Impact of a large deletion in the neuraminidase protein identified in a laninamivir-selected influenza A/Brisbane/10/2007 (H3N2) variant on viral fitness in vitro and in ferrets

Julie Ann,a Yacine Abed,a Edith Beaulieu,b Xavier Bouhy,a Marie-Hélène Joly,b Karen Dubé,b Julie Carbonneau,a Marie-Eve Hamelin,a Corey Mallett,b Guy Boivina

aCHU de Quebec Research Center (CHUL) and Laval University, Québec City, QC, Canada. bGSK Vaccines, Laval, QC, Canada.

Correspondence: Guy Boivin, CHUL, room RC-709, 2705 blvd Laurier, Sainte-Foy, Québec, Canada G1V 4G2.
E-mail: Guy.Boivin@crcchul.ulaval.ca

Accepted 20 October 2015.

Viral fitness of a laninamivir-selected influenza A/Brisbane/10/2007-like (H3N2) isolate (LRVp9) containing a 237-amino acid neuraminidase deletion and a P194L hemagglutinin mutation was evaluated in vitro and in ferrets. LRVp9 and the wild-type (WT) virus showed comparable replication kinetics in MDCK-ST6GalI cells. Cultured virus was recovered between days 2 and 5 post-infection in nasal washes (NW) from the 4 WT-infected ferrets whereas no virus was recovered from the LRVp9-infected animals. There was a ≥1 log reduction in viral RNA copies/μl of NW for LRVp9 compared to WT at most time points. The large neuraminidase deletion compromises viral infectivity in vivo.

Keywords A(H3N2), deletion, influenza, neuraminidase, resistance.

Please cite this paper as: Ann et al. (2016) Impact of a large deletion in the neuraminidase protein identified in a laninamivir-selected influenza A/Brisbane/10/2007 (H3N2) variant on viral fitness in vitro and in ferrets. Influenza and Other Respiratory Viruses 10(2), 122–126.

Introduction

Influenza A(H3N2) viruses have been continuously circulating during seasonal influenza epidemics since 1968, and infections with these viruses have generally been associated with greater morbidity and mortality than A(H1N1) viruses.1 As most seasonal A(H3N2) viruses isolated after 2005 are resistant to adamantanes (amantadine and rimantadine),2 neuraminidase inhibitors (NAIs) constitute the antiviral option of choice against these infections. This class currently includes the globally used oseltamivir phosphate (Tamiflu, Hoffmann-La Roche) and zanamivir (Relenza, GlaxoSmithKline),3 in addition to peramivir (Rapivab, BioCryst) whose parenteral formulation is available in Japan, South Korea, and the USA4,5 and the long-acting NAI, laninamivir octanoate (Inavir, Daiichi Sankyo), which has been approved in Japan.6,8 As for other antivirals, resistance to NAIs in seasonal A (H3N2) viruses may constitute a serious clinical problem. Therefore, studies on the mechanisms of resistance to NAIs and characterization of fitness properties of NAI-resistant A (H3N2) variants are of significant importance.

We recently used an influenza A/Brisbane/10/2007-like (H3N2) virus for in vitro passages in cells overexpressing α2,6 receptors (ST6GalI-MDCK) in presence of laninamivir.9 A selected variant obtained at the 9th passage in presence of laninamivir (LRVp9) had a large deletion (Del 106-342) in the NA that was accompanied by a P194L (H3 numbering) mutation in the receptor binding site (RBS) of the hemagglutinin (HA). This genotype was associated with a loss of NA activity, and the virus growth was not inhibited by the presence of high concentrations of laninamivir and oseltamivir in the agar overlay during susceptibility assessment with plaque reduction assays.9 LRVp9 also had altered binding to turkey and guinea pig red blood cells and grew in ST6GalI-MDCK cells both in presence or absence of laninamivir.9 As little information is available on the viral fitness of laninamivir-resistant A(H3N2) viruses, the aim of this study was to evaluate the impact of laninamivir-induced NA/HA changes on replicative capacity in vitro and infectivity in ferrets.

Methods

The control-passaged A/Brisbane/10/2007-like wild-type (WT) virus and LRVp9 were used to infect ST6GalI-MDCK cells (kindly provided by Dr. Y. Kawaoka, Department of
Pathological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison) at a multiplicity of infection of 0.0001 PFU/cell. The infection medium consisted of Dulbecco’s modified Eagle’s medium (DMEM) containing 1 µg/ml of TPCK-treated trypsin. Supernatants were collected every 12 h until 84 h post-infection (p.i.) and titrated by plaque assays using ST6GalI-MDCK cells.

Groups of 4 seronegative (900- to 1500-g) male ferrets (Marshall BioResources, North Rose, NY) were housed in individual cages separated to prevent cage to cage transmission. Animals were lightly anesthetized by isoflurane and received an intranasal instillation of 4-5 log TCID50/ml in a total volume of 250 µL (125 µL per nostril) of the WT virus or the LRVp9 variant. Nasal wash (NW) samples were collected on a daily basis until day 10 p.i. in awake animals by instillation of 5 ml of PBS into the external nares of animals. To avoid repeated anesthesia during NW sampling that may have an impact on the ferrets welfare, animals were acclimatized to human handling during 10 days prior to viral inoculation. Viral titers of NW samples were determined by plaque assays in ST6GalI-MDCK cells. A quantitative real-time RT-PCR targeting the influenza matrix (M) gene was performed for detection of viral RNA in NW. Sera were collected on days 0 and 14 p.i. to evaluate seroconversion by HA inhibition (HAI) assays.

Ethics statement
Animal procedures were approved by the Institutional Animal Care Committee of Armand Frappier Institute according to the guidelines of the Canadian Council on Animal Care.

Results
The WT and LRVp9 viruses showed comparable replication kinetics in vitro. The peak titer for both viruses (∼10^3 PFU/ml) was reached at 60 h p.i. (Figure 1). Sequencing of the viral HA/NA genes recovered at 72 h p.i. confirmed the genotypes present in the respective inoculum with no additional changes.

All ferrets were seronegative [HAI titers ≤20 for A/Uruguay/716/2007 (H3N2), an A/Brisbane/10/2007-like strain] before viral inoculation. The 4 ferrets inoculated with the WT virus seroconverted with HAI titers of 640–1280 on day 14 p.i. whereas only one ferret from the LRVp9 group seroconverted (HAI titer of 160).

Ferrets inoculated with the WT virus had a mean peak viral titer in NW samples of 3 × 10^8 PFU/ml on day 2/3 p.i. (range 5.2 × 10^8 to 7.2 × 10^8 PFU/ml). The ferrets cleared the virus by day 6 after infection (Figure 2). The ferrets inoculated with LRVp9 did not shed infectious virus. Viral RNA could be detected by quantitative RT-PCR in NW of the four ferrets inoculated with the WT virus on day 1 p.i. (range 1.31 × 10^7 to 7.44 × 10^7 RNA copies/µl) with two animals (ferrets 1 and 2) remaining positive on day 10 p.i. (8.1 × 10^7 and 5.85 × 10^7 RNA copies/µl, respectively) (Figure 3A). By contrast, only one ferret from the mutant group was positive on day 1 p.i. (with 6.59 × 10^7 RNA copies/µl) while no viral RNA could be detected in any ferret after day 8 p.i. (Figure 3B). Although not statistically significant by unpaired t-test, there was a ≥1 log reduction in viral RNA copies/µl for the mutant samples vs respective WT samples at most time points (Figure 3). Of note, viruses sequenced on day 6 p.i. displayed expected genotypes (Figure S1).

Discussion
In this study, we evaluated the viral fitness of an NAI-resistant influenza A/Brisbane/10/2007-like (H3N2) strain containing a large NA deletion and a P194L HA substitution that emerged under in vitro laninamivir pressure. This variant corresponds to the 9th passage in which the growth medium contained 2 µM of laninamivir. In addition to the P194L mutation, the HA protein of this variant contained an S138A substitution, which is unlikely to be linked to the NAI pressure as it was also detected in the virus that was subjected to 9 passages without drug. When blast analyses using GenBank database sequences were performed, the 194L HA genotype was detected in numerous American, Asian, and Australian A(H3N2) viruses that have circulated since 2007 whereas no large NA deletion equivalent to that of LRVp9
could be detected. Residue 194 of influenza A(H3N2) HA is located at the globular head of the molecule and is part of the RBS. Therefore, changes at this position could naturally occur during influenza evolution contrasting to the internal region of NA protein which contains the highly conserved active site whose deletion seems to be strictly linked to the NAI pressure.

Previous studies already described the effect of NAI-resistance framework \(^\text{11}\) and functional \(^\text{12}\) NA substitutions on infectivity of A(H3N2) variants using the ferret model. However, the impact of large NA deletions on viral fitness of A(H3N2) viruses remains poorly studied. Mishin and colleagues previously described the properties of A(H1N1) variants harboring a large NA deletion in ferrets.\(^\text{13}\) Nevertheless, the variant of that study did not contain any HA change. Thus, assessing the impact of the NA/HA changes identified in our A(H3N2) variant on viral fitness in vitro and in ferrets was particularly relevant. Despite efficient replication of the LRVp9 mutant in ST6GalI-MDCK cells, no cytopathic effects could be seen when we used native MDCK cells. Evidence of altered binding to red blood cells of LRVp9 was previously demonstrated using HA-elution assays.\(^\text{9}\) On the other hand, viral infectivity of LRVp9 was severely impaired in ferrets. This could be explained by the absence of NA activity as the catalytic head domain containing the active site of the NA enzyme was missing. Interestingly, previous reports demonstrated that the functional R292K NA substitution, which causes a significant reduction of NA activity, was associated with decreased infectivity and transmissibility of NAI-resistant A(H3N2) viruses in ferrets.\(^\text{11}\)

Therefore, a certain level of NA activity seems to be necessary for efficient infectivity and transmissibility of A(H3N2) viruses in vivo.

A functional balance between the NA and HA of influenza viruses is necessary for both efficient replication and transmission of influenza viruses.\(^\text{14}\) Thus, NA mutations that reduce the NA activity could be compensated by HA mutations that reduce viral binding. This could be the case of the P194L mutation within the RBS of the HA glycoprotein. Notably, the P194L HA substitution was also selected together with the E119A NA substitution in a NAI-resistant influenza A(H5N1) variant under oseltamivir pressure.\(^\text{15}\)

The infectivity of LRVp9 was impaired in vivo, with only 1 of 4 ferrets seroconverting, and this ferret did not shed any infectious virus. Contrasting with in vitro observations, the P194L HA mutation did not restore the viral fitness in vivo. Also, the absence of viral shedding in the ferrets inoculated with the mutant may be explained by the large NA deletion potentially impairing the liberation of new virions and their spread. The number of infectious particles in respiratory tract secretions is a determining factor for transmission by the airborne route or by direct contact.\(^\text{12}\) In that regard, the LRVp9 mutant is unlikely to be transmissible in vivo.

A limitation of our study is that we did not investigate the specific role of the NA and HA mutations that occurred in our drug-selected A(H3N2) variant.

**Conclusions**

In conclusion, we showed that the NA-defective, HA-mutated A(H3N2) variant conserved its viral fitness in vitro, but did not replicate in the upper respiratory tract of ferrets. Constant surveillance of the emergence of drug-resistant virus variants with adequate evaluation of key biological properties in relevant animal models is necessary.
Acknowledgements

This work was supported by a research grant from the Canadian Institutes of Health Research (CIHR-Team Leader) to GB and GlaxoSmithKline Biologicals S.A. GB is the holder of the Canada Research Chair on Emerging Viruses and Antiviral Resistance from the Canadian Institutes of Health Research.

Conflict of interests

GB has received research funds from Biota Scientific Management, GlaxoSmithKline Biologicals S.A., Hoffmann La Roche, and Abbott. EB, MHJ, KD, and CM are employees of the GSK group of companies.

References

1 Kaji M, Watanabe A, Aizawa H. Differences in clinical features between influenza A H1N1, A H3N2, and B in adult patients. Respirology 2003; 8:231–233.
2 Bright RA, Shay DK, Shu B, Cox NJ, Klimov AI. Adamantane resistance among influenza A viruses isolated early during the 2005–2006 influenza season in the United States. JAMA 2006; 295:891–894.
3 Pizzorno A, Abed Y, Boivin G. Influenza drug resistance. Semin Respir Crit Care Med 2011; 32:409–422.
4 Birnkrant D, Cox E. The Emergency Use Authorization of peramivir for treatment of 2009 H1N1 influenza. N Engl J Med 2009; 361:2204–2207.
5 Release FN. FDA approves Rapivab to treat flu infection. Available at http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm427755.htm (accessed 22 December 2014).
6 Watanabe A. A randomized double-blind controlled study of laninamivir compared with oseltamivir for the treatment of influenza
in patients with chronic respiratory diseases. J Infect Chemother 2013; 19:89–97.
7 Watanabe A, Chang SC, Kim MJ, Chu DW, Ohashi Y. Long-acting neuraminidase inhibitor laninamivir octanoate versus oseltamivir for treatment of influenza: a double-blind, randomized, noninferiority clinical trial. Clin Infect Dis 2010; 51:1167–1175.
8 Kubo S, Tomozawa T, Kakuta M, Tokumitsu A, Yamashita M. Laninamivir prodrug CS-8958, a long-acting neuraminidase inhibitor, shows superior anti-influenza virus activity after a single administration. Antimicrob Agents Chemother 2010; 54:1256–1264.
9 Samson M, Abed Y, Desrochers FM et al. Characterization of drug-resistant influenza virus A(H1N1) and A(H3N2) variants selected in vitro with laninamivir. Antimicrob Agents Chemother 2014; 58:5220–5228.
10 Ellis J, Iturriza M, Allen R et al. Evaluation of four real-time PCR assays for detection of influenza A(H1N1)v viruses. Euro Surveill 2009; 14: pii:19230.
11 Herlocher ML, Truscon R, Elias S et al. Influenza viruses resistant to the antiviral drug oseltamivir: transmission studies in ferrets. J Infect Dis 2004; 190:1627–1630.
12 Herlocher ML, Carr J, Ives J et al. Influenza virus carrying an R292K mutation in the neuraminidase gene is not transmitted in ferrets. Antiviral Res 2002; 54:99–111.
13 Mishin VP, Nedyalkova MS, Hayden FG, Gubareva LV. Protection afforded by intranasal immunization with the neuraminidase-lacking mutant of influenza A virus in a ferret model. Vaccine 2005; 23:2922–2927.
14 Zanin M, Marathe B, Wong SS et al. Pandemic Swine H1N1 Influenza viruses with almost undetectable neuraminidase activity are not transmitted via aerosols in ferrets and are inhibited by human mucus but not swine mucus. J Virol 2015; 89:5935–5948.
15 Ilyushina NA, Bozin NV, Webster RG. Decreased neuraminidase activity is important for the adaptation of H5N1 influenza virus to human airway epithelium. J Virol 2012; 86:4724–4733.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Multiple sequence alignment of the neuraminidase (panel A) or hemagglutinin (panel B) protein of the influenza A/Brisbane/10/07-like H3N2 viruses used in this study was performed using clustal W (1.83).