7N9 virus confers resistance to zanamivir, peramivir, and oseltamivir carboxylate. The clinical sample containing a mixed population of R/K may exhibit a phenotype that is sensitive to NA inhibitors, especially when phenotypic enzyme-based NA inhibition assays are used. It is therefore recommended that the emergence of the R292K mutation in H7N9 patients undergoing NA inhibitor treatment should be monitored using genotypic methods in combination with the phenotypic characterization of the virus.

## MATERIALS AND METHODS

**Viruses and cells.** The A/Shanghai/1/2013 and A/Shanghai/2/2013 (H7N9) viruses were provided by CDC, China (2). The viruses were isolated and passaged in embryonic chicken eggs before being passaged twice in Mardin-Darby canine kidney (MDCK) cells. The A/Duck/Jiangxi/3286/2009 (H7N9) virus was isolated and passaged in embryonic chicken eggs and once in MDCK cells. The A(H1N1) pdm09 viruses were prepared as previously described (21). Aliquots of the stock viruses grown in MDCK cells were stored at  $-80^{\circ}\text{C}$ . All experiments were performed in the biosafety level 3 (BSL3) laboratories at the University of Hong Kong and the Shantou University Medical College in compliance with applicable guidelines. Madin-Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection and were maintained in minimum essential medium (MEM) with 10% fetal calf serum. MDCK-SIAT1 cells that overexpress human  $\alpha$ -2,6-sialyltransferase were kindly provided by Mikhail N. Matrosovich (Philipps University, Marburg, Germany) and were maintained as previously described (10). Plaque purification was performed for the A/Shanghai/1/2013 virus in the presence or absence of  $10\ \mu\text{M}$  oseltamivir carboxylate in MDCK cells. The isolated plaques were expanded once in the absence (for isolation of the R292 population) and presence (for isolation of the K292 population) of oseltamivir carboxylate ( $10\ \mu\text{M}$ ) and further expanded once in the absence of oseltamivir carboxylate.

**Sanger and clonal sequencing.** Total RNA was extracted from the viral culture supernatants (RNeasy; Qiagen). The full-length hemagglutinin (HA) and NA genes of the expanded plaques were amplified by reverse transcription PCR (RT-PCR) using universal primers (22) and sequenced by Sanger sequencing. To determine the ratio of R/K at residue 292, a 789-bp PCR product fragment spanning the region of residue 292 was amplified by RT-PCR (Qiagen) (forward, 5'-AACACATGGGCCCGAAA C 3'; reverse, 5'-ATATCGTCTCGTATTAGTAGAAACAAGGGTCTT 3') and cloned into the pCR4-TOPO vector (Invitrogen). Plasmid DNAs were isolated (Miniprep; Qiagen) and were sequenced by Sanger sequencing using the amplification primers.

**Compounds.** The oseltamivir carboxylate and zanamivir compounds were kindly provided by Hoffmann-La Roche Ltd., Switzerland. The stock NA inhibitor aliquots ( $10\ \text{mM}$ ) were stored at  $-20^{\circ}\text{C}$ . Favipiravir (T-705) was purchased from Sigma-Aldrich, dissolved in dimethyl sulfoxide (DMSO), and further diluted with phosphate-buffered saline (PBS) to yield  $2\ \text{mM}$  stock and stored at  $-20^{\circ}\text{C}$ .

**Enzyme-based NA inhibition assay and NA kinetics.** The sensitivities of the H7N9 viruses to zanamivir and oseltamivir carboxylate were determined by an enzyme-based assay using the fluorogenic substrate 2'-(4-methylumbelliferyll)- $\alpha$ -D-N-acetylneuraminic acid (MUNANA) at a final concentration of  $167\ \mu\text{M}$  and incubated at  $37^{\circ}\text{C}$  for 30 min (23, 24). The neuraminidase-cleaved product (4-methylumbelliferone) was detected using an FLUOstar OPTIMA microplate reader (BMG Labtech) at excitation and emission wavelengths of 355 and 460 nm, respectively. The  $\text{IC}_{50}$  for each virus was determined by plotting fluorescence as a function of the compound concentration followed by variable-slope dose-response curve fitting using GraphPad Prism software. The NA enzyme kinetics assays were performed with the MUNANA substrate (final concentration, 0 to  $571.4\ \mu\text{M}$ ) as previously described (21) with viruses diluted to approximately  $10,000\ \text{TCID}_{50}/\text{well}$  in a 96-well plate. The fluorescence of the

released 4-methylumbelliferone was measured every 68 s for 68 min by using a FLUOstar OPTIMA microplate reader (BMG Labtech). The enzyme kinetics data were fitted to the Michaelis-Menten equation to determine the Michaelis constant ( $K_m$ ) and maximum velocity ( $V_{\text{max}}$ ) of substrate conversion using GraphPad Prism.

**Sensitivity to antiviral compounds *in vitro*.** The sensitivity of the human H7N9 viruses to zanamivir (0 to  $500\ \mu\text{M}$ ), oseltamivir carboxylate (0 to  $1,000\ \mu\text{M}$ ), or favipiravir (0 to  $100\ \mu\text{M}$ ) was evaluated in MDCK cells at a multiplicity of infection (MOI) of 0.001  $\text{TCID}_{50}$  per cell. Cells were pretreated with the antiviral compounds for 2 h prior to infection and after the 1-h incubation at  $37^{\circ}\text{C}$ . Supernatants were collected at 48 h postinfection and stored at  $-80^{\circ}\text{C}$  before titration ( $\log_{10}\ \text{TCID}_{50}/\text{ml}$ ; range of dilutions,  $10^{-0.5}$  to  $10^{-8}$ ) in MDCK cells.

**Replication kinetics.** MDCK-SIAT1 cells were infected with the plaque-purified A/Shanghai/1/2013  $\text{NA}^{\text{R292}}$  and  $\text{NA}^{\text{K292}}$  viruses at an MOI of 0.001  $\text{TCID}_{50}/\text{cell}$ . Supernatants were collected at 8 h, 16 h, 24 h, 40 h, 50 h, and 75 h postinfection and stored at  $-80^{\circ}\text{C}$  before titration ( $\log_{10}\ \text{PFU}/\text{ml}$ ; detection limit = 10 PFU) in MDCK cells.

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