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Impact of R152K and R368K neuraminidase catalytic substitutions on *in vitro* properties and virulence of recombinant A(H1N1)pdm09 viruses

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ABSTRACT

Neuraminidase (NA) mutations conferring resistance to NA inhibitors (NAIs) are expected to occur at framework or catalytic residues of the NA enzyme. Numerous clinical and *in vitro* reports already described NAI-resistant A(H1N1)pdm09 variants harboring various framework NA substitutions. By contrast, variants with NA catalytic changes remain poorly documented. Herein, we investigated the effect of R152K and R368K NA catalytic mutations on the NA enzyme properties, *in vitro* replicative capacity and virulence of A(H1N1)pdm09 recombinant viruses. In NA inhibition assays, the R152K and R368K substitutions resulted in reduced inhibition [10- to 100-fold increases in IC_{50} vs the wild-type (WT)] or highly reduced inhibition (> 100-fold increases in IC_{50}) to at least 3 approved NAIs (oseltamivir, zanamivir, peramivir and laninamivir). Such resistance phenotype correlated with a significant reduction of affinity observed for the mutants in enzyme kinetics experiments [increased Km from 20 ± 1.77 for the WT to 200.8 ± 10.54 and 565.2 ± 135 µM ($P < 0.01$) for the R152K and R368K mutants, respectively]. The R152K and R368K variants grew at comparable or even higher titers than the WT in both MDCK and ST6GalI-MDCK cells. In experimentally-infected C57BL/6 mice, the recombinant WT and the R152K and R368K variants induced important signs of infection (weight loss) and resulted in mortality rates of 87.5%, 37.5% and 100%, respectively. The lung viral titers were comparable between the three infected groups. While the NA mutations were stable, an N154I substitution was detected in the HA2 protein of the R152K and R368K variants after *in vitro* passages as well as in lungs of infected mice. Due to the multi-drug resistance phenotypes and conserved fitness, the emergence of NA catalytic mutations accompanied with potential compensatory HA changes should be carefully monitored in A(H1N1)pdm09 viruses.

1. Introduction

Influenza infection is a contagious respiratory disease of significant public health and economic importance worldwide. Each year, seasonal influenza epidemics account for an average of 3–5 million of severe cases and between 250,000 and 500,000 deaths in the world (WHO, 2016). Since the advent of the 2009 pandemic, the influenza A(H1N1) pdm09 virus has become one of the predominant circulating strains during seasonal influenza epidemics globally. The matrix (M)-2 protein of A(H1N1)pdm09 viruses contains the S31N substitution associated with resistance to the adamantanes (i.e. amantadine and rimantadine) (Dong et al., 2015). Consequently, neuraminidase inhibitors (NAIs) that target the active site of the influenza neuraminidase (NA) enzyme are the only class of anti-influenza agents currently recommended for treatment and prophylaxis of influenza A(H1N1)pdm09 infections. Two compounds from this class [i.e., oseltamivir (Tamiflu; Hoffmann-La Roche) and zanamivir (Relenza; GlaxoSmithKline)] are currently licensed worldwide while two others [peramivir (Rapivab, BioCryst) and laninamivir (Inavir, Biota)] have been approved in some countries: Japan, South Korea, USA and Canada (for peramivir) and Japan (for laninamivir) (Birnkrant and Cox, 2009; FDA, 2014; Kubo et al., 2010; Watanabe et al., 2010).

NAIs are derivatives of DANA (2,3-dehydro-2-deoxy-N-acetylneuraminic acid), a sialic acid analogue with a weak NA inhibitory activity (McKimm-Breschkin, 2013). The fact that some structural differences exist between these NAIs (in particular between oseltamivir and zanamivir) suggests that cross-resistant viruses would emerge infrequently (McKimm-Breschkin, 2000). Indeed, the most frequent NA substitutions reported in A(H1N1) (H275Y) and A(H3N2) (E119V) subtypes confer resistance to oseltamivir without altering susceptibility to zanamivir (Abed et al., 2006; Wetherall et al., 2003). However, clinical A(H1N1) pdm09 variants containing the E119D/G NA framework substitutions conferring pan NAI-resistance have been recently identified in immunocompromised patients (L'Huillier et al., 2015; Tamura et al., 2015). In addition, the association of I223R to H275Y, reported in different clinical cases, enhances the level of resistance to oseltamivir

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and peramivir and causes moderate level of resistance to zanamivir (Abed and Boivin, 2017; Nguyen et al., 2010).

Contrasting to the framework NA mutations described above, clinical reports on NAI-resistant variants harboring catalytic NA mutations remain extremely rare in the A(H1N1)pdm09 viral background. Nevertheless, an influenza A(H1N1)pdm09 R152K NA variant was identified under peramivir pressure during clinical trials (Yoshida et al., 2011). The NA gene of this variant also contained a V94I substitution. Similarly, the R368K substitution was also reported in that viral background (FDA, 2016). Using recombinant A(H1N1)pdm09 proteins, we have recently demonstrated that the R152K and R368K NA mutations confer a resistance phenotype to the three tested NAIs (peramivir, oseltamivir and zanamivir) (Fage et al., 2017). The aim of the present study was to assess the enzymatic properties, replicative capacities and virulence of influenza A(H1N1)pdm09 recombinant viruses harbouring the R152K and R368K NA catalytic substitutions.

2. Materials and methods

2.1. Cells and viruses

Madin-Darby canine kidney cells overexpressing the α2,6 sialic acid receptor (ST6-GalI-MDCK) cells were kindly provided by Y. Kawaoka from the University of Wisconsin, Madison, WI. MDCK and human embryonic kidney 293 T cells were purchased from ATCC. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics. The recombinant wild-type (WT) influenza A/Quebec/144147/09 virus (an A/California/07/2009-like A(H1N1) pdm09 strain: GenBank No. FN434457-FN434464) and the single (R152K, R368K, V94I) and double (R152K/V94I) NA variants were generated by transfecting 293 T cells with the 8 bidirectional pLLB-A/G plasmids as previously described (Pizzorno et al., 2011).

2.2. Determination of the phenotype of susceptibility to NA inhibitors

The phenotype of susceptibility of recombinant viruses to oseltamivir carboxylate (Hoffmann-La Roche, Basel, Switzerland), zanamivir (GlaxoSmithKline, Stevenage, United Kingdom), peramivir (BioCryst, Birmingham, USA) and laninamivir (Biota Scientific Management, Notting Hill, Australia) was evaluated by NA inhibition assays as previously reported (Samson et al., 2014). Briefly, viruses were standardized to a NA activity level 10-fold higher than that of the background, as measured by the production of a fluorescent product from Methylumbelliferyl-N-acetylneuraminic acid (MUNANA) (Sigma, St-Louis, MO) substrate. Drug susceptibility profiles were determined by the extent of NA inhibition after incubation with 3-fold serial dilutions of NAIs. The 50% inhibitory concentrations (IC_{50}s) were determined from the dose-response curve.

2.3. NA enzyme kinetics assays

Recombinant viruses were standardized to 10^6 viral RNA copies/ml as determined by the CDC universal influenza A quantitative RT-PCR targeting the viral M2 gene (WHO, 2009) and incubated at 37 °C in 50-µl reactions with different concentrations of MUNANA ranging from 0 to 5000 µM (Yen et al., 2006). Fluorescence was monitored every 90 s for 53 min (35 measures). The Michaelis-Menten constant (Km) and the maximum velocity (Vmax) were calculated with the Prism software (GraphPad, version 6), by fitting the data to the Michaelis-Menten equation using nonlinear regression.

2.4. In vitro replication kinetics experiments

Replicative capacities of the recombinant viruses were evaluated by infecting native MDCK or ST6GalI-MDCK cells with a multiplicity of infection (MOI) of 0.0001 plaque-forming units (PFUs)/cell. Supernatants were collected at 24, 36, 48, 72 and 96 h post-inoculation (p.i.) for determination of $TCID_{50}$ titers using the respective cell line.

2.5. In vitro genetic stability

Recombinant viruses were submitted to four serial passages in ST6GalI-MDCK cells. Confluent cells in 6-well plates were washed with phosphate-buffered saline (PBS) before viral inoculation at an MOI of 0.001 PFUs/cell. After a 1 h adsorption at 37 °C, the supernatant was removed and fresh DMEM-TPCK medium was added. After 3 days, supernatants were used to infect freshly prepared confluent cells. The hemagglutinin (HA) and NA genes from supernatant samples were amplified by RT-PCR and sequenced by using an automated DNA sequencer (ABI Prism 377 DNA sequencer, Applied Biosystems, Foster City, CA).

2.6. Experimental infections of mice

Groups of twelve 6- to 8-week old female C57BL6 mice (Charles River, ON, Canada), housed four per cage and kept under conditions which prevent cage-to-cage transmission, were infected under isoflurane anesthesia by intranasal inoculation of 10^5 $TCID_{50}$ of recombinant WT or NA mutant A(H1N1)pdm09 viruses in 50 µl of PBS. One group of 4 uninfected mice served as control. Animals were weighed daily for 14 days and monitored for clinical signs. Four mice per infected group were sacrificed on day 5 p.i. and lungs were removed aseptically. For determination of lung viral titers (LVTs), harvested lung tissues were homogenized in 1 ml of PBS containing 2 x antibiotic-antimycotic solution (penicillin, streptomycin and amphotericin B) using Omni Tip homogenizer (OMNI International, GA, USA). Cells were pelleted by centrifugation (600 g, 5 min) and supernatants were used for determination of $TCID_{50}$ titers using MDCK cells.

2.7. HA/NA sequencing

The HA and NA genes from genetic stability experiments [i.e., from non-passaged recombinant viruses (P0) and after 4 passages in ST6GalI-MDCK cells (P4)] as well as those from replicative capacity experiments (collected at 96h p.i.) and mouse lung homogenates (collected at day 5 p.i.) were amplified and sequenced using the ABI 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA).

2.8. Statistical analyses

NA kinetic parameters (Km and V_{max} values), *in vitro* and lung viral titers were compared by two-way ANOVA analysis of variance with the Dunnett's multiple comparison post-test. Body weight losses were analyzed by a Student-*t*-test while a Log-Rank (Mantel-Cox) test was used to compare Kaplan–Meier survival plots.

3. Results

3.1. Impact of NA mutations on the susceptibility profiles and NA enzyme kinetics of influenza A(H1N1)pdm09 recombinants

Five recombinant influenza A(H1N1)pdm09 viruses were rescued as part of this study: the WT, R152K, R368K, and V94I single mutants as well as the R152K/V94I double mutant. Based on the WHO guidelines for determination of the phenotype of susceptibility to NAIs, the R152K substitution mediated reduced inhibition (RI; 10- to 100-fold increase in IC_{50} vs the WT) to the 4 tested NAIs (68-, 26-, 74- and 22-fold increases in oseltamivir, zanamivir, peramivir and laninamivir IC_{50} values, respectively) (Table 1). A similar resistance pattern was observed for the double R152K/V94I variant. The R368K substitution resulted into a RI phenotype to oseltamivir and zanamivir (96- and 57-fold

Table 1
Susceptibility profiles of recombinant influenza A(H1N1)pdm09 viruses harboring catalytic neuraminidase (NA) substitutions to NA inhibitors (NAI).

Recombinants	Oseltamivir[a]				Zanamivir[a]				Peramivir[a]				Laninamivir[a]			
	IC_{50} (nM)		Folds	Phenotype[b]	IC_{50} (nM)		Folds	Phenotype[b]	IC_{50} (nM)		Folds	Phenotype[b]	IC_{50} (nM)		Folds	Phenotype[b]
WT	1.48 ± 0.14		1	S	0.68 ± 0.03		1	S	0.20 ± 0.03		1	S	0.36 ± 0.04		1	S
R368K	142.52 ± 9.6		96	RI	39.36 ± 8.48		57	RI	34.40 ± 2.10		172	HRI	1.81 ± 0.26		5	S
R152K	100.48 ± 4.12		68	RI	17.68 ± 2.80		26	RI	14.8 ± 0.80		74	RI	8.04 ± 1.30		22	RI
R152K/V94I	78.12 ± 14.01		53	RI	17.76 ± 0.40		26	RI	11.40 ± 0.16		57	RI	6.49 ± 1.19		18	RI
V94I	1.42 ± 0,00		1	S	0.66 ± 0.04		1	S	0.16 ± 0.00		1	S	0.90 ± 0.09		3	S

WT, wild-type.
[a] Values are mean 50% inhibitory concentration (IC_{50}) ± standard deviation from one experiment performed in duplicate.
[b] The phenotype of susceptibility to NAIs according to WHO guidelines: S, susceptibility or normal inhibition (< 10-fold increase in IC_{50} over WT); RI, reduced inhibition (10- to 100-fold increase in IC_{50} over WT); HRI, highly reduced inhibition (> 100-fold increase in IC_{50} over WT).

Table 2
Enzyme kinetics parameters of recombinant A(H1N1)pdm09 viruses harboring catalytic neuraminidase substitutions.

Recombinants	Km (μM)[a]	Vmax (U/sec)[a]	Vmax ratio
WT	20 ± 1.77	50.8 ± 1.64	1.00
R368K	565.2 ± 135**	81.66 ± 37.89	1.60
R152K	200.8 ± 10.54	175 ± 55.37*	3.44
R152K/V94I	235.5 ± 10.32	5.47 ± 0.55	0.10
V94I	15.06 ± 3.52	22.41 ± 5.87	0.44

[a] Numbers indicate mean Km and relative NA activity (Vmax) values ± standard deviation of a kinetics experiment performed in duplicate, using recombinant viruses standardized to 10^6 RNA copies/ml. *, $P < 0.05$, **, $P < 0.01$ versus the wild-type (WT).

increases in IC_{50} values, respectively) and a highly reduced inhibition (HRI) phenotype to peramivir (172-fold increase in the IC_{50} value). There was only a 5-fold increase in the laninamivir IC_{50} value for this variant (normal inhibition). The V94I variant had a normal inhibition phenotype to all tested NAIs.

The effects of the different mutations selected for this study on NA enzymatic properties are summarized in Table 2. Compared to the recombinant WT virus, the R368K and R152K (alone or combined to the V94I) substitutions reduced the NA affinity [increased Km values from 20 ± 1.77 μM for the WT to 565.2 ± 135 ($p < 0.01$), 200.8 ± 10.54 and 235.5 ± 10.32 μM for the R368K, R152K and R152K/V94I variants, respectively] . By contrast, the V94I substitution did not alter the NA affinity (Km of 15.06 ± 3.52 μM). Nevertheless, this substitution, alone or associated with R152K resulted in Vmax values equivalent to 44% and 10% of that of the WT, respectively.

3.2. Impact of NA mutations on the in vitro replicative capacity of influenza A(H1N1)pdm09 recombinant viruses

In replication kinetics experiments using MDCK cells, the peak viral titers obtained for all tested recombinant viruses were observed at 36–48 h p.i. (Fig. 1A). The peak viral titers for the recombinant WT virus and the single R152K and R368K mutants were comparable ($4.43 \pm 3.92 \times 10^6$, $4.02 \pm 4.23 \times 10^6$ and $3.13 \pm 1.73 \times 10^6$ $TCID_{50}$/ml, respectively) whereas the V94I and R152K/V94I mutants had a significantly lower peak viral titer as compared to the recombinant WT virus ($1.76 \pm 0.97 \times 10^5$ $TCID_{50}$/ml, $p < 0.01$ and $4.43 \pm 3.92 \times 10^3$ $TCID_{50}$/ml, $p < 0.001$). Indeed, contrasting to the R152K and R368K mutants, the viral titers obtained for V94I and R152K/V94I mutants were significantly lower (1- to 3-log reduction) than those observed for the WT at almost all tested time points. Similar patterns were obtained with ST6GalI-MDCK cells where the R152K and R368K recombinants grew at comparable or higher titers vs the WT whereas the V94I and R152K/V94I grew at viral titers that were, for some time points, ≥ 3-Log lower than those obtained for the recombinant WT virus (Fig. 1B).

3.3. In vitro genetic stability of recombinant influenza A(H1N1)pdm09 viruses

The NA gene of recombinant A(H1N1)pdm09 viruses selected after 4 serial passages in ST6GalI-MDCK cells contained the expected mutation with no additional changes (Table 3). By contrast, the gene encoding the HA2 subunit of the HA protein in the R368 variant contained an AAT→ATT change at codon 154 resulting into an N→I amino acid substitution (Table 3). The same substitution was also present at approximately 40% in the HA2 of the R152K variant. Such change was completely absent in the WT as well as in the two remaining mutants.

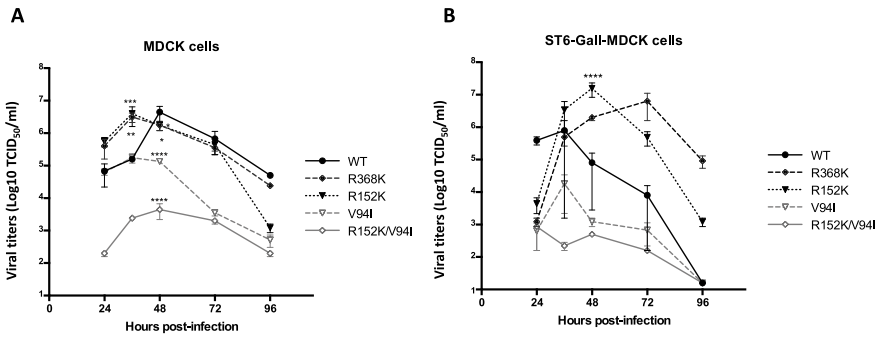

Fig. 1. Replicative properties of recombinant A(H1N1) pdm09 viruses *in vitro*. Confluent MDCK (A) or ST6GalI-MDCK (B) cells were infected with recombinant viruses at a multiplicity of infection (MOI) of 0.0001 PFUs/cell. Supernatants were harvested at the indicated times and titrated by $TCID_{50}$ assays in the respective cell line. Mean viral titers of triplicate ± SD are shown. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$, compared to wild-type (WT).

Table 3
Genetic stability of recombinant A(H1N1)pdm09 viruses harboring catalytic neuraminidase substitutions, *in vitro* and *in vivo*.

Recombinants	P0[a]		P4[a]		Lung (day 5 p.i.)[b]	
	NA[b]	HA[b]	NA[b]	HA[b]	NA[b]	HA[b]
WT	WT (100%)	WT (100%)	WT (100%)	WT (100%)	WT (100%)	WT (100%)
R152K	R152K (100%)	WT (100%)	R152K (100%)	N154I (40%)	R152K (100%)	N154I (20–30%)
R368K	R368K (100%)	WT (100%)	R368K (100%)	N154I (100%)	R368K (100%)	N154I (100%)
V94I	V94I (100%)	WT (100%)	V94I (100%)	WT (100%)	ND	ND
R152K/V94I	R152K/ V94I (100%)	WT (100%)	R152K/ V94I (100%)	WT (100%)	ND	ND

[a] P0: non-passaged recombinant viruses; P4: viruses collected after 4 serial passages in ST6GalI-MDCK cells.
[b] Viruses collected in lung homogenates of infected C57/BL6 mice.

3.4. Impact of NA mutations on the virulence of influenza A(H1N1)pdm09 recombinant viruses in mice

Intranasal inoculation of mice with 10^5 $TCID_{50}$ of the recombinant viruses resulted in clinical signs of infection such as body weight loss, lethargy and mortality that were observed in the three infected groups of mice with no signs being observed in the uninfected group of animals. As shown in Fig. 2A, 7/8 animals (87.5%) infected with the WT recombinant and 3/8 (37.5%) infected with the R152K variant died between day 5 and day 8 p.i. Interestingly, all animals (8/8, 100%) infected with the R368K mutant died on day 5 p.i. Accordingly, important body weight losses, in particular during the first 5 days p.i., were observed in infected animals while no signs of infection were observed in the uninfected group (Fig. 2B). LVTs determined on day 5 p.i. were comparable among the three infected groups (6.67 ± 0.65, 6.26 ± 0.55 and 6.7 ± 0.65 Log_{10} $TCID_{50}$/ml for the WT, R152K and R368K groups, respectively) (Fig. 2C). As described in the *in vitro* genetic stability section, sequence analysis of the NA gene from lung samples of mice confirmed the presence of the R152K and R368K substitutions in the NA gene of the respective groups without other NA changes. On the other hand, these variants contained the N154I HA2 substitution that was present in proportions of 20–30% and 100%, respectively (Table 3).

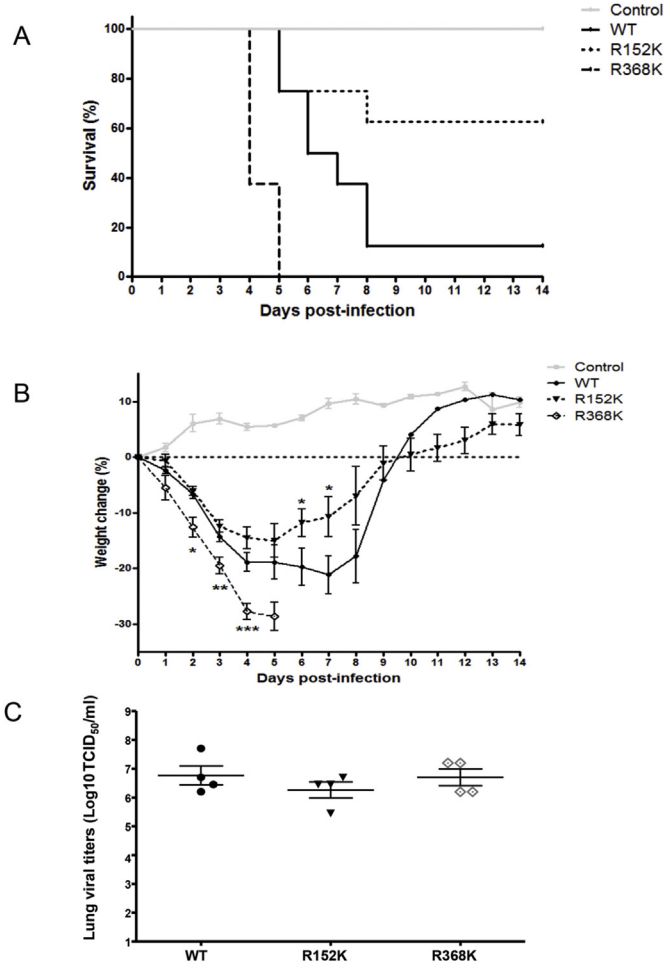

Fig. 2. Kaplan Meier survival curve, body weight losses and lung viral titers of mice infected with recombinant A(H1N1)pdm09 viruses. Groups of 12 mice were infected with 10^5 $TCID_{50}$ of the recombinant A(H1N1)pdm09 wild-type (WT) virus or the R152K or R368K variants. Mortality (A) and mean body weight loss ± standard error of the mean (B) were measured for 8 mice. Percent body weight losses as compared to initial weights were recorded daily until day 14 post-inoculation. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to WT. Mean lung viral titers ± standard error of the mean (C) were determined with $TCID_{50}$ experiments in MDCK cells for groups of 4 mice euthanized on day 5 post-inoculation.

4. Discussion

NAIs are expected to play a major role for the control of severe cases of influenza infections such as those involving immunocompromised subjects or patients with chronic medical conditions (Moscona, 2005). Oseltamivir is the most frequently-used NAI because of its convenient oral formulation whereas zanamivir (delivered by the inhaled route) is considered to be the first-line antiviral option for oseltamivir-resistant viruses, including A(H1N1)pdm09 strains with the H275Y substitution. Therefore, the emergence of influenza viruses harboring NA mutations conferring cross-resistance to both compounds would constitute a serious clinical threat (Abed and Boivin, 2017).

The active center of the influenza NA enzyme is constituted by highly conserved framework and catalytic residues (Colman et al., 1993). Clinical A(H1N1)pdm09 strains containing framework

substitutions, such as the well-known H275Y (Baz et al., 2009) as well as E119G/D (L'Huillier et al., 2015; Tamura et al., 2015) and I223R (LeGoff et al., 2012; van der Vries et al., 2010) variants have been reported. Furthermore, numerous *in vitro* and/or *in vivo* studies already described the impact of such drug-resistant framework mutations in the context of the A(H1N1)pdm09 virus (Abed et al., 2016; Hamelin et al., 2010; van der Vries et al., 2011). By contrast, NA catalytic changes mediating NAI resistance remain poorly characterized in this viral background.

R152K and R368K mutations are among the most important NA catalytic changes with the potential to induce cross resistance to different NAIs. The R152K substitution was initially identified in an influenza B variant (R150K) recovered from an immunocompromised child treated with zanamivir (Gubareva et al., 1998). Such variant was resistant to zanamivir, oseltamivir and peramivir (Gubareva et al., 1998). Since then, R152K cases have remained very rare among influenza A and B viruses, probably in part due to the limited use of zanamivir. The R152K substitution along with the V94I change were recently identified in a A(H1N1)pdm09 virus from a 4-year old patient treated with peramivir during Phase II clinical trials (Yoshida et al., 2011). Exposure of mallards, experimentally-infected with A/Mallard/Sweden/51833/2006 (H1N1) influenza virus, to increasing concentrations of zanamivir also induced the emergence of the R152K NA mutation (Nykvist et al., 2017). More recently, the R152K mutation was the predominant change in the NA gene of an A/California/04/09 (H1N1) virus isolated from nude mice that received laninamivir-favipiravir combined treatment (Kiso et al., 2018).

R368 is another key catalytic residue within the influenza A and B NA enzymes (Colman et al., 1993). As this residue interacts with the carboxylate group of oseltamivir and zanamivir, the R368K change has the potential to mediate cross-resistance to NAIs (Colman et al., 1993). The R368K NA catalytic change was identified in a surveillance study, in which the B/Hong Kong/36/2005-R374K variant exhibited 407- and 29-fold increases in oseltamivir and zanamivir IC_{50} values, respectively, as compared to the matched B/Hong Kong/95/2005 susceptible virus (Sheu et al., 2008). When introduced into the NA gene of a recombinant influenza A/Wuhan/359/95-like (H3N2) virus, the R371K substitution also conferred cross resistance to the two tested NAIs (oseltamivir and zanamivir) (Yen et al., 2006). Of interest, the recombinant A(H3N2)-R371K variant of that study replicated efficiently in MDCK cells and was genetically stable (Yen et al., 2006). The R368K substitution was also part of drug resistance NA changes detected in the A(H1N1) background during surveillance studies and oseltamivir clinical trials (FDA, 2016).

In this report, we assessed the impact of the R152K and R368K substitutions on *in vitro* and *in vivo* properties of recombinant A(H1N1) pdm09 viruses. In addition to the WT and the two NA single catalytic mutants (i.e., R152K and R368K), we also rescued and characterized the R152K/V94I double mutant (as initially reported) as well as the single V94I mutant. The R152K, R368K and R152K/V94I variants were found to alter the NAI susceptibility profile demonstrating RI/HRI phenotypes to at least three different NAIs, including the two most-widely used compounds (i.e. oseltamivir and zanamivir) (Table 1). The V94I substitution had no significant impact on the NAI susceptibility profile using enzymatic assays. The observed alteration in NAI susceptibility was well correlated with a significant reduction in NA affinity observed for the R152K, R368K and R152K/V94I mutants in enzyme kinetics experiments while such effect was not displayed by the single V94I mutant (Table 2). Infections of MDCK cells in replicative experiments led to high viral titers ($> 10^6$ $TCID_{50}$/ml) that were reached at 36–48 h p.i. for the WT and the two single mutants (R152K and R368K). By contrast, the R152K/V94 and, to a lower extent, the V94I variants had replicative defects at most time points (Fig. 1). Similar results were obtained when using ST6GalI-MDCK cells. The apparent deleterious impact of the V94I substitution on viral replication remains unclear since this residue is not part of the NA active site. Nevertheless, although the V94I did not alter the affinity of the enzyme, this change was associated with 46% and 90% decreases of Vmax in the V194I and R152K/V94I, respectively, as compared to the WT (Table 2). Hence, maybe such an effect on NA velocity could be responsible for the replicative defect observed for the single (V94I) and double (R152K/V94I) recombinants.

Because of its poor viral growth *in vitro*, the R152K/V94I mutant was not evaluated in C57/BL/6 mice that were experimentally infected with 10^5 $TCID_{50}$ of single recombinant viruses (WT, R152K and R371K). Similar to the WT recombinant virus, a high virulence potential was observed in animals infected with these two mutants based on body weight losses and mortality rates. Moreover, the infection resulted in high viral titers ($\geq 10^6$ $TCID_{50}$/ml) in the lungs that were comparable among the three infected groups. By sequencing the NA/HA genes from the lungs of infected animals, we further demonstrated that the *in vivo* conserved/enhanced viral fitness was not due to mutation reversion events. This could rather be associated with the N154I HA2 substitution detected in the HA ectodomain region of these variants, potentially affecting the HA structure and flexibility (Keleta et al., 2008). Indeed, N154 is a highly conserved HA2 residue among the 18 influenza A subtypes (Figure S1). This asparagine is involved in a potential glycosylation site (NXT) whose absence was previously described as an important HA change during mouse adaptation of influenza A(H3N2) viruses (Keleta et al., 2008). Thus, this HA substitution may have a possible compensatory effect for the NA catalytic variants contributing to their good viral fitness, which is in contrast to other catalytic mutants such as R292K in the A/H3N2 subtype (Herlocher et al., 2002).

The influenza NA enzyme promotes virion release and spread by removing sialic acid residues from host cell receptors and the newly synthesized viral glycoproteins (McKimm-Breschkin, 2013). The efficiency of such process, which occur at the late stage of the influenza viral cycle, relies on the strength of virus attachment to cells which is mediated by the viral HA protein. If the NA mutation conferring resistance to NAI significantly disrupts the viral NA/HA balance, this would result in a compromised viral fitness. Such feature could probably apply for many NAI-resistant NA variants previously reported in different influenza A subtypes and B viruses (Samson et al., 2013). Of note, different observations were made with regard to the A(H1N1) pdm09-H275Y mutant. We and others previously reported that, unlike old A(H1N1)-H275Y viruses, the H275Y substitution did not compromise the viral fitness in the A(H1N1)pdm09 background (Hamelin et al., 2010; Memoli et al., 2011). By performing biochemical and fitness analyses of different genetic reassortment events between seasonal A(H1N1) viruses and A(H1N1)pdm09 strains, Ferraris et al., suggested that the reduction of the NA affinity due the H275Y mutation could be associated with a better HA/NA balance resulting in enhanced infectivity in mice (Ferraris et al., 2012). Thus, it appears that in the A(H1N1)pdm09 genetic context, some NA mutations with the potential to reduce NA affinity/activity would be advantageous for the virus. Our results suggest that this could be also the case for the R368K and R152K variants with the additional probable contribution of the HA2 N154T substitution. Whether these NA/HA changes may have a significant clinical impact with efficient transmissibility remained unresolved at the moment. However, as these mutants are associated with a cross-resistant phenotype to the major and only effective class of anti-influenza agents, their detection should be carefully monitored.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2018.04.009.

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