Mechanisms of resistance to neuraminidase inhibitors in influenza A viruses and evaluation of combined antiviral therapy

Thèse

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Résumé

Les inhibiteurs de la neuraminidase (INAs) jouent un rôle central dans le contrôle des infections grippales, tant dans le cas des épidémies et des pandémies comme chez les patients immunosuprimés et d'autres patients à risque. Cependant, le développement et la dissémination de la résistance compromettent l'utilité à long terme de cette intervention. En fait, le problème de la résistance aux INAs a été mis en évidence pendant les épidémies de grippe annuelles de 2007-09, avec la dissémination globale d'une variante de la souche A(H1N1) saisonnière résistante à l'oseltamivir. Dans ce cas, les observations préliminaires ont spéculé avec l'existence d'un ensemble de mutations "permissives" qui auraient facilité cette transmission mondiale. Heureusement, l'émergence et la propagation mondiale de la souche pandémique en 2009 a mené au remplacement de la souche saisonnière A/Brisbane/59/2007 (H1N1) résistante à l'oseltamivir, par le virus A(H1N1)pdm09 naturellement sensible aux INA, et, par conséquent, l'oseltamivir a récupéré son utilité clinique. En fait, la plupart des virus A(H1N1)pdm09, A(H3N2) et B circulants à ce jour restent sensibles à l'oseltamivir, avec seulement 1-2% de souches résistantes. Néanmoins, le nombre croissant de souches résistantes récemment détectées en l'absence de traitement fait craindre que ce problème puisse encore augmenter. À cet égard, l'impact de l'émergence et la dissémination de la résistance sur le choix limité des antiviraux actuellement disponibles renforce la nécessité d'une meilleure compréhension des mécanismes sous-jacents à ce phénomène ainsi que de nouvelles approches thérapeutiques. Les différentes études présentées dans le cadre de cette thèse convergent vers l'objectif général de mieux décrire les mécanismes de développement de la résistance aux INAs dans les virus de la grippe. En outre, nous prévoyons que les thérapies combinées pourraient induire une meilleure réponse virologique et immunologique par rapport à la monothérapie antivirale. À la fin, nous nous attendons à ce que notre travail ait un impact sur la gestion des infections grippales en guidant la surveillance mondiale des marqueurs potentiels de résistance, ainsi qu'en proposant des traitements novateurs qui minimisent le développement de souches résistantes.

Abstract

Neuraminidase inhibitors (NAIs) play a central role in the control of influenza infections, with important implications in the management of outbreaks and pandemics as well as in immunocompromised and other at risk patients, with both prophylactic and therapeutic indications. However, the development and dissemination of antiviral drug resistance represents a major limitation that compromises the long-term usefulness of this intervention. Actually, the problem of resistance to NAIs was highlighted by the worldwide dissemination of the oseltamivir-resistant seasonal A(H1N1) neuraminidase H274Y variant during the 2007-09 annual influenza epidemics. In that case, preliminary observations speculated with the existence of a set of “permissive” mutations that could have facilitated this global transmission. Fortunately, the antigenic shift that enabled the emergence of and global spread of the 2009 pandemic strain meant the replacement of the oseltamivir-resistant seasonal A/Brisbane/59/2007 (H1N1) virus by the naturally NAI-susceptible A(H1N1)pdm09 virus, and, consequently, oseltamivir recovered its clinical utility. In fact, most of the circulating A(H1N1)pdm09, A(H3N2) and B viruses remain susceptible to oseltamivir with only 1-2% of tested strains exhibiting phenotypic or genotypic evidence of resistance. Nevertheless, the growing number of resistant strains recently detected in the absence of therapy raises concern that this problem could increase. In that regard, the impact of the emergence and dissemination of resistance on the limited choice of antivirals currently available underscores a better understanding of the mechanisms underlying this phenomenon as well as the necessity for innovative therapeutic approaches. The different studies presented in this thesis converge to the general objective of better describing the mechanisms underlying the development of resistance to NAIs in influenza viruses. Also, we anticipate that combination therapies will induce better virological and immunological responses compared to antiviral monotherapy. In the end, we expect that our work will have an impact on the management of influenza infections by guiding the global surveillance of potential drug resistance markers, as well as proposing innovative ways to improve the clinical outcome and minimizing the development of drug-resistant strains.

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Abbreviation list

a.a. or aa: amino acid
A(H1N1)pdm09 or pH1N1: 2009 pandemic influenza A(H1N1)
ANOVA: analysis of variance
AUC: area under curve
CDC: Centers for Disease Control and Pevention
cDNA: complementary DNA
CNS: central nervous system
CMV: cytomegalovirus
cRNA: complementary RNA
CTLs: cytotoxic lymphocites
Da: dalton
DANA: 2,3-didehydro analogue of the N-acetyl-neuraminic acid
DNA: desoxyribonucleic acid
e.g.: *exempli gratia* (for example)
EDTA: ethylenediaminetetraacetic acid
ER: endoplasmic reticulum
EUA: Emergency Use Authorization
FDA: Food and Drug Administration
g: gram
h: hour
H or HA: hemagglutinin
i.e.: *id est* (that is)
IC_{50}: 50% inhibitory concentration
Ig: immunoglobulin
IFN: interferon
IL: interleukin
IM: intramuscular
IV: intravenous
K: kilo
Km: Michaelis constant
l: liter
LAIV: live attenuated influenza vaccine
LVT: lung viral titer
MDCK: Madin-Darby canine kidney
MDVs: master donor virus
MEM: Modified Eagle Medium
MOI: multiplicity of infection
mm: millimeter
ml: milliliter
mRNA: messenger RNA
MUNANA: methylumbelliferyl-N-acetylneuraminic acid
µg: microgram

μM: micromolar
N or NA: neuraminidase
NAI: neuraminidase inhibitor
NES: nuclear export signal
Neu5Ac: N-acetyl neuraminic acid
NLSs: nuclear localization signals
nM: nanomolar
nt: nucleotide
ORF: open reading frame
p.i. or PI: post-infection
PBS: phosphate-buffered saline
PFU: plaque forming unit
RBS: receptor binding site
RNP: ribonucleoprotein
RNA: ribonucleic acid
RT-PCR: reverse transcriptase-polymerase chain reaction
sec: seconds
sd or SD: standard deviation
SNPs: single nucleotide polymorphisms
svRNA: small viral RNA
TCID50: 50% tissue culture infectious dose
TNF-γ: tumor necrosis factor alpha
US: United States
Vo: substrate conversion velocity
Vmax: maximum velocity
VLPs: virus-like particles
vRNA: viral RNA
WHO: World Health Organization
wt or WT: wild-type

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Foreword

As part of this thesis, I present a review of the most up-to-date literature on influenza viruses and influenza infections. In that regard, general aspects such as the history and evolution of influenza infections, their impact on public health, as well as the epidemiology and clinical aspects are addressed. The structure of the viral particle, the virus replicative cycle and genetic features are also described. Particular attention was paid to the major role of antiviral drugs for the prevention and treatment of influenza virus infections, and the different mechanisms underlying the development of antiviral resistance, which compromise the long-term usefulness of this intervention.

The results of this doctoral project are presented in the form of seven scientific articles (chapters III to IX), all of which are already published in international peer-reviewed journals and available through PubMed. The articles are presented in their final versions accepted for publication. A specific foreword, summarizing the context in which the studies were conducted, the main objectives, author contributions, as well as journal publication details can be found at the beginning of each corresponding chapter.

Chapter I: Introduction

Section I: Influenza viruses

1.1 History

Influenza or flu is a highly contagious respiratory disease that has affected humans since ancient times. In fact, flu symptoms were already described in humans by Hippocrates 2400 years ago [282]; nevertheless, historical data on influenza is quite difficult to interpret because clinical symptoms may be similar to those of a panoply of other diseases, such as diphtheria, pneumonia, dengue and typhoid. The first detailed record of an influenza pandemic occurred in 1850, starting in Asia and then spreading to Europe and Africa. Among the different pandemics that occurred during the 17th and 18th centuries, that of 1830-33 was particularly virulent and caused high morbidity, affecting over a quarter of the population exposed to the virus [349]. Although influenza viruses have killed millions of people in recent centuries, the 1918-19 pandemic, caused by an influenza A virus of the H1N1 subtype (please refer to section 1.2 below for the classification system), was particularly lethal. Since most of the published data available came from Spain, the 1918-19 pandemic would be historically known as the "Spanish flu". Older estimates speak of about 40 to 50 million deaths [332] due to the Spanish flu although the most current approximations agree on a worldwide death toll between 50 and 100 million people [241]. Many hypotheses could explain the extraordinarily high mortality of the Spanish flu, with the high infectivity rate (up to 50% of the exposed population) and the severity of symptoms caused by the induction of a massive production of proinflammatory cytokines ("cytokine storm") as well as bacterial superinfections as the most relevant [332].

In 1933, W. Smith and colleagues became the first to isolate an influenza virus from a human being [469]. Because it was the first human influenza virus, it was named influenza A virus. Recent retrospective seroepidemiological analysis, also known as seroarcheology, suggested that influenza epidemics that occurred in humans during 1889 and 1890 were caused by a virus that was antigenically similar to more contemporary Asian influenza A(H2N2) strains [469]. Moreover, the 1900 influenza epidemic might have been caused by a virus with an HA similar to that the pandemic A/Hong Kong/68 (H3N2) virus but with the NA from A/Equine/Miami/1/63 (H3N8). It was originally believed that the cause of the 1918-19 pandemic was an avian-like A(H1N1) virus and this

seroepidemiological information was then confirmed by sequence analysis of viral RNA in lung tissues collected from people who died at the peak of the pandemic [356, 415]. A few major antigenic changes have also occurred later in the past century, evidenced by the replacement of the circulating A(H1N1) virus by the A(H2N2) subtype (Asian influenza) in 1957, as well as by the emergence of the A/Hong Kong (H3N2) strain in 1968 or the re-emergence of the A(H1N1) virus in 1977. Finally, in late April 2009, a novel A(H1N1) influenza virus began in Mexico before spreading worldwide within six weeks, officially becoming the first pandemic of the 21st century on June 11, 2009 [459]. At least 18449 laboratory-confirmed deaths in 214 countries can be attributed to the 2009-10 influenza A(H1N1) pandemic, almost 450 of which happened in Canada [341]. Nonetheless, the number reported to the World Health Organization (WHO) by its member states is widely considered a gross underestimate of the real burden of the disease, which is believed to have been responsible for up to 400000 deaths worldwide [93], the equivalent of a seasonal epidemic.

1.2 Classification

Influenza viruses belong to the *Orthomyxoviridae* (from the Greek *orthos*, "straight" and *myxo* "mucus") family of RNA viruses, which is composed of six genera: influenzavirus A, B and C, isavirus, thogotovirus, and quaranjavirus [336]. The first three genera contain viruses that cause influenza in vertebrates, including birds, humans, and other mammals. Isaviruses infect salmon; thogotoviruses infect vertebrates and invertebrates, such as mosquitoes and sea lice; and quaranjaviruses infect humans, ticks and seabirds.

Influenza viruses are divided into three types (A, B and C), and current nomenclature system [460] includes the genus (type), the species from which the virus was isolated, location of isolate, number of the isolate and the year of isolation. Also, in the case of the influenza A viruses, the specific type of hemagglutinin (HA) and neuraminidase (NA) proteins, the two major surface antigenic determinants, determine the virus subtype, which is reported between brackets [e.g.: A/Swine/Iowa/15/30 (H1N1)]. By convention, in the case of human isolates, the source host is not included in the nomenclature [e.g.: A/Puerto Rico/8/34 (H1N1)]. Until recently, there were 16 known types of HA (H1 to H16) and 9 known types of NA (N1 to N9), which mathematically gives 144 different potential combinations of these proteins. All H1 to H16 types were found to circulate in

waterfowl, the main natural influenza A reservoir. Nevertheless, two new HA types (H17 and H18) have been recently discovered in bats from Central and South America [425, 426], which could also give rise to two new NA types (N10 and N11) yet this latter point remains to be fully confirmed through sequence-identity analyses.

On the other hand, influenza B viruses are essentially restricted to humans even if a few isolates have also been reported in other mammals, i.e. seals [328]. These viruses carry a single variant of either HA and NA, though the amino acid sequence of both proteins can vary from one B strain to another [259].

Influenza C viruses have been primarily isolated from humans but also from swine in China and the US [169]. Infection of influenza C virus does not cause any severe symptoms in humans or other mammals and therefore it has not been thoroughly studied. However, recent work from Hause et al. [168] claims to have characterized a new variant of influenza C from swine and cattle that could lead to a revision in the genera classification of the *Orthomyxoviridae* family.

1.3 Viral isolation and propagation

Influenza viruses can be grown either in embryonated chicken eggs or in primary cell culture systems. Viral replication in these systems can be indirectly detected and measured by quite a simple test called hemagglutination assay, based on the ability of the viral HA protein to agglutinate erythrocytes, and therefore suggesting the presence of the viral progeny [186], or by use of molecular biology techniques, such as reverse transcriptase (RT)-polymerase chain reaction (PCR). The embryonated egg system was used for the first isolation of an influenza virus in the 1930s and is still the culture system of choice for the production of large amounts of virus and for vaccine manufacturing [469]. Many strains of human influenza A and most of the avian strains can be isolated through direct inoculation into the allantoic cavity of embryonated chicken eggs whereas some influenza A and B viruses must be previously isolated in the amniotic cavity and subsequently adapted to grow in the allantoic cavity of chicken embryos.

On the other hand, while embryonated eggs are used mainly for large scale viral production, the cell culture system is generally used for the primary isolation and laboratory study of influenza

viruses from human and animal biological samples. Influenza viruses replicate on various cell types including monkey kidney, goat, hamster, chicken, as well as cells from the human respiratory epithelium, causing a characteristic cytopathic effect as well as lysis plaques. However, few of these cellular systems are useful for plaque formation by influenza virus unless exogenous trypsin is added to activate the HA molecule. As a result, Madin-Darby canine kidney (MDCK) cells [423] and A549 adenocarcinoma human alveolar basal epithelial cells [141] are currently the two most used cell lines for influenza virus isolation. A decade ago, a variant of the MDCK cell line was developed by Matrosovich and collaborators [286]. The SIAT1-MDCK cell line expresses large amounts of neuraminic acid (known as "sialic acid", the cell receptor for influenza) in the α-2,6 linkage configuration, the predominant configuration in the human upper respiratory tract, and hence shows increased sensitivity for the isolation of human influenza viruses.

1.4 Virion structure

The structure of influenza viruses is rather complex, with A and B viruses being quite similar, whereas C viruses present a distinctive pattern of surface projections. Viral particles can be either spherical or pleomorphic with a diameter between 80-120 nm. Yet, filamentous forms reaching several micrometers have been described occasionally [387].

Influenza viruses are enveloped viruses that possess a lipid membrane derived from the host cell. In the case of influenza A viruses, this envelope harbors three types of proteins: the HA, NA and M2 (Figure 1.1). The rodlike HA and mushroom-shaped NA are anchored to the envelope by short sequences of hydrophobic amino acids and project radially from the surface of the virus forming distinctive spikes, which are readily observable in electron micrographs. These spikes have lengths of about 10-14 nm, with an approximate ratio of 4 HA to 1 NA, and constitute the major viral surface antigens [305]. The matrix protein (M1) lies just underneath the envelope and provides shape and stability to the shell [470]. The core of the virus particle is comprised of the ribonucleoprotein (RNP) complex, from which three main components can be distinguished; 1) the viral genome: a single-stranded, negative sense (the template from which the viral messenger RNA (mRNA) is synthesized) segmented RNA genome; 2) the nucleoprotein (NP), and; 3) the RNA polymerase complex, formed by the polymerase

basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acid (PA) proteins, responsible for replication and transcription of the RNA [387].

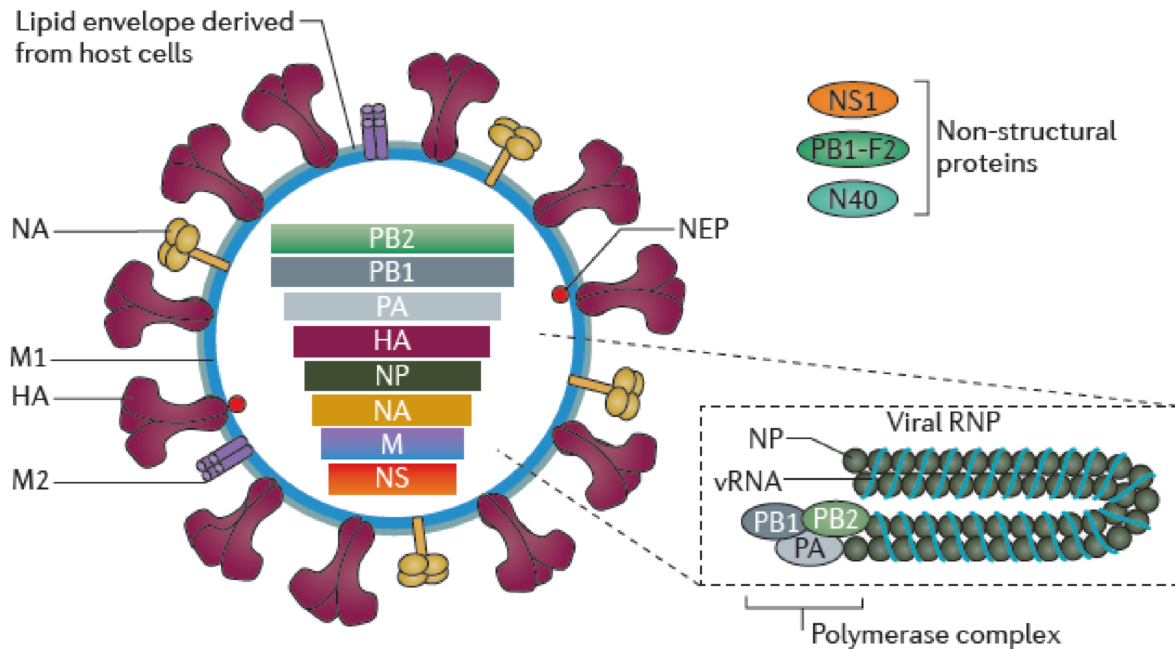

Figure 1.1. Schematic representation of the influenza A virus particle. Taken from [293].

Influenza A and B viruses have 8 RNA segments whereas type C viruses have only 7. The complete genome of influenza A viruses contains about 13600 nucleotides (nt), the influenza B contains 14600 nt and influenza C contains 12900 nt [387]. Each segment is associated with many copies of the NP as well as the three polymerase proteins in the RNP complex and generally encodes for one polypeptide, with the exception of segments M and NS that give rise to spliced messenger RNAs coding for two polypeptides each (M1-M2, and NS1-NEP/NS2, respectively). The two "non-structural" (NS1 and NEP/NS2) proteins encoded by the virus are found in infected cells, whereas NEP/NS2 is also found in the virion. The NEP/NS2 protein, which for a long time was thought to be a non-structural protein, has recently been shown to be a structural component of the viral particle in association with the M1 protein and present in about 130-200 molecules per virion [360]. Of note, two other proteins, PB1-F2 and PB1-N40, are expressed from alternative open

reading frames within the PB1 gene [84, 466], although not all influenza A virus strains encode these proteins, making them true accessory proteins.

1.5 Genome organization

The eight RNA segments that constitute the genome of influenza A viruses are numbered in descending order from the longest (1) to the shortest (8) and, as mentioned, code for up to 12 proteins: PB2, PB1, PB1-F2, PB1-N40, PA, HA, NA, M1, M2, NP, NS1 and NEP/NS2 (Figure 1.2). Each viral segment contains noncoding regions at both 5' and 3' ends. The extreme ends are conserved among all segments, and this is followed by a segment-specific noncoding region [387].

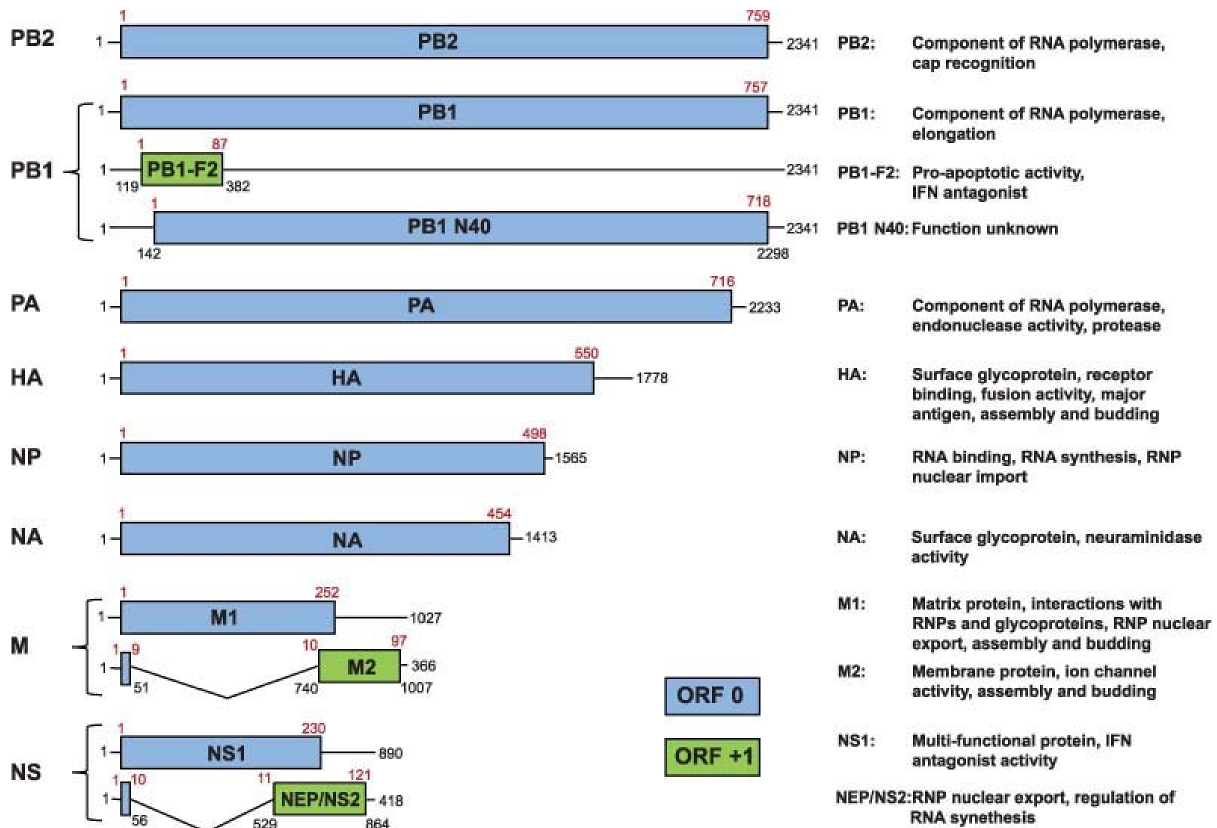

Figure 1.2. Genome segments of influenza A/Puerto Rico/8/34 virus. RNA segments (in nt) are shown in positive sense and their encoded proteins (in aa). The lines at the 5' and 3' termini represent the noncoding regions. The polymerase basic 1 protein (PB1) segment contains a second open reading frame (ORF) in the +1 frame resulting in the PB1-F2 protein and a third ORF in the 0 frame resulting in the PB1-N40 protein. The M2 and NEP/NS2 proteins are encoded by spliced mRNAs. Taken from [387].

1.5.1 Surface glycoproteins

1.5.1.1 Hemagglutinin

The **HA** protein is encoded by viral RNA segment 4 and comprises ~550 aa, although small differences in the exact length of viral proteins can be observed between strains. It is a type I integral membrane protein, and represents the major glycoprotein and subtype-specific protein of influenza A and B viruses. The HA is a rod-shaped homotrimer of non-covalently linked monomers, with the C-terminus inserted into the viral membrane and the hydrophilic end projecting as a spike away from the viral surface ending in a globular head (Figure 1.3) [463, 465]. The HA is synthesized by ribosomes bound to the membrane of the endoplasmic reticulum (ER) of the infected cells and then translocated to the lumen as a single 550-aa polypeptide called HA_0. Then, the HA undergoes three types of post-translational modifications: proteolysis, glycosylation and acylation. Each HA_0 monomer is processed by cellular proteases into two fragments, HA_1 and HA_2, which remain linked by disulfide bonds [406]. The HA_1 is comprised of about 324 aa and mainly occupies the globular head of the spike, bearing a variable carbohydrate moiety that harbors the receptor binding cavity and five epitopes (antigenic determinants). The HA_2 bears approximately 222 aa and is known as the "fusion peptide". It comprises a variable portion of carbohydrates and three palmitate residues that form, along with a small portion of the HA_1, the stalk of the HA protein. The hydrophobic C-terminus of HA_2 is anchored in the viral envelope and is highly conserved among the different strains of influenza, provided its crucial role in the HA fusogenic activity [133].

The HA protein was originally named so given its ability to agglutinate erythrocytes by binding to specific salic acid moieties found in the cell receptors. In fact, this capacity is related to one of the two key roles of the HA in the early stages of infection: 1) the specific binding to sialic acid moieties in the surface receptors of host cell enables the attachment of the viral particle to the host cell, and; 2) the HA, through its HA_2 domain, mediates the fusion of the viral membrane of the endocytosed particle with that of the endosome, therefore enabling the penetration and uncoating of the virus into the cytoplasm [387]. The different stages of the viral replicative cycle are reviewed in detail in section 1.6.

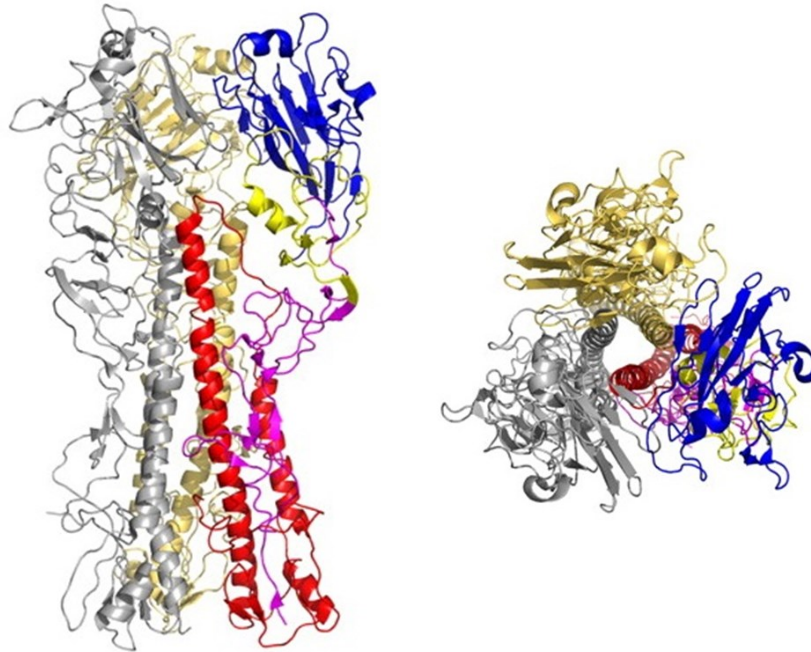

Figure 1.3. Crystal structure of the 1918 influenza A virus HA. Lateral (left) and top (right) views of the HA protein. Two of the monomers from each trimer are in gold and silver, whereas the subunits that make up the third monomer are colored as follows: blue, receptor binding; yellow, vestigial esterase; and magenta and red, fusion subdomains. Adapted from [133].

Structure of the receptor binding site

While evidence of the capacity of influenza viruses to bind oligosaccharides containing sialic acid dates back to the 1950s, it was not until 30 years later with the structural elucidation of the HA ectodomain alone [465] and subsequently complexed with sialic acid analogues [377, 449] that a better comprehension of the interaction between the HA and the receptor was achieved. It was then proposed that the receptor binding site (RBS) consisted of a deep cavity located in the globular domain of each HA monomer of HA [465], and that aa located in this area were highly conserved, while nearby residues were most easily affected by changes due to viral evolution through the years.

Figure 1.4 shows a schematic representation of a sialic acid molecule bound to the RBS of the HA. The base of the RBS is formed mainly by the residues Y98 and W153, which are part of a network of conserved hydrogen bonds comprising H183 and T195. While three (Y98, W153 and H183) of these four residues establish direct contact with the sialic acid molecule, the interactions

established by the four of them contribute to support the architecture of the binding cleft. If the HA is seen from the side, residues 134-138 constitute the front of the RBS, with the hydroxyl group of S136 and residues 135 and 137 forming hydrogen bonds with the sialic acid. The left side of the cleft consists of residues 224-228 and the side chains of residues E190 and L194, which are oriented downwards of the small α-helix in the distal part of the site to make contact with the receptor [77].

As mentioned, the HA is the major surface antigen of influenza viruses, hence inducing the formation of neutralizing antibodies which are very important in protecting the host against infection. Although the aa that form the receptor binding site, as well as the cysteine residues and most of the prolines are highly conserved, the remainder of the HA molecule is highly mutable, with the selection for aa substitutions being directed, at least in part, by the immune pressure.

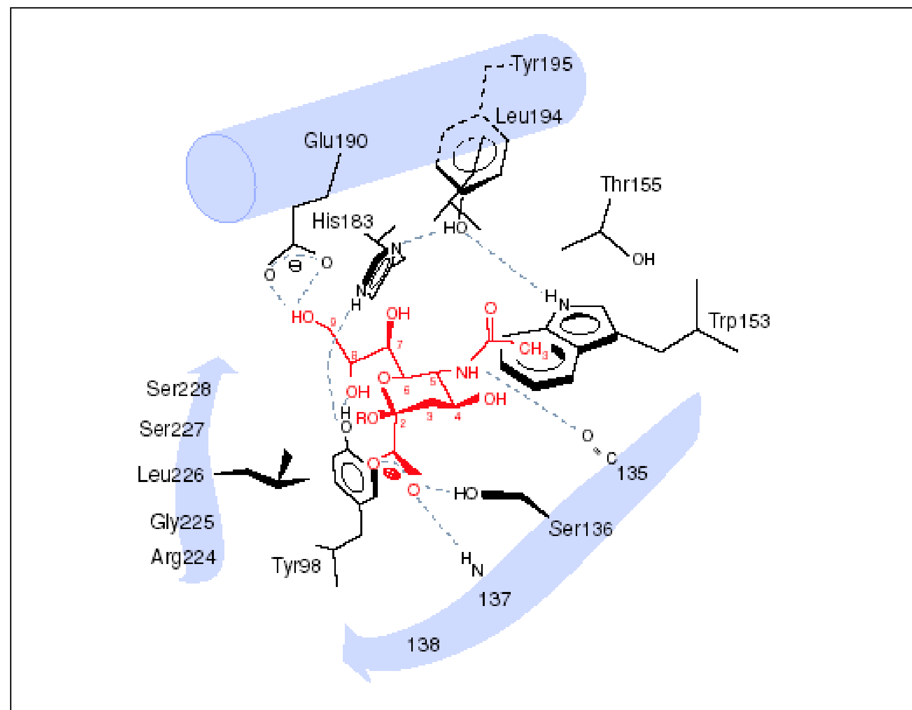

Figure 1.4. Schematic representation of a sialic acid molecule bound to the the RBS of the H1. HA residues are shown in black and the sialic acid molecule in red at the center of the figure. Hydrogen bonds are represented with gray dotted lines and the cylinder represents the small α-helix in the distal part of the binding site. Taken from [77].

Receptor binding specificity

The HAs from different influenza strains harbor different binding properties depending on the type of interaction between the sialic acid and the penultimate galactose of the oligosaccharide receptor, which can be through an α-2,3 (SAα-2,3Gal) or α-2,6 (SAα-2,6Gal) linkage. Although many aa might be involved in receptor-binding specificity, residues 226 and 228 of the HA_1 are the most commonly implicated in H2 and H3 viruses. Strains that bind through α-2,3 linkages usually have a glutamine at position 226 and a glycine at position 228, whereas those strains that bind through α-2,6 linkages tend to have leucine and serine at these positions, respectively [77]. For H1 viruses, aspartate or glutamate at position 190 determines preferential binding to α-2,3 or α-2,6 linkages, respectively [131, 158]. Amino acids at positions 136, 195, and 225 also affect receptor-binding affinity and specificity, although to different extents.

The prevalence of these sialic acid receptors in different tissues reflects the tropism of the virus in the different species, being the receptor-binding specificity of the HA responsible for the host range restriction of influenza virus. Human and classical A(H1N1) swine influenza viruses bind preferentially to α-2,6, whereas most avian and equine viruses have higher binding affinity for α-2,3 [293]. Studies of human respiratory tissue have shown that α-2,6 oligosaccharides are dominant on epithelial cells in nasal mucosa, paranasal sinuses, pharynx, trachea, and bronchi. Alternatively, α-2,3 oligosaccharides are found on nonciliated cuboidal bronchiolar cells at the junction between the respiratory bronchiole and alveolus, and on type II cells lining the alveolar wall [392]. Consequently, viruses with α-2,6 specificity, but not those with α-2,3 specificity bind to the epithelial cells lining the human trachea [211, 392], therefore suggesting that the limited human-to-human transmission of highly pathogenic avian A(H5N1) viruses likely reflects the restrictive replicative efficiency of these viruses in the upper portion of the respiratory tract, where transmission could occur via droplets generated by coughing and sneezing.

On the other hand, the presence of the two types of sialic acid molecules in the epithelial cells of the trachea of pigs accounts for the ability of both avian and human viruses to infect this species [211]. As a result, it has been proposed that pigs can play a central role in the onset of pandemics by acting as "mixing vessels" of antigenically distinct viruses, which might enable the emergence of a new reassortant virus from avian and human origin [276]. Indeed, as described later

in section 1.9.2.2, the A(H1N1)pdm09 virus is a triple reassortant virus originated with genetic material from an avian strain, two swine strains as well as a human strain [135].

HA cleavage and pathogenesis

HA is a critical determinant of the pathogenicity of avian influenza viruses. Cleavage of the precursor HA_0 into HA_1 and HA_2 and the consequent conformational change is a prerequisite to infectivity, with a clear link between HA cleavability and virulence [136, 137, 239].

Two groups of proteases are responsible for HA cleavage, and the tissue tropism of influenza viruses is partly determined by the availability of host proteases to recognize and cleave the two types of aa sequences found at the HA cleavage site. The first group of proteases recognizes a single arginine and cleaves all HAs. Members of this group include plasmin tryptase Clara, miniplasmin, and bacterial proteases [233, 261, 327]. The second group of proteases that cleave HA proteins comprises the ubiquitous intracellular subtilisin-related endoproteases furin and PC6 [191, 407, 442]. These enzymes are calcium dependent, have an acidic pH optimum, and are located in the Golgi and/or trans-Golgi network [443]. The cleavage efficiency of these ubiquitous proteases is determined by the sequence at the cleavage site and the absence or presence of a nearby carbohydrate chain on the HA molecule [190, 228]. The cleavage site of HA_0 comprises 19 residues (323-328 of HA_1, 329 and 1-12 of HA_2, with cleavage occurring specifically at the conserved R329 residue [37]. In avirulent avian as well as non-avian (including human) influenza viruses, the cleavage site consists of several basic residues, forming a consensus R-X-K*R^{329}-R sequence that contains a unique arginine upstream of the cleavage position (*). These viruses are cleaved only by proteases of the first group and therefore produce localized non-severe infection of the respiratory and/or intestinal tract. In contrast, highly pathogenic influenza viruses (HPIV) of the H5 and H7 subtypes contain multiple basic aa at the cleavage site (R-R-R-K-K*R^{329}-R) that is recognized by ubiquitous proteases of the second group. Consequently, these viruses can cause systemic infections in poultry [37, 469], with the exception of some H5 avian strains that have a multibasic recognition site for furin but contain a nearby oligosaccharide that blocks the accessibility of the protease cleavage site, therefore becoming avirulent [226, 227]. Furthermore, HPIV do not need the addition of exogenous proteases to grow and form viral plaques in cell culture as is the case of the viruses cleaved by proteases of the first group, with the A/WSN/33 (H1N1) strain being the only exception. The NA of the A/WSN/33 virus

binds and sequesters plasminogen, and the increased concentration of this plasmin precursor then enhances HA cleavage by this protease [148, 382].

1.5.1.2 Neuraminidase

The **NA** protein is encoded by viral RNA segment 6 and ~454 aa. As explained later in section 1.12.2, the NA constitutes the central protein for the purpose of this thesis, since it is the target of the main class of anti-influenza agents currently in use, namely the neuraminidase inhibitors (NAIs). The NA is a type II integral membrane protein, and represents the second major glycoprotein and subtype-specific protein of influenza A and B viruses. The N-terminus of the protein is oriented towards the interior of the viral particle, with a highly conserved short cytoplasmic tail and a hydrophobic transmembrane region that provides the anchor for the stalk and the head domains [133]. The structure of the head is a homotetramer (Figure 1.5), with each of the four monomers composed of six topologically identical beta sheets arranged in a propeller formation. Sugar residues are attached to four of the five potential glycosylation sites in the head [133]. Each NA monomer shows a deep central cavity on its surface, corresponding to the active site of the protein, therefore conferring catalytic (sialidase) activity to the NA. Sialidases catalyze the hydrolysis of terminal sialic acid residues from the newly formed virions and from the host cell receptors. Indeed, the NA exerts its main function through its sialidase activity by cleaving the α-ketosidic linkage between terminal sialic acid residues and a d-galactose or d-galactosamine from either the HA protein or host cell receptors. This is of vital importance in the context of infection due to 3 reasons [133, 331]:

1) As mentioned in 1.5.1.1, HA binding to sialic acid-containing receptors on the cell surface allows the virus to enter the target cell. However, by the same principle, progeny virions released from the infected cell would remain anchored to the cell surface at the sialic acid level. The enzymatic activity of the NA protein is required to remove the sialic acid, and thereby release the virus from the host cell.

2) Released viral particles are also coated with sialic acid. NA activity removes sialic acid from the carbohydrates present on the viral glycoproteins themselves so the individual virus particles do not aggregate.

3) Respiratory mucus is rich in sialic acid, which causes the HA molecules (and thus the virus) to remain attached to them. The NA thus has a role in cleansing the environment (e.g., mucus and cell surfaces) of sialic acid receptors to allow for virus spread through the respiratory tract.

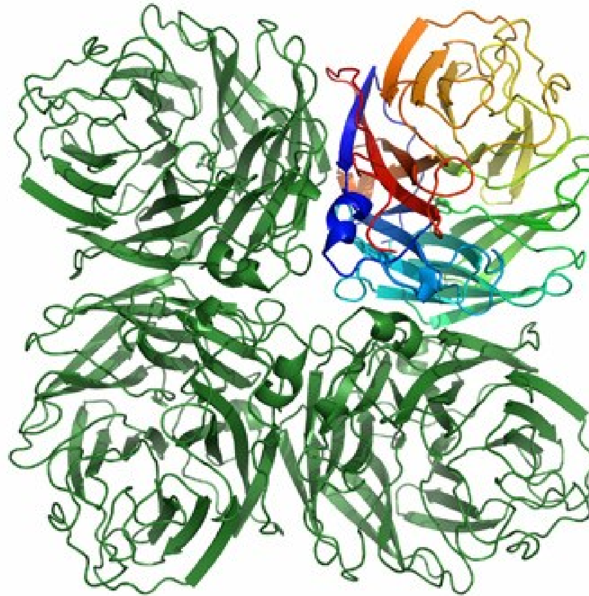

Figure 1.5. Crystal structure of the neuraminidase tetramer of the 1918 influenza A virus (top view). The tetramer is shown in green, with 1 of the 4 subunits colored from blue to red along the chain from the N- terminus to the C-terminus. Taken from [https://www.scripps.edu/news/scientificreports/sk2007/sk07wilson.html].

Evolutionary forces such as natural selection acting upon rapidly mutating viral populations could shape the genetic structure of influenza viruses in different hosts, geographic regions and periods of time (reviewed in section 1.7.4) [117]. Phylogenetic analyses demonstrated that the influenza NA gene diverged first into A and B, followed by the division of influenza A subtypes into two groups of viruses independently adapted to the avian, human, equine and swine hosts, indicating that parallel evolution occurred in these two subgroups (Figure 1.6) [472]. Group I consists of subtypes N1, N4, N5 and N8 whereas the remaining five subtypes (N2, N3, N6, N7 and N9) constitute group II. The phylogenetic tree of N1 NA genes shows two major branches that separate into human and classic swine, or Eurasian swine and avian N1 genes, respectively. The phylogenetic tree of the N2 NA gene can be divided into a North American avian clade, and a second clade that evolved into Eurasian avian and human virus genes at the beginning of the last century [472].

Importantly, this rapid evolution could partially facilitate the ability of influenza viruses to cross host species barriers and successfully emerge in new hosts with often important public health and/or veterinary health implications. As in the case of the HA, NA molecules are antigenic, with four antigenic sites being identified on the NA of influenza A viruses, each comprising multiple epitopes [452]. However, and although antigenic variants are selected in nature, specific antibodies directed against the NA are usually not neutralizing.

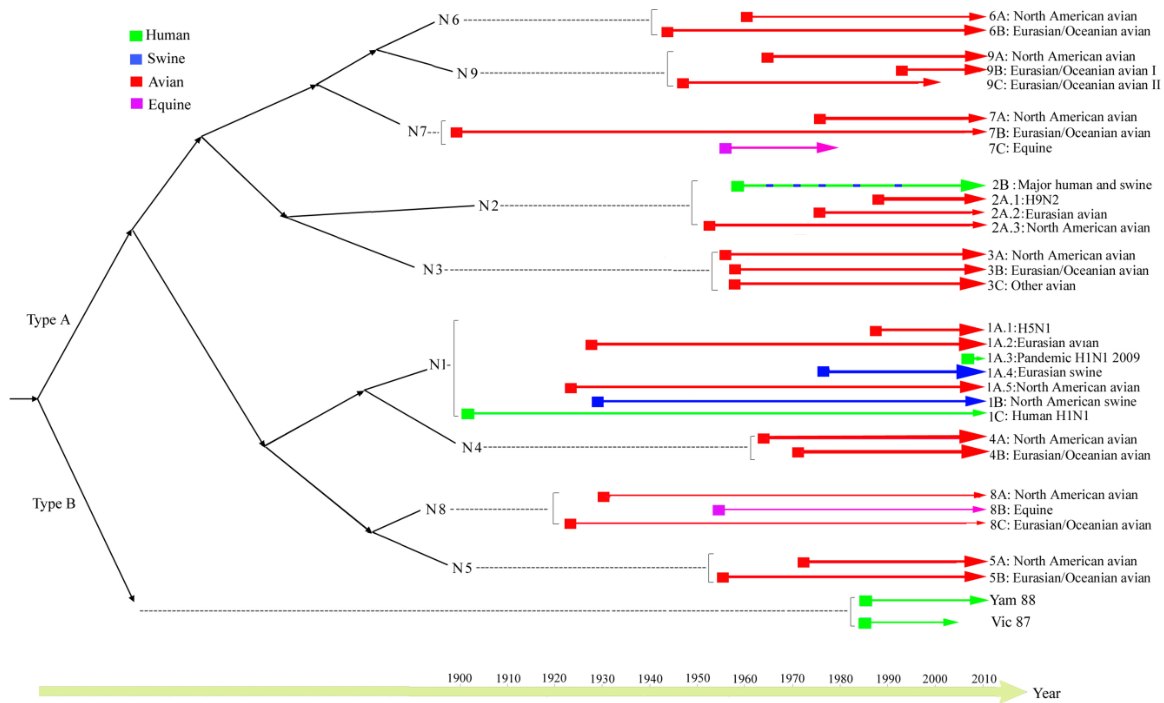

Figure 1.6. The evolutionary dynamics of influenza neuraminidase (NA) over time. The lineages from different hosts are colored, with the emergence times of the lineages represented by the horizontal positions of squared boxes and the mean substitution rates depicted by the degree of line thickness. Taken from [472].

Structure of the NA active site

Amino acids involved in the active site of NA were described based on differences in electron density maps following the interaction between the NA and sialic acid [68]. Results showed that the catalytic site of the NA is constituted of eight functional residues (R118, D151, R152, R224, E276, R292, R371, and Y406; N2 numbering system[1]), surrounded by eleven framework residues (E119,

[1] Unless otherwise noted, the N2 numbering system will be used throughout this document.

R156, W178, S179, D198, I222, E227, H274, E277, N294, and E425) implicated in the stabilization of the active site structure (Figure 1.7) [68]. These residues are well conserved in all influenza A and B viruses [439], and mutagenesis in these specific positions usually results in a loss of enzymatic activity [2, 5, 265]. In fact, given its highly conserved structure as well as the important functional role in viral spread, the NA active site constitutes the target of the NAIs. However, structural analysis revealed that NAs belonging to group I (N1, N4, N5 and N8) have an additional cavity compared to group II NAs. This cavity, the "150-cavity", is located adjacent to the active site, between residues 147 and 152 of the 150-loop. All known influenza NA contain a 150-loop that may be in one of two conformations: an open conformation, which leads to formation of the 150-cavity, or a closed conformation, which leads to an active site lacking a 150-cavity. This is explained by the presence of a tyrosine at position 347 in the group I instead of an asparagine in group II, therefore changing the number and pattern of hydrogen bonds with the aa of the catalytic site [369]. Interestingly, the A(H1N1)pdm09 NA is an atypical group I NA, with some group II-like features in its active site, notably the lack of the 150-cavity [266]. Although the functional significance of this difference is unclear, this feature could have implications on resistance to NAIs as well as provide an additional target for developing other anti-NA drugs.

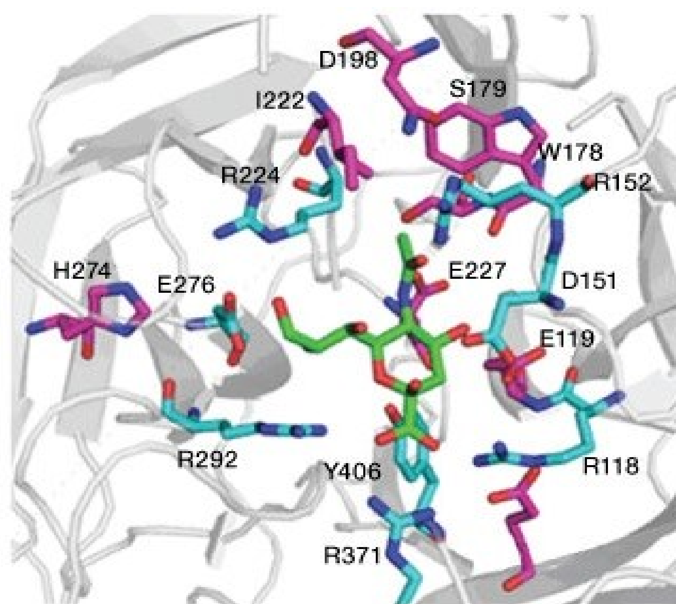

Figure 1.7. Computational modeling of the N9 in complex with sialic acid. Residues from the active site are displayed in stick form and the backbone is in ribbon form. Functional residues are represented in light blue and framework residues in purple, whereas sialic acid is shown in green. Taken from [314].

1.5.2 Other viral proteins

1.5.2.1 The RNA polymerase complex

The influenza viral RNA-dependent RNA polymerase is a 250-KDa complex formed mainly by three proteins (PB2, PB1 and PA) encoded in the three longest genomic RNA segments [206].

The **PB2** protein, encoded in viral RNA segment 1, is the largest viral protein, comprising ~759 aa. This protein is responsible for binding the cap on host nuclear and heterologous pre-mRNA molecules, therefore playing a critical role in the initiation of viral mRNA synthesis [31]. In addition to its transcriptional initiation role, viral endonuclease activity is partly associated with PB2, which is also involved in replication since some specific mutations in PB2 have been shown to affect replication but not transcription [138].

The **PB1** protein is encoded in viral RNA segment 2 and comprises ~757 aa. This protein catalyzes the sequential addition of nucleotides during RNA chain elongation and contains the conserved motifs characteristic of RNA-dependent RNA polymerases [39]. PB1 is also responsible for binding to the terminal ends of both viral and cellular RNAs [146] for the initiation of transcription and replication. Also, influenza A viruses can express an 11th protein, **PB1-F2**, which is encoded by the +1 alternate ORF in the PB1 gene [84]. PB1-F2 is 87 to 90 aa long, depending on the virus strain, and is expressed by most human A(H3N2) viruses, while a large number of human A(H1N1) isolates have a premature stop codon in the PB1-F2 ORF. Of note, the pandemics that occurred in 1918, 1957, and 1968 were all caused by influenza viruses that expressed full-length PB1-F2. The protein has been shown to contribute to influenza virus pathogenicity through several mechanisms. The protein localizes both in the inner and outer side of mitochondrial membranes, hence inducing alteration of mitochondrial morphology, dispersion of mitochondrial membrane potential and even cell death. A role as virulence factor through the induction of apoptosis in immune cells, with a subsequent reduction in viral clearance efficiency has been proposed [84]. In addition to its pro-apoptotic activity, PB1-F2 was reported to have pro-inflammatory properties. Specifically, it was observed that viruses expressing PB1-F2 increase the levels of several cytokines and chemokines, enhance cell infiltration, and exacerbate lung injury in infected mice [70]. Notably, it was found that a serine at position 66 in the PB1-F2 protein dramatically increases immunopathology and mortality caused by the 1918 pandemic strain and by highly pathogenic A(H5N1) viruses [71]. Finally, and as recently described by Wise and colleagues [466], the fifth initiation codon of RNA segment 2 (located

at position +40) can be used to initiate translation of another protein product, the **PB1-N40** protein. Since this alternative initiation codon is in frame with that of the PB1 protein, PB1-N40 could be considered as a truncated form of PB1 lacking the first 39 aa, including the N-terminal region that is essential for the interaction of PB1 with PA. The specific function of PB1-N40 is still unclear, and although it does not appear to be a strictly necessary protein, failure to produce it can impair viral replication under certain conditions.

The **PA** protein is encoded by viral RNA segment 3 and comprises ~716 aa. Like PB2 and PB1, this protein is located in the nucleus of infected cells. Until recently, the specific function of PA was unknown, but crystal structures of the N-terminal domain revealed that the endonuclease activity of the polymerase, which is required to generate the capped primer, resides in the PA protein [101, 484]. Previous work had mistakenly attributed this function to the PB1 protein. In the structure, the fold and position of the active site identifies the PA endonuclease as a member of the PD-(D/E)XK family of nucleases. PA does, however, participate in genome replication as mutations affecting this process have been described [193]. In addition to encoding nuclease function, the N-terminus of PA (aa 1-100) is also reported to be involved in an interaction with PB2, while the C-terminus makes contact with PB1 [179]. Another function ascribed to PA is that it possesses proteolytic activity, as well as it has been shown to be a target for casein kinase II and to be phosphorylated at serine and threonine residues [374].

1.5.2.2 Nucleoprotein

The **NP** protein is encoded by viral RNA segment 5 and comprises ~498 aa. Following synthesis in the cytoplasm, large amounts of NP proteins are transported to the nucleus of the infected cell, where they bind each one of the eight viral RNA segments, coating them individually [38]. The NP is the major viral protein in the RNP complex and the second most abundant in the viral particle. Besides its role as structural element, it constitutes the signal that directs RNA polymerase activity from viral RNA to the synthesis of either mRNA for transcription or complementary RNA (cRNA) for replication. cRNA is a faithful complementary copy of viral RNA and is used as a template for new viral RNA synthesis. At early times post infection, NP is localized predominantly in the nucleus whereas at later times it is found in the cytoplasm, indirectly reflecting the trafficking of RNPs during the virus life cycle [387]. Moreover, NP is a phosphorylated protein and one of the main

targets of the host's cytotoxic T-cell mediated immune response [40]. Antigenic differences in the NP protein (type-specific antigen), together with differences in the M1 protein, underlie the distinction among the genera influenzavirus A, B and C [387].

1.5.2.3 RNA segment 7 and encoded proteins: M1 and M2

The RNA segment 7 of the influenza A virus comprises encodes two proteins: M1 and M2. The 252-aa **M1** protein consists of two globular helical domains that are linked by a protease-sensitive region [12]. Rod-shaped M1 monomers are arranged in an ordered pattern with positive and negatively charged residues on opposite sides of the oligomer [165]. M1 is the most abundant protein in the viral particle and lies just beneath the lipid envelope, conferring rigidity to the membrane and hence receiving its "matrix" name. Apart from associating with the lipid membrane itself, M1 also makes contact with the cytoplasmic tails of the surface glycoproteins as well as with M2 and NEP/NS2 [307]. Moreover, M1 interacts with the RNPs, thereby forming a bridge between membrane proteins and the inner core components of the virion that is postulated to play a vital role in the recruitment of newly synthesized viral components to the site of assembly at the plasma membrane [307].

The 97-aa **M2** protein is a type III integral membrane protein, with a short ectodomain, a transmembrane domain, and a cytoplasmic domain with palmitate and phosphate modifications [343]. In its native form, M2 protein is a homotetramer composed of two dimers linked by disulfide bonds [21, 189]. Although this protein is expressed in large quantities in the plasma membrane of infected cells, only a few molecules (20-60) are incorporated into the viral particle [256, 487].

The M2 protein has been shown to possess ion channel activity, and its major role is conducting protons from the acidified endosomes into the interior of the virus to dissociate the RNP complex from the rest of the viral components, thus facilitating the uncoating process (described in section 1.6). The ion channel activity of M2 has also been implicated in stabilizing HAs from premature low pH transitions in the trans-Golgi network, as in the case of the previously mentioned HPIV [63]. This ion channel is acid gated (but not voltage gated) and highly selective for H^+ ions [343]. While the structure and the precise function of the extracellular portion of the M2 protein remain to be resolved, the structures of the transmembrane and cytoplasmic regions reveal a good understanding of the mechanism of proton conductance, which is controlled by the H37 and Y41

cluster [343]. The transmembrane region, when viewed from the top, shows four helices that sit at an angle in the lipid bilayer, hence forming a pore.

1.5.2.4 RNA segment 8 and encoded proteins: NS1 and NEP/NS2

The RNA segment 8 of the influenza A virus encodes two proteins: NS1 and NEP/NS2, where the former is encoded in a collinear mRNA transcript but the latter is a product of RNA splicing. The **NS1** protein is a dimeric protein that varies between 202-237 aa depending on the virus, and harbors three distinctive domains: a dsRNA-binding domain, an effector domain, and a disordered tail [159]. The dsRNA-binding domain lies within the N-terminal 73 aa and consists of a symmetric homodimer with a six-helical fold showing conserved tracks of basic and hydrophilic residues that mediate the interactions with the phosphate backbone of the RNA, accounting for the observed lack of sequence specificity [353]. The effector domain of NS1 keeps a very similar monomer conformation but with different dimer interfaces, such as strand-strand or helix-helix interactions, and also including binding sites for several host factors [159]. NS1 is a nuclear protein expressed in large amounts in infected cells, and is associated with polysomes [387]. Conversely, this protein has not been detected in the virion, hence its designation as non-structural protein. NS1 has multiple functions, including regulation of splicing, cytoplasmic transport of cellular mRNA and stimulation of translation [255]. However, perhaps the most important role of NS1 is that of suppressing virus-induced host type I interferon (IFN-α/β)-mediated antiviral response, with striking impact on viral pathogenicity. Indeed, NS1 appears dispensable in the absence of an IFN response whereas it is essential in the context of an immune-competent host [419].

The 121-aa **NEP/NS2** protein shares the first 10 N-terminal residues with NS1, whereas the rest of the coding sequence is translated from a different ORF. This protein localizes in both the cell nucleus [153] and cytoplasm [404], and it was originally considered to be a non-structural protein and was named “nonstructural protein 2” (NS2). More recently, however, it has been established that the protein is present in purified virions, where it associates with the M1 protein to mediate the export of RNPs from the nucleus to the cytoplasm [360, 476]. The presence of methionine/leucine-rich nuclear export signal (NES) in the N-terminus of the protein, as well as the fact that injection of anti-NEP/NS2 antibodies into the nucleus of infected cells inhibits RNP export [323] support the proposed role of the

NEP/NS2 protein. As a result, the NS2 protein has been renamed NEP/NS2, standing for "nuclear export protein/non-structural protein 2".

1.6 Replicative cycle

The replication cycle of influenza viruses (Figure 1.8), though a continuous process, could be divided into different stages for study purposes:

Attachment and penetration: In the initial stages of influenza A virus replication, the viral HA attaches to host surface cell receptors that contain terminal α-2,3 or α-2,6 sialic acid moieties. As previously mentioned (section 1.5.1.1), the interaction of influenza viruses with the ubiquitous sialic acid is constrained by the fact that the HAs of viruses that replicate in different species show specificity toward sialic acids with different linkages, in which avian viruses preferentially bind to sialic acid with an α-2,3 linkage and human viruses mostly to α-2,6 linkages [293]. It should be noted, however, that this viral specificity is not absolute and that avian and human cells can contain both neuraminic acid linkages. Attached viral particles are then incorporated by receptor-mediated endocytosis, with clathrin-mediated endocytosis as the preferred mechanism [285], although a non-clathrin, non-caveolae-mediated internalization mechanism has also been described [396].

Fusion and uncoating: Cleavage of HA by cellular proteases is required to expose the HA peptide that is responsible for the fusion between the viral envelope and the endosomal membrane. Acidification of the endocytic vesicle opens the M2 ion channel, resulting in acidification of the inside of the virion, a process that is required for proper uncoating of the RNP complexes that contain the viral genome. Acidification of the endosome triggers the pH-dependent cleavage of the HA_0 precursor into HA_1 and HA_2. Precisely, the HA_1 portion of the protein remains intact during the low pH-induced conformational change, whereas HA_2 subunit undergoes a refolding process that exposes the fusion peptide at its N-terminus, enabling it to interact with the membrane of the endosome [88, 104]. The transmembrane domain of the HA_2 (inserted into the viral membrane) and the fusion peptide (inserted into the endosomal membrane) are in juxtaposition in the low pH-induced HA structure. The concerted structural change of several hemagglutinin molecules then opens up a pore, which releases the contents of the virion (i.e., viral RNPs) into the cytoplasm of the cell.

Replication and protein synthesis: All proteins in the RNP complex possess nuclear localization signals (NLSs), which mediate their interaction with the nuclear import machinery for translocation [218]. Once in the nucleus, the RNA-dependent RNA polymerase complex transcribes and replicates the incoming negative-sense viral RNA (vRNA) by a primer-dependent mechanism. Transcription gives rise to capped and polyadenylated viral mRNAs, which are then exported to the cytoplasm for translation. Replication takes place in a two-step process by which a complementary positive-sense cRNA is first made and used as template to generate more vRNA. Produced viral proteins that are needed in replication and transcription are translocated back to the nucleus, and progeny RNPs are then exported to the cytoplasm for packaging, assisted by M1 and NEP/NS2. In fact, the accumulation of soluble NP and NEP/NS2 in the nucleus seems to be related to the production of another RNA product by the RNA polymerase complex. The 22-27 nt negative-sense small viral RNAs (svRNAs) correspond to the 5' end of each viral RNA segment and are thought to regulate the switch from transcription to replication [219, 337].

Assembly, maturation and release: Newly synthesized RNPs containing genomic vRNAs are exported to the cytoplasm through RNP-M1-NEP/NS2 complexes. The three integral membrane proteins (HA, NA and M2) are transported by the trans-Golgi secretory pathway, where they are folded and glycosylated (except for M2) and where HA is assembled into a trimer and NA and M2 into tetramers [105]. The mature proteins then arrive at the apical side of the plasma membrane, where they become anchored to the membrane through their transmembrane domains and where HA and NA associate with lipid rafts forming "patches" [18]. Then, the accumulation of M1 protein beneath the membrane and its association with the RNPs and NEP/NS2 assists in the assembly of virus particles. In parallel, each of the eight vRNA segments is independently incorporated through unique "packaging signals" present in the 5' and 3' coding and non-coding regions, ensuring that every virion possesses a full complement of the eight vRNA segments [203, 308]. After the assembly of all viral components, the plasma membrane curves outwards until both extremes fuse at the base of the bud, enveloping the mature viral particle and separating it from the cell membrane [308]. Emerging viral particles are coated with sialic acid, therefore staying anchored to the cell membrane due to the interaction with the HA. As mentioned in section 1.5.4, the enzymatic activity of the NA protein is required to remove the sialic acid and thereby release the virus from its host cell, in an active process. NA activity is also required to remove sialic acid from the carbohydrates present on the viral glycoproteins themselves so that the individual virus particles do not aggregate. Since both HA and

NA recognize the same molecule (sialic acid) but have opposing effects (receptor binding and receptor destroying) a delicate balance between the two glycoproteins is essential to optimize viral fitness [441].

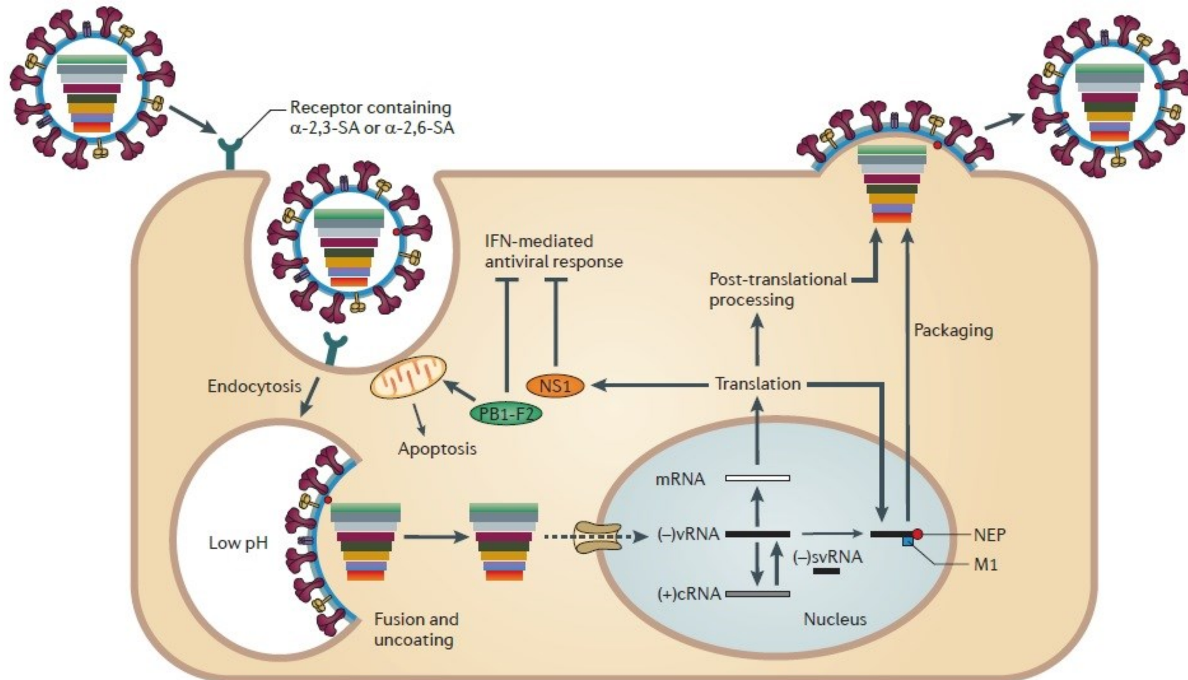

Figure 1.8. Influenza A virus replicative cycle. SA: sialic acid, (–)vRNA: negative-sense viral RNA, (+)cRNA: complementary positive-sense RNA, svRNA: negative-sense small viral RNAs, mRNA: viral messenger RNA. Taken from [293].

1.7 Viral Genetics

Influenza viruses have evolved through three very efficient mechanisms to generate genetic diversity, which are summarized in Figure 1.9. Point mutations, recombination, and segment reassortment can work independently or even combine themselves to affect viral replication, antigenicity, virulence, tropism and many other determinants of virus-host coevolution.

1.7.1 RNA mutations and viral quasispecies

As other RNA viruses, influenza polymerases have few or no proofreading mechanisms and many mutations are introduced during replication. In fact, a mutation rate of 10^{-5} to 10^{-6} nt per position could occur on each viral generation, which is significantly higher than that of mammalian DNA genomes (10^{-8} to 10^{-11}). The high error rate of the replication complex of influenza viruses results in the generation and co-circulation of different genetic variants within a host organism, called "quasispecies" [103]. If the biologic fitness of a quasispecies is comparable to that of the predominant variant, the minor variant may be maintained in virus populations at low frequencies [258]. In the event of host or environmental pressure (including innate and adaptive immune responses, and selective pressure resulting from a host change or antiviral pressure), greater genetic diversity may increase the probability for variants that are better adapted to the changed environment. In such a scenario, quasispecies may be selected and become the dominant virus population.

In the past, the detection of quasispecies was cumbersome due to the detection limits of conventional sequencing techniques. With advances in deep sequencing and mass spectrometry analyses, minor sequence variants can be detected more easily, therefore determining the different levels of quasispecies in mixed infections [140, 249, 371], which may provide critical information on the emergence of novel variants. As it will be discussed in chapter VII, this is of special importance in the context of antiviral treatment, for the rapid detection of drug-resistant subpopulations could guide the implementation of the most adequate therapy.

1.7.2 Viral RNA recombination

Contrary to the relatively minor changes resulting from the introduction of point mutations during replication, influenza viruses can acquire large portions of genomic material through recombination. Intragenic recombination between different RNA segments, commonly referred to as nonhomologous recombination [326], as well as intragenic recombination between viral RNA and exogenous RNA [231], has been observed and may possibly play a role in determining pathogenicity. For instance, the insertion of 60 nt of the RNA segment encoding the NP into the A/turkey/Oregon/71 HA has been shown to enhance protein cleavability [326]. Template switching (or "copy choice") during RNA replication seems to be the principal mechanism driving this process. However, although

recombination is often observed in positive-stranded RNA viruses such as picornaviruses and coronaviruses [424], it remains a very rare phenomenon in influenza viruses.

1.7.3 Viral RNA segment reassortment

Simultaneous infection of a single cell by two distinct influenza A viruses can lead to the rearrangement of gene segments, the so-called “reassortment”. This phenomenon can markedly change the virus phenotype, with intra- or inter-subtypic events having the potential to generate a novel influenza virus strain, and even favor viral transmission between species. In fact, and as discussed in detail in section 1.9.2, the contribution of genetic reassortment to the generation of new influenza virus strains is strikingly exemplified in the case of the last three influenza pandemics of 1957, 1968 and 2009, all of which were caused by reassortant strains from at least human, avian and occasionally swine origins [135, 225, 378]. Although many studies describe the selective incorporation of vRNA segments during packaging [127, 203], the mechanism underlying genetic reassortment is still unclear underscoring further investigation.

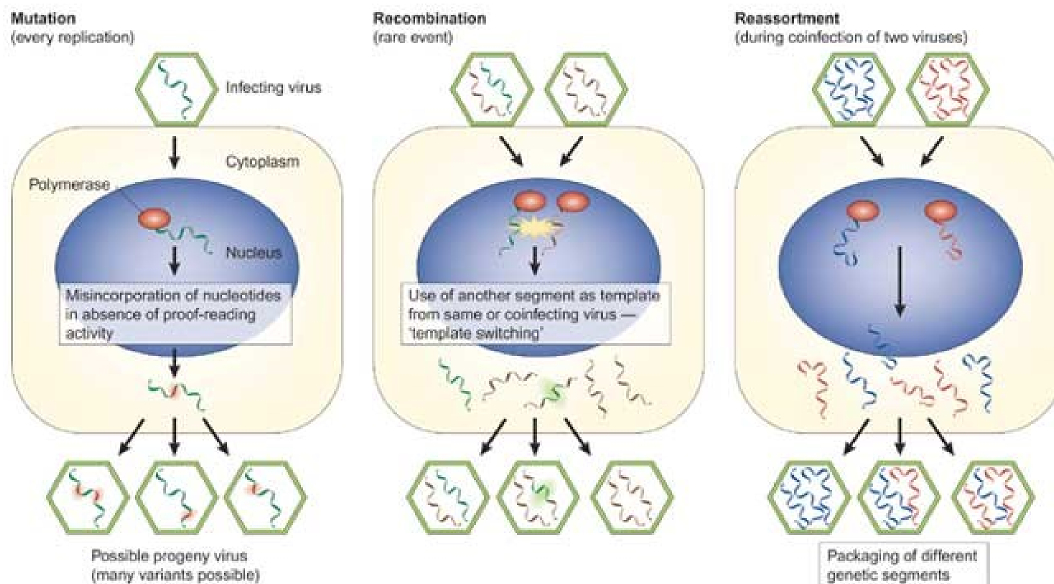

Figure 1.9. Molecular mechanisms involved in influenza viral diversity. a) During replication, single point mutations are incorporated into one or more genomic positions as a result of a lack of proofreading activity of the viral polymerase. b) During recombination, foreign genetic material is incorporated into the viral genome through mechanisms such as template switching during replication. c) During reassortment, which occurs on dual infection of a cell with segmented genome viruses, whole gene segments can be swapped. Taken from [451].

1.7.4 <u>Antigenic changes</u>

As mentioned above, influenza viruses are subject to significant genetic variation. Genetic changes affecting the two major viral antigens, the HA and NA surface glycoproteins, can therefore have an impact on viral antigenic properties. Indeed, these changes in viral antigenicity are directly responsible for the continuous circulation of influenza viruses in the population, as well as for their somehow unpredictable behavior [75]. Two types of antigenic variation have been described in influenza viruses: antigenic drift and antigenic shift. While influenza A viruses undergo both kinds of changes, type B viruses change only by the more gradual process of antigenic drift.

1.7.4.1 Antigenic drift

Antigenic drift can be defined as a gradual change in genotype mainly due to antibody-mediated immune selection pressure, driving the progressive accumulation of mutations. Indeed, the mutation frequency in the HA and NA aa sequences over 1 year is estimated to be <1% [6]. These small genetic changes usually produce viruses that are pretty closely related to one another, therefore sharing the same antigenic properties. But these small genetic changes can accumulate over time and result in viruses that are antigenically different. Since these new viruses are not completely neutralized by antibodies to pre-existing virus strains, they can cause new epidemics, that typically prevail for 2-5 years before being replaced by a new variant [243, 401]. Epidemics due to new "drifted" strains are not as great as those caused by "shifted" strains, given the presence of partial immunity from previous infection (cross-protection). However, antigenic drift has great impact on vaccination, being the main reason why the influenza vaccine composition must be reviewed each year, and updated as needed to keep up with evolving viruses.

HA antigenic drift

As already mentioned, the HA protein is the main antigenic determinant of influenza viruses. Combined crystallography, sequence analysis and escape mutant experiments have enabled the identification of 5 antigenic sites in both H1 (named Ca1, Ca2, Cb, Sa, and Sb) and H3 (named A-E) HA proteins (Figure 1.10) [469]. Antigenic drifts occur by accumulation of a series of point mutations resulting in aa substitutions in the antigenic sites of the HA. These substitutions prevent the binding of the antibodies induced by previous infections, and consequently the virus can efficiently infect the

host. Antigenicity changes appear to depend not only on the nature and position of the aa substitution in the antigenic site but also on other aa at key positions of the HA [464]. Single point mutations in one HA antigenic site can be sufficient for antigenic variation, although a combination of mutations in at least two antigenic sites is normally needed [462, 463]. H3 proteins have drifted more rapidly than their H1 counterparts [117], therefore resulting in the frequent replacement of antigenic variants. Of note, the HA of A(H1N1)pdm09 viruses has not yet drifted significantly [128], presumably due to the lack of selective pressure in a predominantly naïve population.

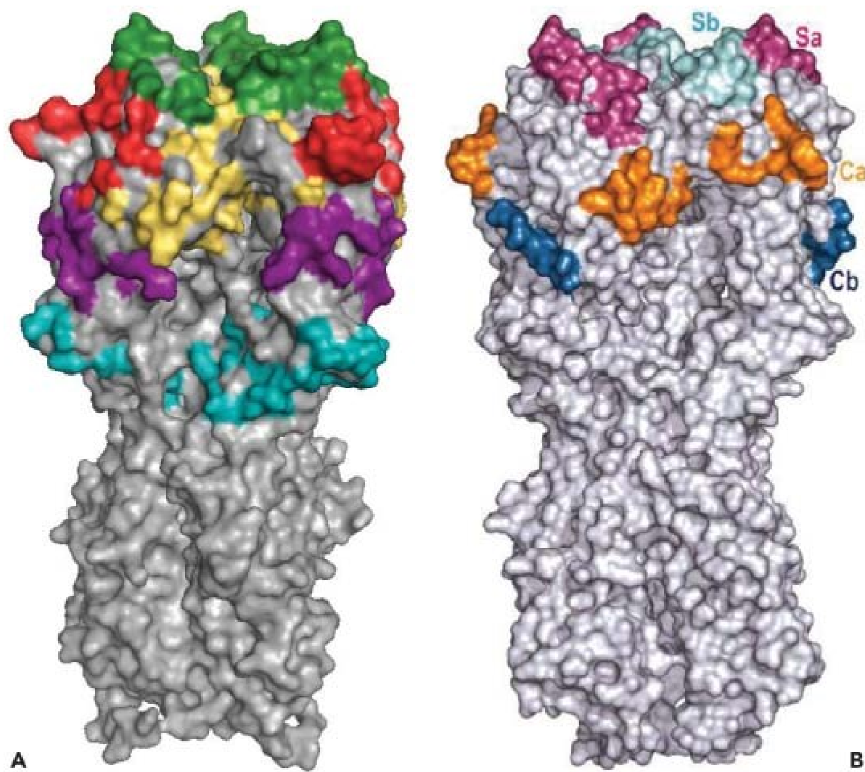

Figure 1.10. Crystallographic structures of influenza A virus H3 and H1 HA proteins. A) Location of the five antigenic epitopes of the H3 protein: site A (red), site B (green), site C (blue), site D (yellow) and site E (purple). B) Location of the five antigenic epitopes of the H1 protein: overlapping sites Ca1 and Ca2 (orange), site Cb (dark blue), site Sa (red), site Sb (light blue). Taken from [473].

NA antigenic drift

The NA protein and represents the second major glycoprotein and, with the HA, determines the strain-specific serotype. Two major antigenic sites have been identified on the upper surface of the NA flanking the sialic acid binding site, with at least one other site sitting at the bottom of the

globular head of the protein [8]. Antigenic drift also occurs in the NA protein and the emergence of new variants is correlated with differences in the aa sequence although with a lesser overall impact than that of HA antigenic drift [469]. Although they do not neutralize viral infectivity, anti-NA antibodies may affect pathogenicity by reducing pulmonary virus titers and the extent of lung lesions [234]. Therefore, antigenic variability of the NA protein should also be considered when analyzing the epidemic impact of influenza strains and to predict newly emerging viruses

1.7.4.2 Antigenic shift

Antigenic shift is the process by which two or more different strains of a virus, or strains of two or more different viruses, combine through genetic reassortment to form a new subtype having a mixture of the surface antigens of the two or more original strains. While influenza viruses are changing by antigenic drift all the time, antigenic shift happens only occasionally. This process involves major antigenic changes in the surface glycoproteins of influenza A viruses, to which pre-existing antibody response provides little or no cross-protection, although cytotoxic T-lymphocyte responses that target the conserved peptides encoded in viral internal proteins may provide protection [469]. The new strain would then have an evolutionary advantage over older strains and, provided that the new virus also harbors other properties, such as virulence and efficient transmissibility, a worldwide outbreak (or "pandemic") could occur [435]. Although genetic reassortment is the main process leading to antigenic shift, this may also occur as a result of continuous adaptation of an avian influenza strain resulting in a new subtype of influenza A virus with sustained human-to-human transmissibility [427, 481]. In that regard, the K627 residue of PB2 protein confers the ability to replicate at 33 °C and hence viruses harboring this aa grow efficiently in the upper respiratory tract of humans [293]. An aspartic acid-to-asparagine (D701N) substitution in PB2 has also been shown to enable the adaptation of avian influenza viruses to grow in mammalian cells [293]. Other recent studies have found that human-adapted PB2 and HA can confer replication competence and transmissibility to some avian viruses in the ferret model [438]. In fact, a genetically modified A(H5N1) virus was shown to have acquired mutations during passage in ferrets, ultimately becoming airborne transmissible in ferrets without the need of any reassortment events or previous adaptation in an intermediate host. Four aa substitutions (H103Y, T156A, Q222L and G224S) in the HA and one (E627K) in the PB2 proteins were identified to be consistently present in airborne-

transmitted viruses [180, 367]. These studies raised great controversy in the mass media and public opinion, and prompted in January 2012 the interruption of all studies involving the potential generation of A(H5N1) HPIV with enhanced human-to-human transmissibility, in order to evaluate whether the benefits of this sort of studies outweighed the risks. One year later, and after relevant authorities in several countries have reviewed the biosafety, biosecurity, and funding conditions under which further research would be conducted on the laboratory-modified A(H5N1) viruses, studies were resumed [121]. A schematic representation of the different types of antigenic variation is presented in Figure 1.11.

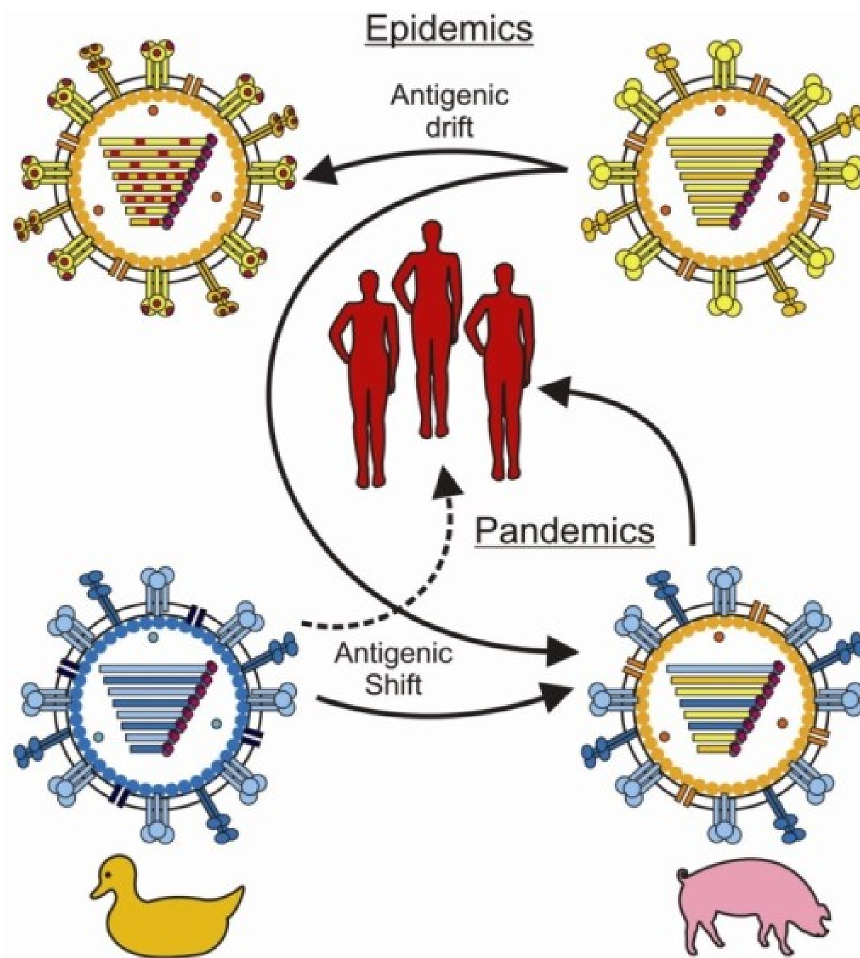

Figure 1.11. Antigenic drift and antigenic shift of influenza viruses. In antigenic drift, pre-existing antibody response against the HA and NA glycoproteins selects antigenic variants with aa changes modifying the antigenic structure that allow influenza A and B viruses to evade immunity. Antigenic drift is a result of both immune and natural selection. In antigenic shift, reassortment between avian and human influenza A virus or continued adaptation of an avian influenza virus may result in a new subtype of influenza A virus with sustained human-to-human transmissibility. Taken from [435].

1.7.5 Reverse genetics

Since neither the vRNA nor the cRNA are infectious in their "naked" form, genetic manipulation of influenza viruses represents a quite safe yet very important experimental technique for both the expression of viral proteins and artificial generation of complete viral particles. These reverse genetics systems rely on the intracellular synthesis of influenza viral RNAs by a cellular enzyme, RNA polymerase I, that transcribes ribosomal RNA in the nucleus of eukaryotic cells. The influenza viral segments are encoded by cDNAs flanked by the RNA polymerase I promoter and the RNA polymerase I terminator or a ribozyme sequence. RNA polymerase I transcription in transfected cells results in the efficient synthesis of RNA transcripts with defined 5' ends, whereas the integrity of the 3' ends is achieved by using the nucleotide-specific RNA polymerase I terminator or a self-cleaving ribozyme [312]. Former systems developed in the late 1990s [119, 312] required the co-transfection of the cells with a set of 12 plasmids; eight containing each of the vRNA segments and four expression plasmids coding for the NP and polymerase proteins. This system was then modified [188] to reduce to 8 the number of required plasmids. In this system, RNA segments are flanked by the polymerase I promoter while the terminator sequences are flanked by a CMV (polymerase II) promoter and polyadenylation signal in the opposite orientation. As a result, both the negative-sense vRNA and the mRNA can be transcribed from the same plasmid, hence abrogating the need of the four expression plasmids. In another modification [310], the eight RNA polymerase I transcription units for the eight viral RNAs are combined, allowing the generation of the entire viral genome from a single plasmid.

Overall, reverse genetics systems are highly effective, enabling the recovery of 10^7-10^8 plaque-forming units (PFU) of influenza A virus per ml of cell culture supernatant. These systems have been widely exploited for the functional study of viral proteins, the study of non-coding sequences involved in transcription and replication, the expression of foreign antigens as well as for the generation of influenza virus vaccines [311]. In many cases, the definitive role of a gene or of a domain (or even of a single aa) can only be explored by introducing appropriate mutations into the genome of the virus and then analyzing the phenotype of the rescued virus. In that regard, and as it will be demonstrated in many of the studies presented in this thesis, the use of recombinant viruses and/or viral proteins does represent an invaluable tool for the characterization of the mechanisms involved in antiviral resistance.

1.8 Clinical features

1.8.1 Immune response

Following influenza infection in immunocompetent individuals, the first response against the virus is that of innate immunity, which is characterized by the production of interleukin 6 (IL-6) and type I interferons (IFN-α/β) by infected epithelial cells and monocytes/macrophages. This proinflammatory cytokine production reaches its peak on day 2 post-infection [174], consistent with the highest levels of fever and other clinical symptoms, mucus production, and viral load observed at that time point. Noteworthy, the role of the viral protein NS1 as suppressor of the IFN-α/β-mediated antiviral response (section 1.5.2.4) highlights the importance of this host protein as negative regulator of viral replication [134, 419]. Monocyte/macrophage-induced IL-1 β, IL-8 and tumor necrosis factor alpha (TNF-α) appear later in the respiratory secretions and/or in the serum, but the production IL-β, IL-2 and the transforming growth factor beta (TGF-β) are normally not affected in the course of viral infection [252]. Furthermore, in addition to the initial non-specific innate immune response, the adaptive cellular and humoral responses are necessary to control and eliminate the infection caused by influenza virus.

The adaptive cellular response is initiated by cytotoxic T-CD8$^+$ lymphocytes (CTLs), which detect and lyse infected cells. This first CTL response can be detectable in the blood 6-14 days after infection and last until day 21 [46]. The specificity of CTLs is directed mainly against the HA, NP, M and PB2 viral proteins, which have highly conserved peptide epitopes. Since viral replication is confined to the respiratory epithelium, the antiviral capacity of CTLs depends on their capacity to migrate to the lungs and the infected respiratory tract, where they arrive 5-7 days post-infection [62, 260]. T-CD4$^+$ lymphocytes, in turn, lyse infected cells less efficiently than T-CD8$^+$ cells. However, they play an important role as “helper” T cells providing accessory signals for CTL proliferation and for antibody production by B cells, the central players of humoral response [253, 254]. In that regard, the humoral response reduces viral load and prevents reinfection. This response is conducted by antibodies secreted in the respiratory mucosa such as IgA, which primarily protect the upper respiratory airways, as well as IgM and IgG that neutralize the antigens in the lower respiratory tract. Between the first and second week following a primary infection, neutralizing IgM, IgA and IgG antibodies directed against the HA protein (the main viral antigen) can be detected in the serum of adults, whereas after a secondary infection there is a predominance of IgG and IgA antibodies.

These antibodies are mainly produced by B cells in the peripheral blood of both naïve and infected individuals, playing an important role in virus neutralization and strain-specific disease prevention. Indeed, hemagglutination inhibition titers ≥1:40 generally constitute a good correlate of immune protection against the influenza virus (except in healthy young adults), with such a response being detected in 50% of immunized subjects and roughly 80% of people with naturally-acquired infections [73]. On the other hand, neutralizing IgA antibodies can also be produced locally in the respiratory mucosa, where they can act intracellularly during viral transcytosis to the apical surface to inhibit virus replication [288, 359].

1.8.2 Pathogenesis

Infection by influenza A viruses induces pathological changes throughout the respiratory tract, but primarily in the lower respiratory tract. The progression of changes in the cells of the respiratory epithelium suggests that they begin in the tracheal bronchial epithelium and then ascend, with acute and diffuse inflammation of the larynx, trachea and bronchi as the first visible signs of uncomplicated influenza [183, 416]. Histological studies on nasal exudate cells and tracheal biopsies have indicated that the major site of virus infection is the ciliated columnar epithelial cells. Following infection, these cells become progressively rounded and swollen, and the nucleus appears contracted, with the chromatin irreversibly condensed (pyknosis). Approximately 24 h after the onset of symptoms, the cytoplasm becomes vacuolated, the nucleus degenerates and ciliation is lost. As a result, necrotic cells start to detach and the ciliated epithelium desquamates down to a one-cell-thick basal layer. This layer is significantly more sensitive than the outer epithelium and is also unable to retain mucus, so the consequent infiltration of neutrophils and mononuclear cells provokes submucosal edema and hyperemia, closely related to most of the observed respiratory symptoms [280, 416]. In the event of the infection progressing to a viral pneumonia (inflammation of the alveoli in the lung parenchyma), the alveoli walls become thickened with edema and infiltration, as well as coated with connective tissue. The resulting interstitial pneumonitis, with predominantly mononuclear leukocyte infiltration, capillary dilation and thrombosis seriously compromises oxygen exchange between the lung and the blood. Depending on the severity, this situation can lead to tissue hypoxia and functional impairment [391]. Of note, diffuse alveolar damage, hemorrhagic interstitial pneumonitis, and peribronchiolar and perivascular lymphocytic infiltrates have already been

described in patients infected with the A(H1N1)pdm09 virus [142, 391]. Finally, on the third to fifth day of symptom onset, signs of focal mitotic activity begin to appear focally in the basal cell layer, then starting the regeneration of epithelial tissue. The beginning of reparative (angiogenic) processes may overlap with the final stages of tissue destruction, taking up to one month to completely heal the epithelial damage [416].

To conclude, the balance between viral replication and the host immune response is a major determinant of the outcome of infection. In that sense, many A(H5N1) viruses have a remarkable capacity to resist the antiviral effects of host cytokines [384], as well as to induce high levels of cytokine expression (i.e. IL-6, TNF-α and IFN-γ) through the infection of human macrophages, ultimately leading to tissue damage in the infected host [86]. Indeed, studies performed in mice with reassortant viruses harboring the HA protein of the 1918 A(H1N1) virus showed a high induction of macrophage-derived cytokines that stimulated inflammatory cell infiltration and hemorrhage, two hallmarks of the Spanish flu [223, 242].

1.8.3 Laboratory diagnosis

The diagnosis of influenza in clinical and public health laboratories facilitates patient management decisions and provides outbreak and surveillance data for public health policy and guidelines. Several diagnostic tests are available to detect influenza A and B viruses, including viral culture, reverse transcriptase-polymerase chain reaction (RT-PCR), rapid antigen testing, and serology. The tests can differ in their sensitivity and specificity for different types of specimens. In general, nasopharyngeal specimens collected with a swab are more sensitive than throat swab samples [54].

Viral isolation in cell culture (usually MDCK cells) used to be considered the “gold standard” for virus diagnosis [54]. This test has the advantage of identifying which viruses (influenza A, B, or another respiratory virus) and which strains of virus are present. A faster culture method, known as shell vial culture, may detect the presence of a respiratory virus in 24-48 h; however, a traditional viral culture normally requires 3-10 days. As a result, this method is usually of limited use in clinical settings for the prompt diagnosis of influenza [340]. RT-PCR assays are considered to be the most sensitive, specific and versatile tests for the diagnosis of influenza and, given their rapid response

time (within 1 day), they are replacing viral isolation as the reference standard [54]. Viral RNA can be used in RT-PCR not only to identify the virus as influenza, but also to further determine the subtype and even the strain by sequence analysis, although genotyping of the virus directly from patient specimens may require some level of amplification in cell culture. Most RT-PCR assays for influenza A and B viruses use primers complementary to the relatively stable gene segment 7, which encodes the conserved matrix protein, and can successfully detect all viral strains observed to date. HA-specific RT-PCR allows identification of the HA subtype of influenza A virus [120]. In addition, multiplexing is possible to test multiple samples at once, or to test for more than one viral RNA segment in one reaction. Of note, the detection of influenza viral RNA by these assays does not always indicate detection of viable virus or on-going influenza viral replication.

Rapid diagnostic tests are used to detect viral antigens in nasal secretions, and represent one of the most common methods to help differentiate influenza from other viral and bacterial infections with similar symptoms. Depending on the method, it may be completed in the doctor's office in less than 15 minutes or be sent to a laboratory, with the results available the same day. Rapid tests vary in their ability to detect influenza, and are best used within the first 48 h of the onset of symptoms. Some types can only detect influenza A; others can detect both A and B but not distinguish between the two. Still, others can detect and distinguish between influenza A and B. However, none of them are able to differentiate between different influenza A strains. Rapid tests will generally detect 50-70% of influenza cases, and their main disadvantage is the considerable rate of false-negative results [54]. Therefore, the CDC recommends not withholding treatment from people with suspected influenza, even if they test negative. If someone needs confirmation from a laboratory diagnosis, it will be necessary to follow a negative rapid test with a viral culture or RT-PCR test. Finally, serologic testing is based on the presence of influenza-specific antibodies, and is typically carried out by using hemagglutination inhibition or microneutralization assays. These assays are used by the WHO because of their reliability for typing, subtyping and further determining the antigenic characteristics (i.e. HA drifts) of influenza viral isolates, but are not recommended for standard diagnostic testing because of their complexity [340].

To conclude, it is up to each laboratory to assess the optimal methods for its situation and the best application of each technique, taking into account numerous factors including budget,

equipment, staff expertise, patient population served and needs of submitting clinicians, as well as its surveillance and public health responsibilities.

1.9 Epidemiology

Infections caused by influenza A viruses have a short incubation period (average 2 days, range 1-5 days), with patients presenting high viral concentrations in respiratory secretions during the initial phase of the disease [263]. Influenza can be spread in three main ways: by direct contact (e.g.: when an infected person sneezes directly into the eyes, nose or mouth of another person); by the airborne route (e.g.: when someone inhales the aerosols produced by an infected person coughing, sneezing or even breathing); and through hand-to-eye, hand-to-nose, or hand-to-mouth transmission, either from contaminated surfaces or from direct personal contact such as a hand-shake [161]. Nevertheless, aerosolized droplets constitute the most effective means of transmission [418]. While the largest droplets produced by breathing and speaking fall to the ground within a few meters and will transmit an infection only to those in the immediate vicinity, other droplets travel a distance determined by their size. Most aerosol droplets produced during sneezing or coughing are 1-4 μm in diameter; these so called "droplet nuclei" or aerosol remain suspended in the air for very long periods and may not only travel long distances, but can reach the respiratory tract, with those less than 2 μm in diameter being preferentially deposited in the lower airways of the lung [83, 268]. Following aerosolization, influenza A virus retains its infectivity for 24 hours (h) or more in conditions of low relative humidity (17-24%) but just for 1 h in high relative humidity conditions. On nonporous hard surfaces, viruses retain their infectivity up to 24-48 h, with low relative humidity and a low UV radiation (typical winter conditions) again facilitating virus survival [161].

The epidemiology of human influenza viruses is defined by their constant antigenic variation to escape the host immune response. Influenza viruses have global distribution and infections in humans may occur in two epidemiologic forms: epidemics and pandemics. Seasonal annual epidemics may be relatively localized and of varying intensity throughout the year. One of the most important characteristics of epidemics caused by influenza virus is their rapid emergence and spread resulting in millions of human infections worldwide that entail significant health and economic burdens. On the other hand, an influenza pandemic is a large-scale global outbreak of the disease, which may have devastating effects, resulting in millions of deaths [301]. Before discussing influenza epidemics and pandemics later in this chapter, it should be considered that the prevalence of

different groups of influenza viruses varies geographically and temporally. In fact, since 1977, seasonal A(H1N1) and A(H3N2) viruses have been circulating together with influenza B viruses. Moreover, in 2009, the seasonal A(H1N1) viruses were largely replaced by A(H1N1)pdm09 viruses, therefore making influenza virus epidemiology quite complex [75, 472].

1.9.1 Influenza epidemics

Although infections with influenza viruses are reported throughout the year, the intensity of influenza activity varies annually in temperate areas, reaching peak prevalence in winter and early spring [75, 368]. Since the northern and southern hemispheres have winter at different times of the year, there are actually two different flu seasons each year, with the highest incidence reported between December and March and between July and August, respectively. In tropical regions, epidemics can also occur throughout the year, usually during the rainy season [368]. As already explained, the co-circulation of many influenza strains accounts for this seasonal pattern yet population determinants such as pre-existing immunity as well as environmental factors influencing exposure are also important. Cold temperatures lead to drier air, which may dehydrate mucus, preventing the body from effectively expelling virus particles. The virus also survives longer on surfaces at colder temperatures and aerosol transmission of the virus is the highest in cold environments; as lower temperatures usually lead to lower air humidity in winter, this seems to be the main cause of increased seasonal influenza transmission in temperate regions [385, 386]. Likewise, people stay indoors more often during winter, therefore increasing the frequency of person-to-person contact events.

Even if the incidence of influenza infections can vary widely between years and the mortality rate of seasonal influenza viruses can be considered low (<0.1%), 3-5 million cases of severe illness are reported in a typical influenza season worldwide, with about 500000 deaths, mainly among children, the elderly and individuals with cardiopulmonary diseases [273]. In the US, more than 200000 hospitalizations and approximately 36000 deaths are directly associated with influenza every year [420, 421]. In Canada, influenza viruses are responsible for more morbidity and mortality each year than all other respiratory viral diseases combined, with an attack rate of 10-20%, resulting in 1.5 million days of absenteeism, 50000 hospitalizations and about 3000 deaths. This figure makes influenza infections the sixth leading cause of death in this country [408].

Most influenza virus strains are not very infectious, with each infected person infecting on average one or two other individuals (basic infectious rate of ~1.4). However, the generation time for influenza is extremely short: the time from a person becoming infected to when he infects the next person is only two days, meaning that influenza epidemics generally peak at around 2 months and burn out after 3 months [116]. Influenza infection is characterized by non-specific symptoms such as fever (>90% of cases), cough (>80%), congestion (>80%), rhinorrhea, headache, myalgia and anorexia. Symptoms usually appear two days after infection and might be accompanied by nausea and vomiting. In healthy adults, symptoms disappear after five or seven days in the absence of treatment, but complications such as viral or bacterial pneumonia can occur mainly among high-risk groups, namely young children, the elderly, immunocompromised patients, and pregnant women in the 2nd and 3rd trimester of pregnancy [35, 455]. While the duration of viral excretion depends on patient age, clinical studies performed in both children and adults have shown that viral shedding may persist up to 7-8 days after the onset of symptoms [124, 263, 375], with prolonged times in the case of immunocompromised patients [24, 147]. School-aged children play a key role in virus spreading in the community through household transmission. Attack rates can peak up to 60-75% in this population group, progressively decreasing with age to become four times lower in people aged 60 or older [214, 313]. Increases in school absenteeism also increase doctor's visits and hospitalizations, and are typically followed by increases in workplace absenteeism. Influenza A(H1N1) and A(H3N2) viruses, as well as influenza B viruses, cause similar symptoms [125, 405]; nevertheless, the frequency of severe infections is higher in the case of A(H3N2) infections [421]. Reinfection with a closely related variant can occur, though the symptoms are usually less severe than those that follow the initial encounter with a particular virus strain [91, 92].

1.9.2 Influenza pandemics

Contrary to relatively localized epidemics, pandemics are outbreaks that impact large geographic areas and large portions of the population in a short period of time. Fortunately, pandemics occur irregularly (10- to 40-year intervals), with about three influenza pandemics in each century for the last 300 years although reliable records only date back to the 1918 pandemic. Pandemics are the most dramatic manifestation of influenza, usually attacking 20-40% of the world population and usually causing significant mortality; however, the cumulative death toll of epidemics

in interpandemic periods, although less dramatic, parallel those of pandemics. Temporal curves of individual pandemics are comparable in the sense that virus introduction into a community is followed by a relatively sharp, single peak that represents school and workplace absenteeism, which is then followed by increased mortality slightly later [321]. Typically, the pandemic virus spreads worldwide over a period of 6-9 months with two waves of infection separated in time by some months, although sometimes three waves of infection may occur.

As discussed before (section 1.7.4.2), influenza pandemics occur when an antigenic shift - either by genetic reassortment or by adaptation to a new host- enables the zoonotic transmission of a new animal strain to the human population. Pigs, chickens and ducks are the most important species related to the emergence of pandemic strains with efficient inter-human transmission. These novel strains are thus slightly or completely unaffected by previous population immunity to older strains of human influenza and can therefore spread rapidly with extremely high attack rates, causing significant morbidity and mortality [321]. However, the severity of a pandemic may be dependent on the composition of the virus, as cytotoxic T lymphocyte responses that target the relatively conserved internal proteins may provide protection [362]. In that case, a mild pandemic is possible when the pandemic virus emerges through genetic reassortment and by acquiring internal gene segments from previously circulated human influenza virus.

1.9.2.1 Pandemics of the 20th century

Three influenza A pandemics occurred during the 20th century: the 1918 "Spanish flu", caused by an A(H1N1) virus; the 1957 "Asian flu" caused by an A(H2N2) virus; and the 1968 "Hong Kong flu", caused by an A(H3N2) virus [349]. These pandemics have occurred at irregular intervals and have led to high rates of morbidity and mortality in susceptible world population, particularly among young people.

The Spanish flu started in the spring of 1918 as a mild respiratory disease that disseminated in the US, eventually reaching Europe through the American World War I troops [332]. The 1918 pandemic (spanning three waves until the early 1920) remains unprecedented in its severity, with a mortality rate over 2% compared to the <0.1% of seasonal epidemics. Also in contrast to seasonal influenza, which disproportionately affects the very young and old, those aged 20-40 years were

especially affected by the pandemic virus [241, 299]. The huge death toll (50-100 million deaths) was caused by a particularly high infection rate of up to 50% as well as the extreme severity of the symptoms. The majority of deaths occurred as a result of bacterial pneumonia, a secondary infection caused by influenza, but the virus also killed people directly, by triggering a disproportionate immune response with the so called "cytokine storms" that led to massive hemorrhages and lung edema [332, 415]. In 2005, Taubenberger and colleagues managed to recover genomic RNA of the 1918 A(H1N1) pandemic influenza virus from a paraffin-fixed tissue collection, as well as form the corpse of a victim buried in the Alaskan *permafrost* (a layer of permanently frozen ground found in periglacial zones) [417]. Their phylogenetic analysis revealed both "avian-like" and "human-like" signature aa but concluded that the 1918 pandemic virus was entirely an avian virus that adapted to human infection, and then to human-to-human transmission, possibly by successive mutations but without genetic reassortment (Figure 1.12) [28, 417]. Further analysis suggested that the 1918 virus genes were not directly transmitted from an avian species, and likely circulated in a mammalian host for several years before causing the pandemic outbreak. However, it is still not clear either how long it took for the avian-originated influenza virus to become adapted in mammals or in which mammalian reservoirs the adaptations occurred [9, 402].

The Asian flu originated in southern China in February 1957 and rapidly spread to the neighboring Singapore, Hong Kong and then Japan, where the causative A(H2N2) strain was finally isolated [145]. Almost nine months later, the first wave reached Europe and the US, followed by a second wave in January 1958. Once again, the infection rate was highest among young people, exceeding 50% in those aged between 5 and 19 years [145]. Both waves were characterized by significant mortality, with a fatality rate of 0.13% and 1-1.5 million deaths worldwide [397]. Likewise, the Hong Kong flu of 1968 [A(H3N2)] emerged in southern Asia during summer and rapidly spread around the world [65]. Although the attack rates reached 40% and were highest in those aged between 10 and 14 years, the fatality rate was the lowest (0.1%) of the three pandemics, with 0.75-1 million deaths worldwide [397]. Preexisting neutralizing antibodies to the N2 protein in the human population might have accounted for the moderate severity of the outbreak. Unlike the 1918 pandemic virus, genetic analyses showed that both the 1957 A(H2N2) and 1968 A(H3N2) viruses emerged as a result of genetic reassortment events involving two viruses. In 1957, the mixed infection of the same animal (probably human but also possibly swine) with an avian A(H2N2) and a human A(H1N1) strains resulted in a new influenza virus. This virus acquired three gene segments

(PB1, HA, and NA) from an avian reservoir while keeping five other gene segments from the A(H1N1) human strain circulating prior to 1957. This new reassortant virus circulated in humans until 1968, when its PB1 and HA segments were again replaced with two new avian genes, therefore giving birth to the reassortant 1968 A(H3N2) pandemic virus (Figure 1.12) [28].

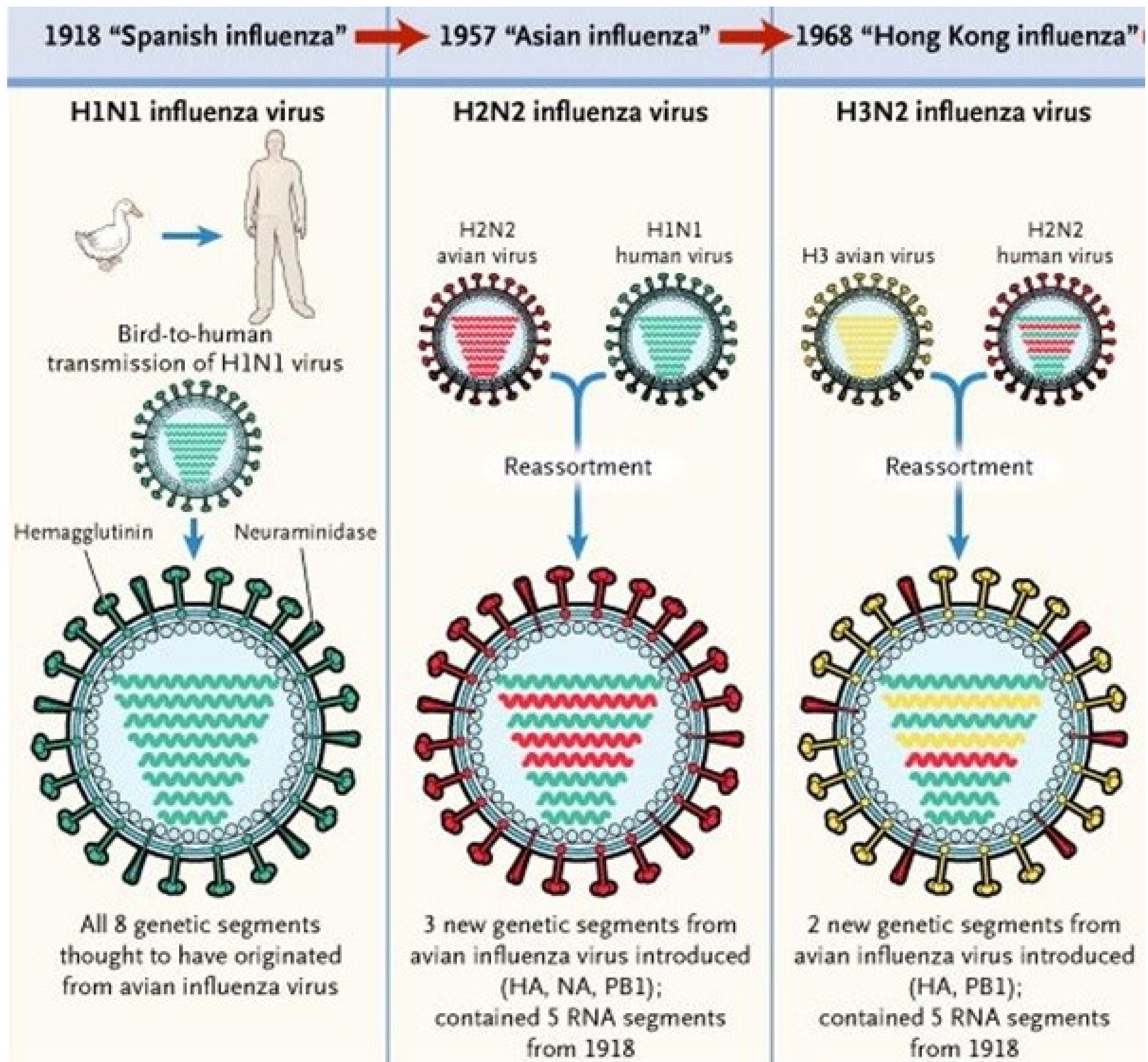

Figure 1.12. The origin of the three pandemics of the 20th century. In 1918 (left), an A(H1N1) virus closely related to avian viruses adapted to replicate efficiently in humans. In 1957 (center) and in 1968 (right), reassortment events led to new viruses that resulted in pandemic influenza. The 1957 influenza virus acquired 3 genetic segments (HA, NA and PB1) from an avian species, whereas the 1968 influenza virus acquired 2 (HA and PB1). Adapted from [28].

1.9.2.2 The 2009 A(H1N1) pandemic

In April 2009, infections with genetically similar A(H1N1) viruses of swine origin were detected in southern California and Mexico, and a potential outbreak was reported [94, 459]. In the days that followed, the number of human cases continued to increase across different continents. As evidence of human-to-human transmission was more than tangible, the WHO declared the first pandemic alert of the 21st century on June 11, 2009 [66, 486]. Northern countries experienced a first wave of illnesses in the spring followed by a second wave during the fall. However, a number of other countries, especially in the southern hemisphere, only experienced a single wave of illness [44]. At least 18449 laboratory-confirmed deaths in 214 countries can be attributed to the 2009-10 influenza pandemic, almost 450 of which happened in Canada [341]. Nonetheless, the number reported to the WHO by its member states is widely considered a gross underestimate of the real burden of the disease, which is believed to have been responsible for up to 400000 deaths worldwide [93]. Even if the WHO has ceased the pandemic alert in August 2010, the A(H1N1)pdm09 virus continues to be the predominant A(H1N1) influenza strain circulating to date.

Infection with the A(H1N1)pdm09 influenza virus was characterized by a self-limited febrile upper respiratory illness with symptoms similar to those observed in seasonal influenza infections, such as fever (94%), cough (92%), sore throat (66%), rhinorrhea, headache, and muscle pain. Interestingly, vomiting (25%) and diarrhea (25%) were also frequently observed though none of them is a typical symptom of seasonal influenza [48, 55]. In more severe cases, some patients have shown severe pneumonia with multifocal infiltrates, acute respiratory distress syndrome and multiple organ failure [325, 333]. In general, the 2009 pandemic has mainly affected children aged less than one year, adults between 25 and 64 years and pregnant women. People with comorbidities such as overweight, diabetes, and cardiac/respiratory underlying diseases were more susceptible to develop complications leading to hospitalization. In pregnant women, infections were particularly severe during the second and third trimester of pregnancy [216, 257], with estimates that over one third of pregnant women infected by the A(H1N1)pdm09 virus in the US were hospitalized [376]. Overall, the death rate among hospital patients infected with the A(H1N1)pdm09 virus was 16% [450]. Somehow as a reminder of the 1918 pandemic, young adults were particularly affected by the A(H1N1)pdm09 virus, with 69% of deaths being reported among adults aged 25-64 years. Indeed, while an estimated 90% of deaths associated with seasonal strains are observed among those aged over 65 years, only 14% of deaths were observed for the same age group during the 2009 pandemic [123].

Despite increased surveillance of avian viruses in Southeast Asia since the A(H5N1) HPIV outbreaks, the emergence of an swine-origin A(H1N1) pandemic virus was largely unexpected. Nevertheless, prolonged coordinated international efforts to avoid a potential A(H5N1) pandemic allowed the unprecedented prompt detection, characterization and continuous surveillance of the novel A(H1N1)pdm09 strain as it spread worldwide [126, 135, 403]. Genomic analyses showed that the A(H1N1)pdm09 virus harbors a complex gene distribution, issued from a series of reassortment events that are illustrated in Figure 1.13. In the early 1990s, three different influenza A strains circulated in North America: the "classical" swine A(H1N1), a seasonal human A(H3N2) and an avian virus of unknown subtype. Following reassortment events, these three viruses gave origin to the triple reassortant A(H3N2) North American swine virus, which then reassorted again with a "classical" swine A(H1N1) circulating virus, creating the A(H1N2) North American swine virus. This A(H1N2) virus finally combined through another reassortment event with an A(H1N1) swine virus from the Eurasian lineage, therefore generating the human A(H1N1)pdm09 virus [135, 333, 430]. As a result, the A(H1N1)pdm09 genome is composed of the PB2 and PA segments from North American avian viruses, the PB1 segment of the human A(H3N2) viruses, the HA from ancient human A(H1N1) viruses, NP and NS segments derived from classical swine A(H1N1) viruses, and finally the NA and M segments of Eurasian 'avian-like' swine viruses. Interestingly, sequence and antigenic analyses also revealed shared antigenic epitopes between the HA protein of the A(H1N1)pdm09 strain and those of the A(H1N1) viruses that circulated sometime between 1918 and the 1950s [135, 151, 473, 475]. It has then been postulated that the presence of partial immunity issued from exposure of the elderly population during the first half of the 20th century to close descendants of the 1918 A(H1N1) pandemic strain may account for the low infection rate with the A(H1N1)pdm09 virus this age group [87, 366].

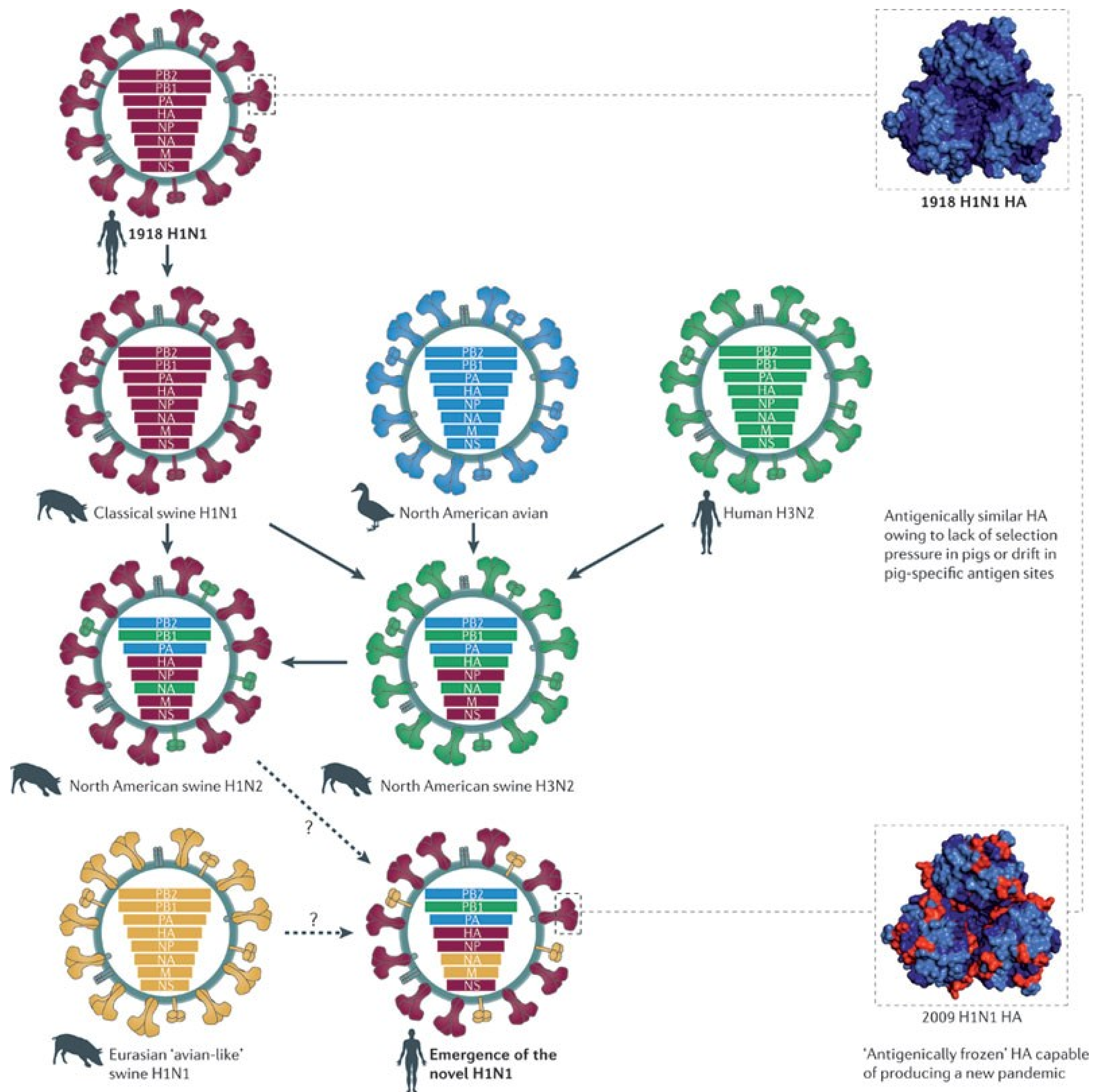

Figure 1.13. The origin of the A(H1N1)pdm09 virus. Triple reassortant swine origin influenza viruses of different strains and subtypes emerged and became predominant among North American pig herds in the 1990s, hence providing the genetic pool for the genesis of the A(H1N1)pdm09 virus. The antigenic similarities between the 1918 A(H1N1) and the A(H1N1)pdm09 HA proteins are represented in the crystal structure models, as seen from a top view. The antigenic sites are shown in light blue whereas sites that differ between both HA proteins are depicted in red. Taken from [293].

1.9.3 Zoonotic infections

Influenza virus can infect a variety of animals, including humans, birds, swine, horses, and dogs. Except for the newly described H17 and H18 in bats, all other known types of HA have been already isolated from wild waterfowl (ducks, gulls, shorebirds) in Eurasia, which has therefore been recognized as the primary reservoir of influenza A viruses [482]. Influenza viruses can cause zoonotic infections, meaning they can be transmitted between species, notably from animals or birds to humans. Fortunately, these events are relatively rare and usually bear viruses with inefficient human-to-human transmission. However, this phenomenon is of special importance in southern China owing to the proximity of dense populations of people, pigs, and wild and domestic birds, thereby facilitating genetic reassortment of viruses from different species, or the emergence of drift variants, given the high human population density and year-round virus circulation [321]. The examples described below (in chronological order) summarize the most relevant cases of zoonotic influenza infections in the last 20 years and also underscore the need of virological surveillance of the human-animal-ecosystem interface for the early detection of influenza viruses with zoonotic and/or pandemic potential.

1.9.3.1 H5N1

In May 1997, the first case of human infection with an influenza A(H5N1) virus was reported in Hong Kong and, in August of the same year, the first A(H5N1) HPIV to cause a fatal infection in humans was identified [95, 246, 411]. By November-December, a total of 18 cases were identified in people living in the Hong Kong area. This outbreak followed serious outbreaks of avian A(H5N1) influenza in chicken farms and, at the time, signaled the possibility of an incipient pandemic. Genetic analysis of the human influenza isolates confirmed their avian origin and that they were not derived by reassortment [64]. The high mortality rate (6 out of 18 cases) showed acute respiratory distress syndrome or multiple organ failure mainly among otherwise healthy young adults [79], hence suggested an unusually aggressive clinical course. Fortunately, there were few if any secondary infections, and the A(H5N1) outbreak ceased with the slaughter of all chickens in Hong Kong (about 1.5 million). The re-emergence of the A/Hong Kong/97 (H5N1) HPIV in flocks in May 2001, and February-April 2002, precipitated the depopulation of the territory's poultry stocks once again. However, no A(H5N1) human cases were identified until February 2003, when two cases (1 death) were confirmed in a family of Hong Kong residents. Genetic analysis of the two H5N1 isolates

showed that the virus genes were purely avian in origin, but differed from the 1997 strains that infected human beings [321].

In May 2010, 498 laboratory-confirmed human cases (294 fatal) of A(H5N1) infections were reported in 15 countries: Azerbaijan, Bangladesh, Cambodia, China, Djibouti, Egypt, Indonesia, Iraq, Laos, Myanmar, Nigeria, Pakistan, Thailand, Turkey and Vietnam [456], hence representing the most important zoonotic influenza virus infection to date. In most of the cases, infection was acquired through direct contact with infected poultry and, although human-to-human transmission is quite rare, it has still been reported [222, 433]. Once again, the highest mortality rates occurred in patients 10 to 19 years old due to severe pneumonia and acute respiratory distress syndrome, with patients aged 50 years or more showing the lowest mortality [1]. In terms of pathogenicity, enhanced mortality was related to high viral load, lymphopenia, and the virus-induced excessive expression of certain proinflammatory cytokines and chemokines, a phenomenon known as “cytokine storm” [1, 96]. As of October 2014, the A(H5N1) toll raised to 668 human cases and 393 deaths, with Bangladesh, Cambodia, China, Egypt, and Indonesia also reporting widespread and ongoing infections in their poultry [456].

1.9.3.2 H9N2

The increase in surveillance following the A(H5N1) outbreak in 1997, led to the identification in 1998 of 9 human isolates of an A(H9N2) virus in the Chinese Guandong province and, in March 1999, this virus was again isolated from 2 children in the same region [269]. In both cases, the disease was mild and self-limited [334]. No serological evidence of H9N2 infection was found in family members or health personnel who were in contact with the children suggesting that the A/Hong Kong/99 (H9N2) virus cannot be easily transmitted from person to person [434]. However, antibodies against H9 viruses were found in about 4% of blood donors in Hong Kong [334] implying that limited human infection with A(H9N2) may occur in this locality. Surveillance studies in pigs from the same region (southern China) revealed that A(H9N2) viruses co-circulate with human A/Sydney/97-like (H3N2) viruses as well as other swine A(H1N1) and A(H3N2) viruses. Together, these observations highlight the pandemic potential of the currently circulating A(H9N2) viruses.

1.9.3.3 H7N2, H7N3, H7N7, H7N9 and H10N8

In November 2003, a patient with severe respiratory symptoms was admitted to a hospital in New York. Initially, laboratory studies confirmed that the patient had been infected by an influenza A(H1N1) virus but months later an avian A(H7N2) was identified as the etiologic agent of that infection [330]. Although the patient recovered within a few weeks of onset of symptoms, laboratory analysis concluded that the A(H7N2) possessed a receptor-binding affinity more characteristic of human than avian viruses [27]. In May 2007, an A(H7N2) virus was confirmed as responsible for a lethal outbreak at a poultry farm in Wales. Nine people who were associated with the infected or dead poultry and reported flu-like symptoms were screened, with 4 of them testing positive for infection A(H7N2) and being successfully treated for mild flu [112].

In February 2004, an outbreak caused by a A(H7N3) HPIV occurred in British Colombia, Canada, and surveillance systems identified mild conjunctivitis and influenza-like illness in two poultry workers [431]. This situation was similar to that of the A(H7N7) HPIV outbreak reported in poultry farms in the Netherlands in February 2003, which was associated with fatal respiratory illness in one of 89 human cases. Most patients presented with conjunctivitis, and only 7 had respiratory illness. Transmission of A(H7N7) influenza from poultry workers to family members was found on three occasions [122]. Furthermore, in March 2013 an A(H7N9) virus was first reported to cause human infection in China. Most cases had illness onset during the month of April, resulting in severe respiratory illness, with unusual prevalence of older males among infected patients [11, 267]. While several environmental, behavioral, and biological explanations for this pattern have been proposed [398], the reason is still unknown. By October 2014, the outbreak's overall total reached 453 cases, with 175 deaths over two years [457]. Most of the cases that were reported had poultry exposure and lived in areas where the A(H7N9) virus had been found previously, and although epidemiological investigations are ongoing for some of the more recent cases, currently no evidence has been found that indicates sustained human-to-human transmission. Finally, the first confirmed human infection with an influenza A(H10N8) virus was reported in mainland China in December 2013. The patient, a 73-year-old woman with multiple comorbidities, died one week after being hospitalized. Two other cases of human infection with A(H10N8) were subsequently confirmed, one of which was fatal [488]. Phylogenetic analysis showed that the A(H10N8) virus was a novel reassortant strain, with the HA and NA segments derived from different influenza viruses from wild birds, and the six internal genes most likely originated from A(H9N2) viruses in poultry [488].

Section II: Prevention and control

As already discussed in Section I, influenza viruses represent a major public health priority. As a result, both the scientific community and public health authorities face constant challenges when it comes to preventing and treating influenza infections. This section reviews the two main types of interventions available: vaccines and antiviral drugs.

1.10 Influenza vaccines

Annual vaccination constitutes the most effective way to reduce the morbidity and mortality of influenza viruses, especially in people with high risk of developing complications. Influenza vaccines have been available since 1945, however, unlike some other vaccine-preventable diseases, the highly variable nature of influenza virus from season to season requires the annual adjustment of vaccine composition. In view of this, and because it usually takes about 6 months to produce enough doses of a new vaccine, each year the WHO devotes significant resources to maintain a global surveillance program to predict the dominant strains of influenza that will most probably circulate the following season. Influenza reaches peak prevalence in winter, and because the northern and southern hemispheres have winter at different times of the year, there are actually two different flu seasons each year. This is why the WHO (assisted by the National Influenza Centers) makes recommendations for two different vaccine formulations every year; one for each hemisphere. The efficacy of such vaccines depends then on how closely the vaccine strains match the circulating strains. Because the main influenza A(H1N1), A(H3N2) and B viruses co-circulate in the same season, traditional vaccines formulations include one strain of each subtype, the so called "trivalent" vaccines. In addition, new "quadrivalent" vaccines harboring two influenza B strains from different lineages are also available.

Two different types of vaccines have already successfully proven their efficacy and safety, and are therefore on the market: inactivated vaccines and live attenuated vaccines (LAIV). Many advances in the field of vaccines have improved their production, immunogenicity and tolerability [321], with current vaccines having an overall mean efficacy in reducing the risk of disease of 67% in young adults, assuming a good match between vaccine and circulating strains [329]. Although the WHO recommends yearly influenza vaccination to be routinely offered to everybody aged 6 months

and older with rare exceptions, priority is given to people at high risk of developing influenza-related complications. This group includes those aged ≥65 years (especially nursing-home residents), people with chronic medical conditions (i.e. asthma, COPD, heart disease, diabetes), immunocompromised patients, pregnant women and children 6-24 months old, as well as those who live with or care for high-risk individuals [59].

1.10.1 Inactivated vaccines

Inactivated vaccines are composed either of whole viruses inactivated with formaldehyde or β-propiolactone, viruses fragmented by chemicals or detergents ("split-antigen"), or purified antigen preparations. Antigens for inactivated vaccines are produced by serial passage of wild-type strains or high-yield reassortant viruses in embryonated chicken eggs. They are harvested from fetal or allantoic liquid and then concentrated and purified. In the case of influenza inactivated vaccines, the "split-antigen" strategy is used and the antigen content is standardized. Indeed, current inactivated seasonal vaccines contain 15 µg of antigen (HA) from each of the three viral subtypes, i.e. H1, H3 and B [321]. Oil-in-water adjuvants such as MF59 or ASO3 can also be added to the vaccine composition in order to boost the immune response mounted by the vaccine, increasing hemagglutination inhibition antibody responses particularly in older people with chronic diseases, and being well tolerated despite slightly higher rates of transient mild local reactions than with non-adjuvanted vaccines [321]. In fact, both adjuvanted and non-adjuvanted inactivated seasonal influenza vaccines are currently available [152, 365, 489].

Although inactivated vaccines are quite safe and effective, there is always room for improvement. Vaccine production time can vary between 3-6 months, which is long enough for an emerging pandemic strain to spread worldwide and/or for circulating seasonal strains to undergo antigenic drifts hence mismatching the strains selected for the vaccine. Moreover, because of the vaccine production method, the final formulation can contain traces of residual egg protein that might, although rarely, cause hypersensitivity reactions in allergic individuals upon injection. An interesting alternative to reduce production time as well as avoid egg-related allergic reactions would be to produce inactivated vaccines in cell culture (e.g.: in MDCK cells), yet further development is still needed [160].

1.10.2 Live attenuated vaccines

The main idea underlying the production of a live attenuated influenza vaccine (LAIV) is that of stimulating not only the humoral immune response as in the case of inactivated vaccines but also the cellular response. In fact, LAIVs are administered intranasally (aerosolization of 0.25 ml per nostril) to mimic natural infection and therefore provide a broader immunological response and longer protection than inactivated vaccines. The process for vaccine production is similar to that of inactivated vaccines in the sense that viruses are grown in embryonated chicken eggs, but using attenuated master donor viruses (MDVs). These viruses acquired an attenuated temperature-sensitive phenotype through serial passages in cell culture at low temperature (25 °C), therefore replicating relatively well in the nasopharynx (~33 °C) but not in the lower airways (~37 °C). Reassortant vaccine strains contain six gene segments (PB1, PB2, PA, M, NP and NS) from these MDVs and the remaining two segments (HA and NA) from the selected circulating strains. Attenuated viruses commonly used in the production of this type of vaccines are A/Ann Arbor/6/60 (H2N2) for influenza A and B/Ann Arbor/1/66 for influenza B [76].

Administration of LAIVs is recommended for healthy children and adults aged 2-49 years [114], showing slightly higher protection than inactivated vaccines, mainly among young children [429]. However, LAIVs are not recommended for children <24 months of age due to increased risk of wheezing, people with medical conditions such as chronic heart or lung disease, asthma or reactive airways disease, pregnant women, the elderly and immunocompromised persons [56]. This aspect raises significant concern, considering that population groups most likely to develop complications derived from influenza infection are not covered by this vaccine.

1.10.3 Other strategies for vaccine development

Nucleic-acid based vaccines are prepared from specific plasmids that enable the expression of encoded influenza HA and NA glycoproteins, as well as internal proteins such as M1 and NP. These DNA vaccines are commonly administered via two routes: intramuscular injection or bombardment of the skin using a gene gun [467]. The major advantage of these vaccines is that they can induce both humoral and cellular immune responses. However, although studies have shown

that certain formulations effectively induced protection in mice, chickens and ferrets [270, 338, 363, 432], the safety and immunogenicity of these vaccines in humans remain to be evaluated [410].

Recombinant subunit influenza vaccines are composed of HA and NA proteins expressed in insect cells by the rapid and highly efficient recombinant baculovirus system. Despite the fact that these recombinant proteins are well tolerated in adults and the elderly [321], studies with recombinant HA, NA and M2 proteins showed that these proteins were not very immunogenic and therefore, several doses as well as the use of adjuvants would be required to improve efficacy [98, 361].

An alternative to the latter approach would be to co-express multiple influenza proteins in insect cells. In that sense, studies have shown that co-expression of either influenza HA and M proteins, or HA, NA, and M proteins result in the generation of virus-like particles (VLPs) which are morphologically comparable to natural virions but without nucleic acids [351, 354]. These VLP-based vaccines, either enhanced by a liposomal adjuvant or not, have proved to be more immunogenic and protective than recombinant subunit vaccines in mice and ferrets [42, 352, 354].

Finally, the ideal influenza vaccine would be a universal vaccine, that is, one that confers cross-protection against a range of influenza viruses. To that end, researchers focused on the use of either an antigenically stable protein, or an antigenically stable portion of an antigenically variable protein essential for viral replication [410]. In that sense, recent phase 1 clinical studies using a vaccine formulation based on a non-glycosylated highly conserved 23-aa ectodomain of the M2 protein have shown immunogenicity in humans [379]. Furthermore, the recent characterization of human broadly neutralizing antibodies directed against highly conserved epitopes in the stem region of influenza virus HA [468], coupled with the successful stable expression of an HA stem domain trimer [274], will enable the exploration of potential HA-based broadly protective vaccines.

1.11 Antivirals

Although annual vaccination constitutes the main approach for the prevention and control of influenza infections, its efficacy is limited by frequent mismatches between circulating and vaccine viral strains, inadequate immune responses mounted by some individuals and poor vaccination uptake [52]. As a consequence, antiviral drugs play an important role in the management of influenza

outbreaks and pandemics, with both prophylactic and therapeutic indications. People with severe influenza infections as well as those in the high-risk groups already mentioned (section 1.10) constitute the main target population for antiviral therapy. So far, only two classes of anti-influenza agents have been approved for clinical use: adamantanes and neuraminidase inhibitors (NAIs), the latter constituting the main focus of this thesis.

1.11.1 Adamantanes

1.11.1.1 Mechanism of action

Amantadine (1-adamantamine hydrochloride), commercially available as Symmetrel, and its analogue rimantadine (α-methyl-1-adamantanemethylamine) available as Flumadine, constitute the first antivirals approved for the management of influenza infections [303]. Adamantanes inhibit an early step of the influenza A virus replication cycle by interfering with the function of the M2 viral protein. As previously mentioned (section 1.5.3), the M2 protein is an ion channel through which the hydrogen ions pass into the interstices of the viral particle, causing the dissociation of the M1 matrix protein from the RNP complex. The RNP can then enter the cell nucleus and initiate replication. The M2 ion channel also plays an important role during viral maturation, by regulating the pH of the Golgi lumen to prevent premature rearrangement of the HA to its fusion-active conformation [170, 281]. Since influenza B viruses do not possess M2 ion channel, adamantanes are only effective against type A influenza viruses.

Even if their exact mechanism of action is still a matter of debate, low doses of amantadine and rimantadine effectively block M2, thus interfering with viral internalization and uncoating (Figure 1.8, section 1.6) [175, 342]. In cell culture experiments, amantadine inhibits influenza A virus plaque formation by 50% (IC_{50}) at a concentration of 0.1 to 0.4 µg/ml [175].

1.11.1.2 Clinical indications and pharmacokinetics

As summarized by Couch et al., adamantanes can prevent about one-half of influenza A virus infections and 70-90% of illnesses when used as prophylaxis [72]. The therapeutic benefit of adamantanes has also been demonstrated, being associated with an average 1-day reduction in the duration of illness, when the drugs are administered within 48 h of the onset of symptoms [72, 224].

Both amantadine and rimantadine are administered orally as tablets or syrup at a dose of 5 mg/kg of body weight for children and up to 200 mg/day for adults. Maximal plasma concentrations of amantadine are achieved within 2 h of drug intake (4 h for rimantadine), with half-lives of about 15 h in young adults and 30 h in the elderly (30 h in both age groups for rimantadine). Both drugs are almost completely metabolized and highly excreted in urine [72].

On the other hand, the use of adamantanes has been associated with several central nervous system (CNS) side effects, such as anxiety, depression and insomnia. These toxic effects have been reported with higher frequency for amantadine (6% of adults and 15-19% of elderly patients) than rimantadine (2% of patients) [72, 230]. As a result, treatment with a 100 mg/day dose of rimantadine is preferred, especially in the elderly, since it is as effective as the 200 mg/day dose, but with less toxicity [275]. Apart from CNS symptoms, transient gastrointestinal disorders (nausea, vomiting and dyspepsia) can be caused by both amantadine and rimantadine [72].

1.11.1.3 Mechanisms of resistance to adamantanes

Genotypic studies demonstrated that resistance to adamantanes results from a single substitution at one of 5 codons at positions 26, 27, 30, 31, and 34 in the transmembrane region of the M2 protein (Figure 1.14) [34, 240, 284, 370]. It has also been reported that two or more different M2 mutants could be recovered from amantadine-treated patients [34, 393]. Specific M2 mutations have been associated with certain influenza subtypes, with the predominance of the V27A mutation in seasonal A(H1N1) viruses and the S31N substitution in A(H3N2) isolates [370]. Of note, all characterized A(H1N1)pdm09 viruses have been shown to contain the S31N mutation in their M2 gene, already present in the parental A(H1N1) of swine origin [60].

Although almost all influenza viruses were naturally susceptible to the adamantanes before 2004, rates of resistance greater than 30% and as high as 80% have been reported after only a few days of therapy in both immunocompetent and immunocompromised patients [240, 393]. Surveillance programs further demonstrated that the rate of natural resistance to adamantanes evolved from 1% to 12.3% in 2004 among A(H3N2) viruses collected worldwide [43, 490]. Subsequently, >90% of A(H3N2) viruses have exhibited resistance to amantadine whereas a lower rate of resistance (15.5%) has been reported for A(H1N1) viruses before the 2009 pandemic [99].

Noteworthy, high rates of resistance (>90%) were also reported among clade 1 A(H5N1) viruses from Southeast Asia as well as in all characterized A(H1N1)pdm09 viruses [60, 85].

Amantadine-resistant A(H3N2) variants do not show growth impairment *in vitro* or reduced virulence in experimentally-infected ferrets [413]. Also, our group reported that recombinant A(H1N1) viruses harboring different mutations of resistance to adamantanes (L26F, V27A, A30T, S31N, G34E and V27A/S31N) retained their replication properties *in vitro* and were at least as virulent as the recombinant wild-type (WT) virus in mice [4]. Accordingly, adamantane-resistant variants were readily transmitted in families or during nosocomial outbreaks [172]. Due to their activity against influenza A viruses only, their adverse effects, and the rapid emergence of resistance either during treatment or in the absence of drug pressure, the Centers for Disease Control and Prevention (CDC) has strongly advised against the use of this class of drugs unless susceptibility is formerly established with laboratory testing. Hence, since 2010, NAIs are the only class of antivirals recommended by the WHO for the treatment and prophylaxis of influenza A and B infections [461].

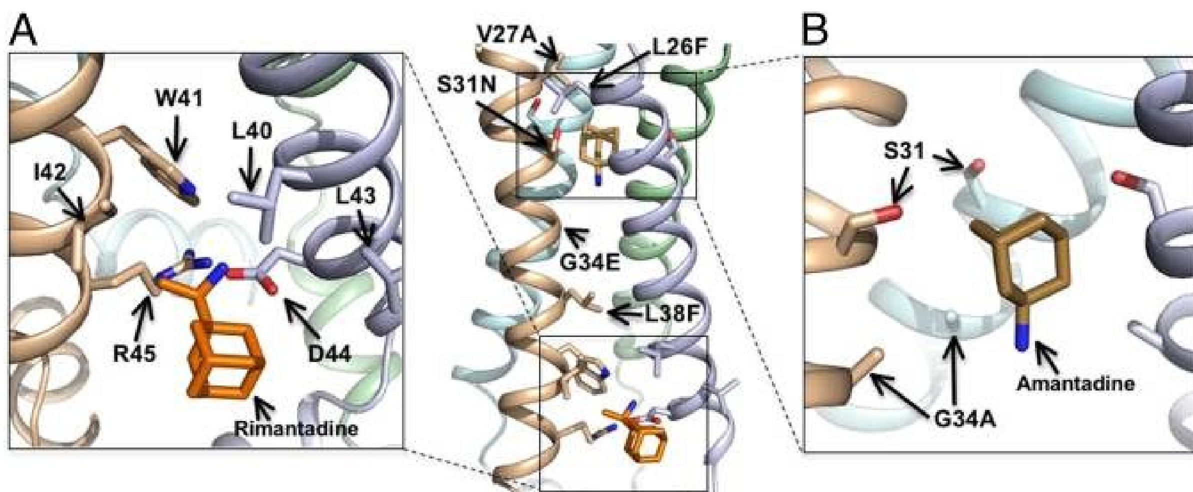

Figure 1.14. Proposed rimantadine (A) and amantadine (B) binding sites of the M2 ion channel. Taken from [344].

1.11.2 Neuraminidase inhibitors (NAIs)

Two NAIs are currently licensed worldwide for therapeutic and prophylactic uses: the oral agent oseltamivir phosphate, commercially available as Tamiflu (F. Hoffmann-La Roche) and the

inhaled drug zanamivir, which is commercially available as Relenza (GlaxoSmithKline). During the 2009 influenza pandemic, the US Food and Drug Administration (FDA) issued an Emergency Use Authorization (EUA) for the parenteral drug peramivir (BioCryst) for the treatment of hospitalized patients with known or suspected influenza A(H1N1)pdm09 infection [30]. Peramivir is already approved in Japan as Rapiacta and also available in South Korea as Peramiflu. Laninamivir octanoate, a prodrug of laninamivir (another inhaled NAI with long-acting properties), has also been approved in Japan and is commercially available under the name of Inavir (Daiichi Sankyo Company Ltd.). The latter two NAIs are currently in clinical evaluation in the US and other countries.

1.11.2.1 Mechanism of action

While the HA protein is responsible for virus attachment to the sialic acid receptors on the host cell, the catalytic activity of the NA cleaves off the terminal N-acetyl neuraminic acid (Neu5Ac) on these α-2,3 and α-2,6 sialic acid moieties. Hence, NA enzymatic activity plays a key role in releasing progeny virions from the host cell and also in facilitating viral spread throughout the upper airways by cleaving off the sialic acid on the mucin of respiratory mucus (reviewed in section 1.5.1.2). Given its catalytic function, the structure of the NA active site is highly conserved among influenza A and B viruses, and hence constitutes an attractive target for antiviral therapy. Crystallographic data of NAs from N2, N9 and B viral backgrounds [15, 45, 68] contributed to the design and synthesis of a series of compounds able to mimic the natural substrate of the NA enzyme and compete for the binding to the active site (Figure 1.15). Since these NAIs are based on the structure of the 2,3-didehydro analogue of the N-acetyl-neuraminic acid (DANA), they possess higher binding affinity than the Neu5Ac thus preventing the cleavage of the natural substrate. As a result, progeny virions fail to be released from the sialic acid receptors and aggregate on the surface of the infected cell, hampering the spread of infection to other non-infected cells (Figure 1.16).

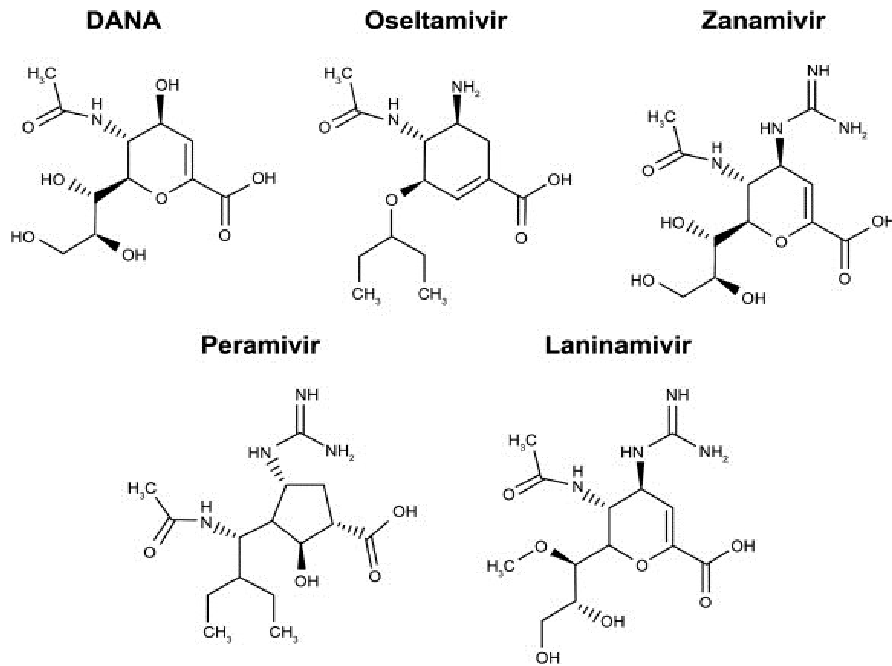

Figure 1.15. Chemical structure of neuraminidase inhibitors (NAIs). All these agents are based on the structure of the 2,3-didehydro analog of the N-acetyl-neuraminic acid (DANA). The bioavailable prodrug of oseltamivir is an ethyl ester that is converted into the active carboxylate by hepatic esterases. Zanamivir is a 4-deoxy-4-guanidino analog of DANA. Peramivir is a cyclopentane derivative with a guanidinyl group and a lipophilic chain. Laninamivir is the active product of the esterified octanoate CS-8958. These molecules interact differently within the enzyme active site, which may influence their antiviral activity.

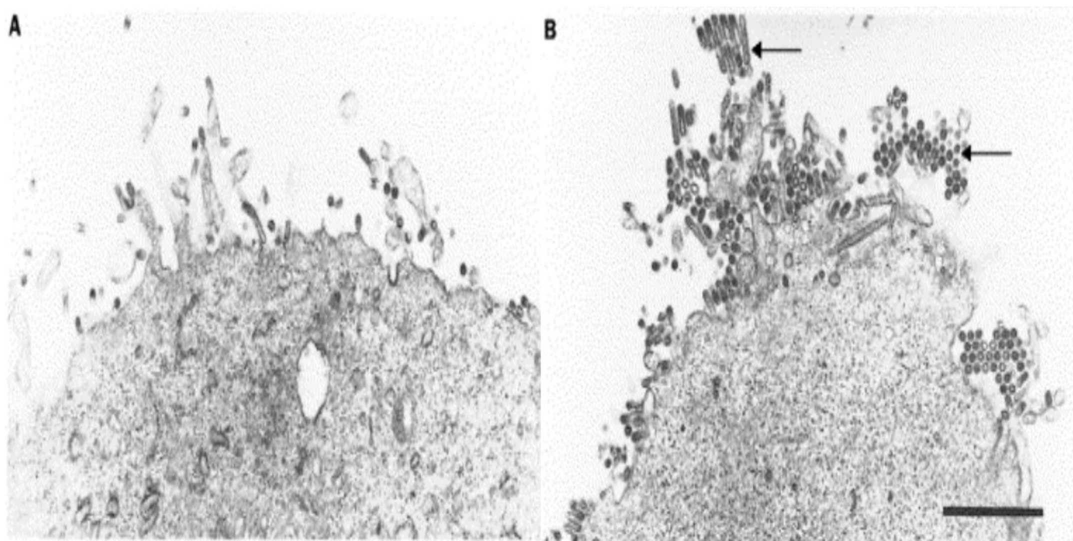

Figure 1.16. Electron micrographs of MDCK cells infected with influenza A virus. (A) Normal assembly and budding of virus in absence of NA inhibitor. (B) Lateral aggregation and formation of large bundles by virus in presence of NA inhibitor. Bar=1 μm. Adapted from [156].

1.11.2.2 Clinical indications and pharmacokinetics

Oseltamivir

Oseltamivir is an ethyl ester prodrug which requires ester hydrolysis to be converted to the active form oseltamivir carboxylate. This compound was developed through modifications of the sialic acid analogue framework, including the addition of a bulky lipophilic side chain that allows the drug to be given orally [235]. Like other NAIs, oseltamivir acts as a competitive inhibitor of the sialic acid and, accordingly, it binds to the influenza viral NA active site and blocks the activity of the enzyme. Oseltamivir has shown *in vitro* activity against influenza A and B types and different influenza A subtypes, including human and avian viruses. Oseltamivir is approved for prophylaxis or treatment of uncomplicated acute illness due to influenza infection in patients 1 year and older who have been symptomatic for less than 2 days. The drug is administered twice daily for 5 days using the dosages indicated in Table 1. Following oral administration, oseltamivir phosphate is rapidly absorbed from the gastrointestinal tract and converted predominantly by hepatic esterases into the active metabolite oseltamivir carboxylate. The absolute bioavailability of this compound is 80% and the active metabolite is detectable in plasma within 30 min reaching maximal concentrations after 3 to 4 h. After peak plasma concentrations are achieved, the concentration declines by renal excretion with an apparent half-life of 6 to 10 h [90, 106, 178]. The use of intravenous (IV) oseltamivir, which is currently evaluated in clinical trials, should be considered only for patients with severe influenza who cannot take oral or inhaled medication [41]. Exposure to the active metabolite, oseltamivir carboxylate, after dosing with 100 mg intravenously over 2 h was comparable to the usual 75 mg dose administered orally [41].

Zanamivir

Zanamivir is a 4-deoxy-4-guanidino analogue of DANA that was approved as an anti-influenza agent in 1999. Due to its poor oral bioavailability, zanamivir is formulated as a dry powder which has to be delivered with an inhaler device (Diskhaler®) that entails the cooperation of the patient [102]. As a result, the amount of drug delivered to the respiratory tract may depend on the inspiration flow, which represents a limitation for the use of this formulation in some individuals including intubated patients. In addition, adverse events such as cough, decreased pulmonary function or fatal bronchospasm have been seldom reported, particularly in patients with underlying

pulmonary disease [209]. In general, zanamivir has shown higher *in vitro* activity than oseltamivir against influenza A(H1N1), A(H1N1)pdm09, and B viruses as shown by lower mean IC_{50} values in NA inhibition assays [57, 390]. Conversely, A(H3N2) strains are more sensitive to oseltamivir than to zanamivir [118]. A zanamivir treatment dose of 10 mg twice daily for 5 days has been licensed for patients 7 years of age and older, whereas the recommended household prophylactic dose is 10 mg once daily for 10 days in individuals ≥5 years old (Table 1). In the case of community prophylaxis, the regimen can be extended for up to 28 days in adolescents and adults [115]. After a 10 mg dose inhalation, zanamivir is rapidly deposited mainly in the oropharynx (78%) and in the lungs (13%). Only 10 to 12% of the dose is systemically absorbed. The drug is eliminated unchanged in the urine within 24 h whereas the unabsorbed drug is excreted via the gastrointestinal tract. Direct measurement of zanamivir concentrations in sputum and nasal wash samples after a single 10 mg inhaled dose supports the twice daily treatment regimen since drug concentrations remain above the IC_{50} values after 24 and 12 h, respectively [335]. An IV formulation of zanamivir (300 and 600 mg doses) is currently in phase III clinical trial and is also available for treatment under compassionate use authorization. Available pharmacokinetics data have established that the median plasma elimination half-life is 2 h [53]. The presence of the drug in the respiratory tract was confirmed in nasal wash samples of infected adults on days 2 and 4 after twice daily dosing of 600 mg intravenously [47].

Peramivir

Peramivir is a cyclopentane derivative with a negatively-charged carboxylate group, a positively-charged guanidino group and a lipophilic side chain [13]. Due to its low bioavailability, this drug needs to be delivered parenterally [16, 485]. Peramivir has demonstrated activity *in vitro* and in animal models against various influenza A and B viruses, including the A(H1N1)pdm09 virus and highly pathogenic A(H5N1) viruses [13, 36, 238]. Peramivir was first developed for oral administration and showed *in vivo* activity in both mice and ferrets [17, 394, 395]. However, peramivir did not demonstrate a statistically significant clinical benefits in controlled trials of prophylaxis and treatment, despite the fact that oral peramivir was associated with significant reduction in viral titers. These results have been attributed to a low oral bioavailability in humans [20]. To improve the bioavailability, subsequent studies have been performed using IV or intramuscular (IM) injections of peramivir. A

study has shown that IM administration was associated with a higher survival rate than oral administration in mice (100% versus 50%) after infection with the influenza A/NWS/33 strain [16]. Accordingly, our mouse studies revealed that a single IM dose of peramivir provided important prophylactic and therapeutic benefit against the lethal A/WSN/33 (H1N1) virus and its oseltamivir-resistant variant [7]. Moreover, preclinical studies in mice and ferrets demonstrated that parenteral administrations of peramivir rapidly produced high plasma concentrations [485]. In clinical trials, the use of IV peramivir at a single 300 or 600 mg dose was associated with clinical benefits that were comparable to those provided by oseltamivir at a dose of 75 mg twice a day [244]. In addition, the overall incidence of adverse effects in the 300 mg IV peramivir group was significantly lower compared to the oseltamivir one. These results contributed to the approval of IV peramivir for adults in Japan and South Korea in 2010.

The availability of IV NAIs offers an alternative route of administration, which is especially important for patients who cannot take oral or inhaled medication, such as mechanically-ventilated patients. The standard peramivir adult dose is 600 mg once a day, administered intravenously for 5 to 10 days (Table 1). The IV administration of peramivir (600 mg daily for 5 days) resulted in a peak plasma concentration of 45200 ng/ml [244]. Peramivir is approximately 90% eliminated as an unchanged drug by the kidneys with an apparent half-life of 7.7 to 20.8 h in adults with normal renal function [113].

Laninamivir

Laninamivir octanoate is an inhaled prodrug which is processed in the lungs into laninamivir. Laninamivir contains a 4-guanidino group, like zanamivir, in addition to a 7-methoxy group. Laninamivir has shown good inhibition against a broad range of influenza A (N1 to N9) and influenza B strains [247, 474]. A single nasal administration leads to long retention of the drug in the lungs conferring a long-lasting anti-NA activity. As for zanamivir, laninamivir octanoate is formulated as a dry powder that has to be administered with a specific inhaler device. The recommended treatment for pediatric patients ≤10 years old is a single 20 mg inhalation dose, whereas for patients ≥10 years old the dose is doubled to 40 mg (Table 1) [204]. Once inhaled, this inactive prodrug is converted to the active metabolite laninamivir in the respiratory tract within 24 h. Following a single 40 mg dose, the concentration of laninamivir in the lungs peaks to a median of 30.7 ng/ml within 4 h, and

subsequently decreases with a half-life of 67 h [78]. This high retention time confers long-lasting NA inhibition [245, 474], an added value in terms of treatment planning and drug stockpiling. In mouse and ferret models, the efficacy of laninamivir in either prophylaxis or treatment was superior to those of oseltamivir and zanamivir [247]. Even at 120 h post dose, the concentration of laninamivir in the lungs of treated mice was far beyond the mean IC_{50} values for the different influenza A and B viruses [204]. In clinical trials, the efficacy and tolerability of a single 20 or 40 mg inhaled dose of laninamivir octanoate in pediatric and adult patients infected with influenza were comparable to that of a 5-day treatment with oseltamivir 75 mg twice daily [29, 207, 208, 412, 448]. It is noteworthy that a single inhalation of 20 or 40 mg of laninamivir octanoate was effective for the treatment of seasonal influenza A(H1N1) infections including oseltamivir-resistant variants [448].

Table 1. Recommended dosage of NAIs for chemoprophylaxis and therapeutic use.

NAIs	Age	Prophylaxis	Treatment
Oseltamivir (Tamiflu)	Children 1 - 12 years	Oral Suspension	
	≤ 15 kg	30 mg once daily / 10 days	30 mg twice daily / 5 days
	> 15 kg to 23 kg	45 mg once daily / 10 days	45 mg twice daily / 5 days
	> 23 kg to 40 kg	60 mg once daily / 10 days	60 mg twice daily / 5 days
	> 40 kg	75 mg once daily / 10 days	75 mg twice daily / 5 days
	Adolescents and adults	Oral Capsule	
	≥ 13	75 mg once daily / 10 days	75 mg twice daily / 5 days
Zanamivir (Relenza)	Children and adults	Oral Inhalation	
	≥ 5 years	**10 mg once daily/10 days**	___
	≥ 7 years	10 mg once daily/10 days	10 mg twice daily/5 days
Peramivir (Peramiflu) (Rapiacta)	Children	Intravenous (max. daily dose 600 mg)	
	0 - 30 days	___	6 mg/kg once daily / 5 days
	31 - 90 days	___	8 mg/kg once daily / 5 days
	91 - 180 days	___	10 mg/kg once daily / 5 days
	≥ 181 days- 5 years	___	12 mg/kg once daily / 5 days
	≥ 6 years	___	10 mg/kg once daily / 5 days
	Adolescents and adults	Intravenous (max. daily dose 600 mg)	
	≤ 17 years	___	10 mg/kg once daily / 5 days
	≥ 17 years	___	600 mg once daily / 5 days
Laninamivir (Inavir)	Children and adults	Oral Inhalation	
	≤ 10 years	___	20 mg Single inhalation
	≥ 10 years	___	40 mg Single inhalation

1.11.2.3 Resistance to NAIs due to NA mutations

Influenza viruses with reduced sensitivity to NAI typically contain mutations in the NA which directly or indirectly alter the shape of the NA catalytic site, thus reducing the inhibitor binding ability. As stated in section 1.5.1.2, the catalytic site of the NA is constituted of eight functional residues (R118, D151, R152, R224, E276, R292, R371, and Y406), surrounded by eleven framework residues (E119, R156, W178, S179, D198, I222, E227, H274, E277, N294, and E425) implicated in the stabilization of the active site structure. Although these residues are conserved in all influenza A and B viruses [67], resistance mutations can differ according to the viral subtype and also to the type of NAI used, provided the differential interactions between NAIs and the active site of the NA (Figure 1.17).

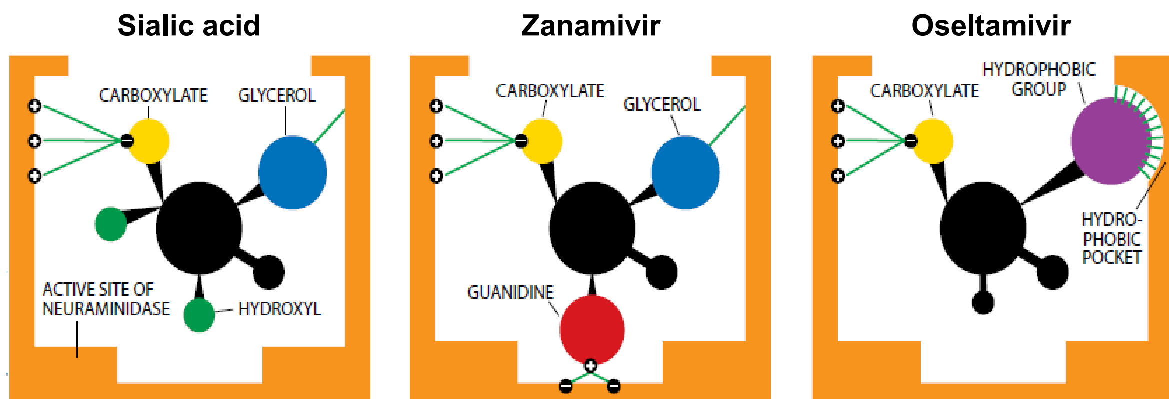

Figure 1.17. Differential binding of sialic acid and NAIs to the NA active site. Sialic acid (left) is held in the active site cleft mainly through its glycerol and carboxylate groups, which form bonds (green lines) with aa in the active site. Zanamivir (center) adds other bonds by replacing the hydroxyl of a sialic acid derivative with a large, positively charged guanidine, forming strong attachments to two negatively charged aa at the bottom of the cleft. Oseltamivir (right) retains the carboxylate bonds made by sialic acid but also makes use of a hydrophobic group, inducing the binding cleft to form a similarly hydrophobic pocket, which holds the drug in place through hydrophobic attractions (short green lines). Adapted from [259].

Because oseltamivir has a large hydrophobic side chain, the NA must undergo rearrangements to accommodate drug binding. To form this pocket, the aa E276 must rotate and bind to R224 [69, 279]. Any mutations that affect this rearrangement may reduce the binding affinity of oseltamivir leading to lower efficiency (Figure 1.18). Up to now, the histidine-to-tyrosine H274Y

substitution (H275Y in the N1 numbering system), as well as mutations R292K and N294S (N295S in N1 numbering) have been shown to inhibit the rotation of the E276 residue and to prevent pocket formation, therefore conferring reduced sensitivity to oseltamivir [445]. The molecular structure of zanamivir includes a guanidino group, instead of the hydrophobic group found in oseltamivir. As a result, mutations affecting the pocket formation do not confer resistance to zanamivir. On the other hand, the guanidino group of zanamivir interacts with the conserved E119 residue in the active center pocket [491]. As with oseltamivir, resistance to zanamivir can develop as a result of mutations in framework or catalytic residues of the NA protein that affect binding affinity between the enzyme and the inhibitor [154]. Peramivir possesses a guanidino group similar to that of zanamivir and a hydrophobic group similar to that of oseltamivir. Therefore, as it is the case for oseltamivir, structural rearrangements are necessary to accommodate the peramivir hydrophobic side chain [13] and mutations that affect the activity of oseltamivir and zanamivir can also affect peramivir activity. No laninamivir-resistant mutations have been reported in the clinic yet. However, since zanamivir and laninamivir share highly similar binding properties with the NA protein [440], mutations conferring reduced susceptibility to zanamivir are expected to have similar effect on laninamivir, something that has recently been confirmed by our group [372].

The NA inhibition assay is the universally adopted laboratory method to test the susceptibility of influenza viruses to NAIs. While chemoluminescent NAI assays need less input virus for testing, fluorometric assays allow a better discrimination between susceptible and resistant viruses [316]. The current version of this test is a refined version of the one originally developed by Potier and colleagues in 1979 [348]. In this test, the NA activity of a viral sample can be measured through the production of a fluorescent product by using either a fluorogenic or chemoluminiscent sialic acid analog as substrate. Susceptibility profiles for each drug can be determined by the extent of NA inhibition (loss of fluorescence) observed following incubation of the sample with serial dilutions of different NAIs, therefore obtaining an IC_{50} value from the dose-response curve. The WHO guidelines on the determination of NAI resistance for influenza A virus isolates define “reduced inhibition” by a 10-100-fold increase in IC_{50} values, and “highly reduced inhibition” by a >100-fold increase in IC_{50} values, when compared to reference susceptible strains. In the case of influenza B viruses thresholds are set in 5-50-fold and >50-fold increase in IC_{50} values, respectively [458]. However, there is no established cutoff IC_{50} value that discriminates between viruses susceptible to NAIs and viruses with clinically-relevant resistance, except for the H274Y mutant, which is considered clinically resistant.

Genotypic assays constitute a more rapid alternative to detect drug resistance mutations directly from the clinical samples after RT-PCR amplification. Although comprehensive, this approach does not determine the level of resistance, and its utility is limited to the detection of mutations already known to confer reduced susceptibility to NAIs. Moreover, a mutant variant must be in excess of 15-20% of the total viral population in order to be identified by conventional (Sanger) DNA sequencing. In that regard, the advent of pyrosequencing and especially next-generation deep sequencing has allowed the detection of minor variants in excess of 1-2% [139]. The importance of this increased sensitivity will be discussed in the study presented in chapter VI.

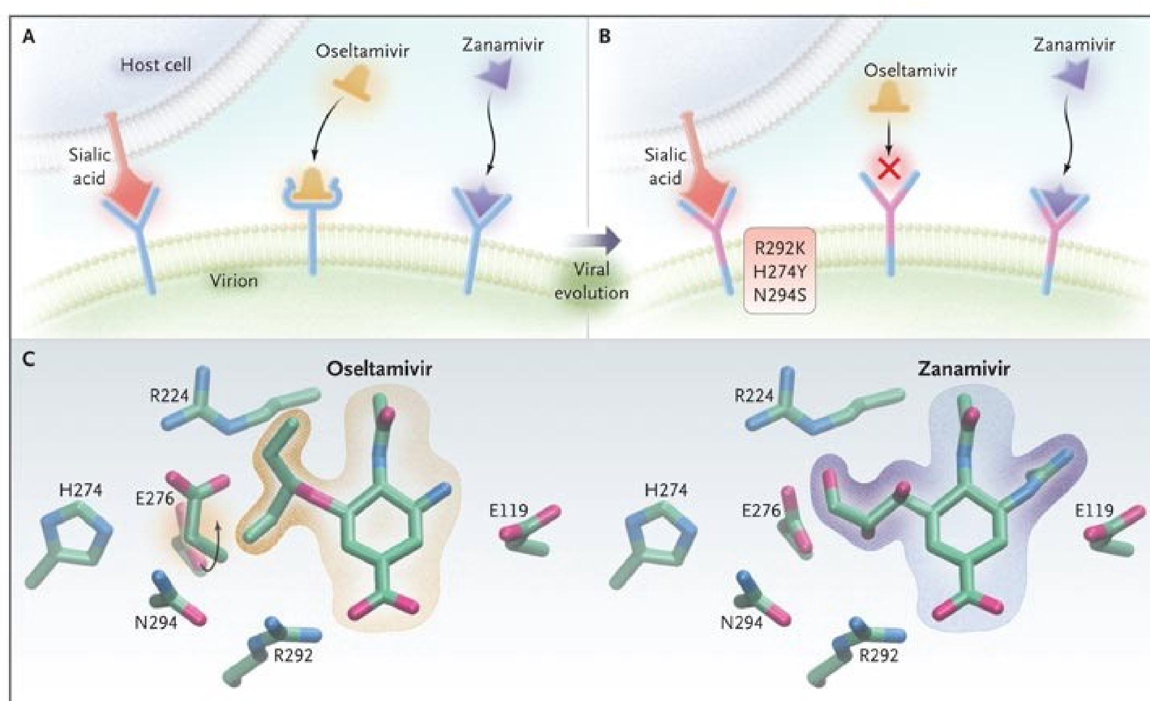

Figure 1.18. Mechanism of Resistance to Oseltamivir. (A) The NA active site changes shape to create a pocket for oseltamivir, whereas it accommodates zanamivir without such a change. (B) Any of several mutations may prevent the binding of oseltamivir by preventing the formation of this pocket without altering binding to sialic acid and to zanamivir. (C) The pocket for oseltamivir is created by the rotation of E276 and bonding to R224; R292K, N294S, and H274Y mutations prevent rotation and therefore result in resistance to oseltamivir. Taken from [304].

Resistance to oseltamivir

Oseltamivir-resistant influenza viruses have been rarely detected in clinical samples before the availability of NAIs. During the first 5 years (1999-04) following the introduction of oseltamivir and

zanamivir, the incidence of oseltamivir resistance seen in clinical trial samples was 0.33% in adults (≥13 years) and 4.0% in children (≤12 years) [10, 302, 447]. However, more significant resistance levels were observed in some clinical therapeutic settings, such as in young hospitalized children (up to 18%) [237], immunocompromised patients, [24, 210, 453] and human cases of influenza A(H5N1) infections [97, 262]. Unexpectedly, high rates of natural resistance to oseltamivir were reported worldwide during the 2007-08 influenza season. During the subsequent season, almost all characterized influenza A/Brisbane/59/2007(H1N1)-like strains from North America and Europe were reported to be oseltamivir resistant due to the H274Y mutation [61]. Epidemiological studies reported no evidence of an association between the development of resistance and oseltamivir use [100, 167, 295].

Since the first wave of the 2009 pandemic and with the disappearance of the A/Brisbane/59/2007 strain, the overall level oseltamivir resistance amongst A(H1N1)pdm09 variants has remained relatively low [357]: ~1% in the US [409], <1% in Canada [341], ~2.5% in Europe and <1.6% worldwide [194]. Whereas the percentage of sporadic cases of oseltamivir-resistant A(H1N1)pdm09 viruses in North America has remained relatively stable among immunocompromised patients and healthy children who had received oseltamivir prophylaxis or treatment [25, 150], the fraction of drug-resistant cases not associated with oseltamivir exposure has increased significantly in US, from 11% in the 2009-10 season to 74% in 2010-11 [409]. In addition, a few clusters of oseltamivir-resistant cases, not associated to treatment and likely involving transmission of A(H1N1)pdm09 mutant strains, have been reported in United Kingdom, Australia and Vietnam [196, 197, 251, 278].

In influenza viruses of the N1 subtype, including seasonal A(H1N1), A(H1N1)pdm09 [25, 297] and highly pathogenic A(H5N1) [97, 262] strains, oseltamivir-resistant clinical isolates typically contain the H274Y mutation. Additional NA mutations in oseltamivir-resistant A(H1N1)pdm09 isolates, such as I222R/K/V (I223R/K/V in N1 numbering) [57, 264, 317, 436], S246N (S247N in N1 numbering) [201] and I117V [200] were shown to cause a synergistic effect on drug resistance when combined with H274Y. Furthermore, the I222R mutation has been reported to confer reduced susceptibility to multiple NAIs by itself, in the absence of the H274Y mutation. It should be indicated that only the H274Y and, to a lesser extent, the N294S NA mutations have been shown to confer resistance to oseltamivir in the human A(H5N1) background so far [262]. Moreover, the reduced

sensitivity to oseltamivir conferred by the N294S mutation, has been shown to occur without NAI pressure in humans infected with highly pathogenic A(H5N1) viruses [108, 262].

In influenza viruses of the N2 subtype, the R292K substitution in the catalytic site is rather common and markedly reduces sensitivity to oseltamivir. Decreased sensitivity to oseltamivir is also frequently conferred by the E119V mutation; in the latter case, the mutated NA may accommodate a water molecule, which would interfere with the binding of oseltamivir to the active site. As predicted, the R292K and E119V mutations predominate in clinical A(H3N2) isolates [237, 454], with the former mutation also being reported in recent oseltamivir-resistant A(H7N9) viruses [192]. The N294S variant also showed decreased susceptibility to oseltamivir in seasonal A(H3N2) viruses [2, 237]. It should be noted that, in addition to aa substitutions, small NA deletion mutations conferring reduced susceptibility to NAIs have also been reported in A(H3N2) viruses [3, 298].

Surveillance data from the 2010 and 2011 seasons in mainland China revealed that four influenza B viruses exhibited reduced susceptibilities to oseltamivir and shared the amino acid substitution I222T (I221T in B numbering). Additionally, a single virus with reduced susceptibility to oseltamivir contained the amino acid substitution D198N (D197N in B numbering) [444]. Various NA mutations identified in clinical isolates seem to be specific to type B viruses: E105K, R152K (R150K in B numbering), D198N (D197N in B numbering) and R371K [210, 300, 390]. An influenza B isolate with the H274Y mutation (H273Y in B numbering) has also been recovered from a 33-year old patient with no known history of NAI treatment [185] whereas a N294S variant was detected in a 7-year old patient with cancer prior to oseltamivir therapy [50].

Resistance to zanamivir

So far, resistance to zanamivir has remained quite infrequent in the clinical setting for both seasonal and pandemic viruses compared to oseltamivir [422]. The greater structural homology with the NA natural substrate (sialic acid) as well as the lower use of this drug compared to oseltamivir are the most probable factors that account for this observation.

Surveillance data from the 2006-08 seasons in Australia and Southeast Asia identified zanamivir-resistant influenza A(H1N1) viral isolates harboring the Q136K substitution [199]. However, this mutation could not be identified in the matching clinical specimens from Australia, suggesting

either the presence of this variant at a very low level before cell culture amplification, or its emergence as a result of *in vitro* viral propagation in MDCK cells [324]. Hence, the clinical relevance of the Q136K NA mutation is still a matter of debate. Of note, an influenza A(H5N1) variant carrying the Q136L mutation was recovered from nasal wash samples of a ferret treated with zanamivir. This variant showed high and moderate levels of resistance to zanamivir and oseltamivir, respectively [202]. Other studies have revealed the presence of A(H1N1)pdm09 viruses with I222R and I222K (I223R and I223K in N1 numbering) mutations conferring reduced susceptibility to zanamivir [110, 315, 436]. Substitutions at residues 119 (E119G) and 198 (D198G; D199G in N1 numbering) were selected *in vitro* under zanamivir pressure among influenza A(H5N1) viruses [198]. In addition, zanamivir resistance was observed for recombinant influenza A/WSN/33 (H1N1) carrying the E119V mutation although the fitness of this mutant was severely compromised [5].

Influenza A(H3N2) variants containing zanamivir resistant D151A/E/G/V mutations were identified in A(H3N2) variants during NAI resistance surveillance programs [289, 390]. Nevertheless, there are uncertainties about the clinical relevance of such changes. In contrast with the situation in A(H1N1) viruses, where the Q136K mutation was only detected in supernatants of infected cells, the Q136K mutation has been detected in two clinical samples of influenza A(H3N2) viruses isolated in Myanmar in 2007 and 2008 with reduced susceptibility to zanamivir [89]. Several other A(H3N2) variants such as R224K, R292K and R371K that exhibit reduced susceptibility or resistance to zanamivir and at least one other NAI were generated *in vitro* by using drug pressure or rescued by reverse genetics [478, 479]. Zanamivir-resistant recombinant H3N2 viruses harboring different substitutions at residue 119 (E119G/D/A) were also rescued by reverse genetics [491]. The fitness of these recombinant variants was significantly impaired due to altered NA enzymatic activity/affinity and stability.

In clinic, an influenza B variant with the R152K substitution (R150K in B numbering) has been recovered from an immunocompromised child treated with zanamivir [157]. Also, the E119G (E116G in B numbering), R152K (R150K in B numbering) and R292K variants with reduced susceptibility to zanamivir have been generated *in vitro* [215]. Using reverse genetics, Jackson and collaborators observed that the E119G/D/A substitutions conferred highly reduced or reduced susceptibility to zanamivir, oseltamivir and peramivir in the influenza B background. Of note, the fitness of the E119G mutant was not impaired. Interestingly, in the influenza B background, the

E119V mutation conferred highly reduced susceptibility to oseltamivir and peramivir with no change in zanamivir susceptibility [215].

Overall, available data on zanamivir resistance is quite limited compared to that on oseltamivir. In that regard, the study presented in chapter V of this document partially addresses this issue by characterizing the potential impact of the E119G and Q136K NA mutations on A(H1N1)pdm09 viruses.

Resistance to peramivir

In clinic, emergence of peramivir-resistant viruses was first observed following prophylaxis or treatment with oseltamivir. Early during the 2009 pandemic, the CDC reported two unrelated cases with peramivir-resistant A(H1N1)pdm09 strains that contained the H274Y mutation following treatment with oseltamivir [58]. These strains showed high levels of resistance to oseltamivir and lower level resistance to peramivir [194]. Other cases of peramivir resistance have been reported after shorter courses (9 and 14 days) of oseltamivir treatment [297].

Peramivir resistance is mainly conferred by the H274Y mutation in influenza viruses of the N1 subtype. For instance, a H274Y mutant emerged in a patient with severe A(H1N1)pdm09 infection during IV peramivir therapy [358]. During the 2007-10 influenza epidemics in Japan, clinical isolates from four peramivir clinical studies in adult and pediatric patients were tested. Reduced susceptibility to peramivir was found in 2.6% (2007-08), 0.1% (2008-09) and 6.1% (2009-10) of cases. Almost all resistant viruses carried the H274Y mutation and exhibited a 10- to 50-fold reduction in susceptibility to peramivir. *In vitro* passages of the influenza A/WSN/33(H1N1) strain under peramivir pressure resulted in the emergence of the H274Y mutation [23]. Nevertheless, the clinical relevance of the H274Y mutation in conferring resistance to permavir is still a matter of debate due to the excellent pharmacokinetic properties of the drug. Indeed, our group has shown the prophylactic and therapeutic benefit of a single IM dose of peramivir in mice infected with either WT or H274Y A/WSN/33 (H1N1) viruses [7]. Of note, there are no reports on peramivir resistance in influenza viruses of the N2 subtype.

Two NA mutations (V94I and R152K: respectively V89I and R150K in B numbering) were detected in an influenza B isolate from a pediatric patient. These mutants were associated with a

reduction in susceptibility to peramivir [483]. As described previously for oseltamivir resistance, an influenza B virus with the H274Y mutation (H273Y in B numbering system) has been recovered from a patient who had no known history of previous NAI treatment. This mutation was shown to confer cross-resistance to peramivir and oseltamivir [185]. The influenza B H274Y mutant was also generated *in vitro* under peramivir pressure [22]. Moreover, mutated recombinant B viruses with the E119D/A/V/G, R152K and R292K mutations (E116D/A/V/G, R150K and R191K in B numbering), were shown to increase peramivir IC_{50} values by >1598-, >1598-, 531-, >1598-, 214- and 502-fold, respectively, compared to the WT strain [215]. In another study, drug-selected R152K variants also exhibited a reduction in susceptibility to peramivir [300]. Of note, a comprehensive table of the resistance pattern to osetamivir, zanamivir and peramivir due to NA mutations found *in vitro*, in the clinic and in surveillance programs is presented in the annex of this thesis.

Resistance to laninamivir

As mentioned, no laninamivir-resistant mutations have been reported in clinic to date. However, an *in vitro* study performed by our group confirmed that, as expected due to their structural similarities, the susceptibility profile of laninamivir is very similar to that of zanamivir [372]. Moreover, it is legitimate to infer that both zanamivir and laninamivir might be effective against the most frequent oseltamivir-resistant mutants, namely H274Y in A(H1N1) and E119V in A(H3N2). Nevertheless, it is important to note the higher potency of laninamivir against N1 viruses in which the more open configuration of the 150-loop of the NA might facilitate access to the active site for laninamivir, contrasting to the relatively closed configuration of the 150-loop in group 2 NAs that may hinder the entry and binding of zanamivir and laninamivir [440].

1.11.2.4 Resistance to NAIs due to HA mutations

Besides NA mutations, resistance to NAIs might also emerge due to mutations in the HA protein, though these mutations have been reported mainly in viruses isolated in cell culture and their role in clinical isolates is still unknown. These mutations occur in or near of the receptor binding site of the HA protein and reduce binding affinity between the HA and sialic acid [144]. As a result, newly formed virions do not bind strongly to cell receptors and viral release is possible even if NA activity is

reduced by the action of NAIs. In fact, HA mutations reduce the viral dependence on NA activity, then leading to decreased sensitivity to the NAIs [291]. In that regard, HA mutations have been reported in NAI-resistant viruses carrying NA deletions conferring partial or even complete loss of NA activity [23, 309, 372].

Decreased sensitivity due to HA mutations has been demonstrated by growing the mutants under drug concentrations sufficiently high to inhibit the replication of the WT susceptible virus. However, alterations in HA binding to cell receptors are also dependent on the receptor phenotype of the cell type used. Thus, a clinical isolate may not show altered sensitivity in traditional MDCK cells, which mostly express α-2,3 sialic acid. Also, in a phenomenon known as "drug dependence", certain viruses that have such an important decrease in HA affinity that significantly reduces their replicative capacity will have major difficulty to adsorb to the host cell before the NA cleaves the sialic acid receptor. As a result, these viruses will infect the cell more efficiently if the NA activity is inhibited [19, 23, 155, 292]. These type of mutants generally confer cross-resistance to all types of NAIs as the effect of the low binding affinity is independent of how the NA is inhibited [292].

1.11.2.5 Resistance mutations and viral fitness

Mutations of resistance to NAIs that involve functional residues, such as R292K in the A(H3N2) background, were associated with a significant decrease of NA activity resulting in a reduced replicative capacity in cell culture [479] as well as severely reduced transmissibility in ferrets [181]. A similar effect was observed when the R292K mutation was introduced in the NA of recombinant influenza B viruses [215]. In contrast, the impact of framework NA substitutions, including H274Y and E119V varied depending on the subtype and even between strains of the same subtype. For instance, our group previously demonstrated that the E119V mutation severely compromised the replication of A/WSN/33 (H1N1) [5] recombinants, in contrast with A(H3N2) E119V variants, whose infectivity and transmissibility in ferrets were not significantly altered [181]. On the other hand, while the H274Y mutation impaired the replicative capacity of old influenza A(H1N1) viruses, including A/Texas/36/1991 and A/New Caledonia/99/2001 variants [181, 213], there was no compromising impact of this mutation on viral fitness of A/Brisbane/59/2007-like viruses [26, 355]. Consequently, A/Brisbane/59/2007-like viruses completely replaced their WT counterparts during the 2007-09 annual influenza epidemics, and showed clinical and epidemiological characteristics that

were comparable to NAI-sensitive A(H1N1) strains [195, 250]. Further phylogenetic and functional studies presented in chapter VII of this thesis revealed that, besides the H274Y mutation, the NA protein of oseltamivir-resistant A/Brisbane/59/2007-like isolates contained permissive substitutions (R222Q, V234M and D344N in N1 numbering), with the potential to counteract the deleterious effect on NA folding and/or transport caused by the H274Y change, hence improving the viral fitness of this variant [32]. In addtion, the H274Y mutation was shown to cause only slight or no significant impact on the fitness of A(H1N1)pdm09 viruses, being at least as virulent as the WT virus in mice and ferrets [162], although airborne transmission might be less efficient [163]. Accordingly, evidence of sustained transmission of H274Y A(H1N1)pdm09 variants in humans has been relatively low [194]. However, recent reports on community outbreaks show a high prevalence of oseltamivir-resistant variants recovered from individuals with no known exposure to NAIs [195, 294]. Here again, another set of permissive NA mutations might be implicated (see chapter VIII of this thesis), therefore highlighting the importance of continuous monitoring the evolution of antiviral resistance in influenza viruses.

In that regard, the impact of the emergence and dissemination of resistance on the limited choice of antivirals currently available for the treatment and prevention of influenza infections underscores a better understanding of the mechanisms underlying this phenomenon as well as the necessity for innovative therapeutic approaches, the two main points addressed in this work.

1.11.3 Investigational agents and combination therapy

As discussed above, the development and spread of resistance to existing anti-influenza agents may compromise their usefulness for the control of future influenza infections during epidemics and occasional pandemics. The lack of efficacy of adamantanes, for which influenza A(H1N1)pdm09 and A(H3N2) strains are resistant, as well as the issue of NAI-resistant variants that may emerge either during drug pressure or as a result of natural genetic evolution particularly emphasize the need to develop additional anti-influenza strategies. In that regard, new anti-influenza targets involving the polymerase (T-705 or favipiravir) [129, 130] or host cells receptors (DAS181 or Fludase) [80, 428] are at various stages of clinical development.

Furthermore, one strategy that could potentially improve the effectiveness of anti-influenza agents while reducing the emergence of drug-resistant variants is to use a combination of

compounds which target different viral functions or with different interactions with the same substrate, notably the NA. Indeed, the concept of combined antiviral therapy against influenza is not new with initial studies performed in the 1980s [171, 173] and may be particularly useful in immunocompromised patients for synergistic or at least additive antiviral effects [149, 177]. Recently, a triple combination regimen of oseltamivir, amantadine and the nucleoside analogue ribavirin (a RNA polymerase inhibitor), exhibited a strong synergistic activity against seasonal and A(H1N1)pdm09 viruses *in vitro* and *in vivo* [318-320]. In addition, lethal mouse studies demonstrated a significant increase in survival for animals that received peramivir combined to ribavirin [399] and rimantadine combined to oseltamivir [132] versus respective monotherapies. Interestingly, the combination of oseltamivir to ribavirin or to amantadine [205] showed benefits against highly pathogenic influenza A(H5N1) infections in mice. On the other hand, a French randomized clinical trial [107] in adults infected with A(H3N2) viruses showed that the oseltamivir-zanamivir combination was less effective than oseltamivir monotherapy, and not significantly more effective than zanamivir monotherapy. In addition, combined oral oseltamivir and inhaled zanamivir proved to be ineffective for the treatment of a series of intensive care patients with severe and complicated A(H1N1)pdm09 infections [339], highlighting the need of additional studies before recommending combined NAI therapy.

Chapter II: Hypotheses and objectives

2.1 Hypotheses

H1: Different NAI-resistant mutations can emerge and disseminate in both seasonal and pandemic influenza viruses, potentially compromising the usefulness of the restricted number of anti-influenza agents available.

H2: Most NAI-resistant variants are compromised in terms of viral fitness; however, certain mutants can retain virulence, replicative capacity and transmissibility due to compensatory/permissive mechanisms.

H3: The outcome of severe influenza infections can be improved whereas the likelihood of emergence of resistance can be reduced by combining antivirals with differential interactions with the viral target protein.

2.2 Objectives

2.2.1 General objective

The different studies presented in this thesis converge on the general objective of this doctoral project, which is to better describe the mechanisms underlying the development of resistance to NAIs in influenza viruses.

2.2.2 Specific objectives

i) To generate recombinant A(H1N1)pdm09 viruses with specific NA mutations associated with resistance to NAIs in other influenza subtypes and determine their impact on the antiviral resistance phenotype, NA enzymatic activity, viral fitness, virulence and transmissibility.

ii) To determine the causes of the differential effect on NAI resistance and the impact on viral fitness of NA substitutions at residue 223 in A(H1N1)pdm09 viruses.

iii) To predict the potential emergence and dissemination of A(H1N1)pdm09 viruses with reduced sensitivity to zanamivir.

iv) To study the influence of suboptimal-dose treatments with oseltamivir on the selection of oseltamivir-resistant viruses in mice.

v) To confirm the role of permissive NA mutations in both seasonal A(H1N1) and A(H1N1)pdm09 viruses.

vi) To evaluate the efficacy of combining the two approved NAIs (oseltamivir and zanamivir) in the treatment of experimental infections with NAI-sensitive and NAI-resistant A(H1N1)pdm09 as well as A(H3N2) viruses.

Chapter III: "Generation and characterization of recombinant pandemic influenza A(H1N1) viruses resistant to neuraminidase inhibitors"

3.1 Foreword

This chapter contains the text of the article **"Generation and characterization of recombinant pandemic influenza A(H1N1) viruses resistant to neuraminidase inhibitors"**, written by Andrés Pizzorno, Xavier Bouhy, Yacine Abed and Guy Boivin. In this study, carried out in the early stages of the 2009 influenza pandemic, we investigated the effect of several NA mutations reported to confer resistance to NAIs in different influenza subtypes on the susceptibility phenotype, enzymatic properties and replicative capacity of A(H1N1)pdm09 viruses, with the aim to anticipate their potential emergence and impact. AP, YA and GB conceived and designed the experiments; AP performed the experiments; AP and XB optimized the enzyme kinetic experiments, AP, YA and GB analyzed the data; AP, YA and GB wrote the article and responded to the reviewer's comments. The article is presented in its final version accepted for publication in **The Journal of Infectious Diseases** in 2011 (Jan 1;203(1):25-31).

3.2 Article

Generation and characterization of recombinant pandemic influenza A/H1N1 viruses resistant to neuraminidase inhibitors

Running title: Drug-resistant recombinant pH1N1 viruses

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ABSTRACT

Background. Neuraminidase inhibitors (NAIs) play a key role in the management of influenza epidemics and pandemics. Given the novel pandemic A/H1N1 (pH1N1) virus and the restricted number of approved anti-influenza drugs, evaluation of potential drug-resistant variants is of high priority.

Methods. Recombinant pH1N1 viruses were generated by reverse-genetics, expressing either the wild-type or nine mutant NA proteins (N2 numbering: E119G/V, D198G, I222V, H274Y, N294S, S334N, I222V-H274Y and H274Y-S334N). These recombinant viruses were evaluated for their resistance phenotype to four NAIs (oseltamivir, zanamivir, peramivir and A-315675), NA enzymatic activity and replicative capacity.

Results. The E119G/V mutations conferred a multi-drug resistance phenotype to many NAIs, but severely compromised viral fitness. The oseltamivir- and peramivir-resistance phenotype was confirmed for the H274Y and N294S mutants, while both viruses remained susceptible to zanamivir. Remarkably, the I222V mutation had a synergistic effect on the oseltamivir- and peramivir-resistance phenotype of H274Y and also compensated its reduced viral fitness, raising concern about the potential emergence and dissemination of this double mutant virus.

Conclusions. This study highlights the importance of a continuous monitoring of antiviral drug resistance in clinical samples, as well as the need to develop new agents and combination strategies.

Keywords. Influenza, pandemic, H1N1, resistance, oseltamivir, zanamivir, peramivir, recombinant viruses.

INTRODUCTION

Antiviral therapy plays an important role in the management of influenza outbreaks and pandemics, with both prophylactic and therapeutic indications. In that regard, two classes of drugs have been approved for clinical use: the adamantanes and the neuraminidase inhibitors (NAIs). The global circulation of adamantane-resistant virus variants has led to the use of NAIs as the anti-influenza agents of choice [1, 2]. Apart from the two commercially-available compounds oseltamivir and zanamivir, some experimental NAIs such as the cyclopentane analogue peramivir and the pyrrolidine-based agent A-315675 have been tested with promising results, with the former being approved in Japan [3-5]. NAIs target the active site of influenza A and B neuraminidase (NA) enzyme, preventing cleavage of terminal sialic acid residues on the membrane of the infected cell, and thus hampering viral propagation. Several subtype-specific mutations in framework or catalytic residues of the NA that confer resistance to these drugs have been described *in vitro* and *in vivo* [6-11]. In influenza A/H3N2 viruses, oseltamivir-resistant variants with the R292K substitution have been reported in the clinic [6, 12]. Furthermore, mutation E119V has also been detected in drug-resistant strains from patients treated with oseltamivir [6, 12, 13]. Another substitution at position 119 (E119G) has been described in viruses of the N2 subtype after serial passages in the presence of zanamivir [14]. On the other hand, the predominant mutation conferring resistance to oseltamivir in the A/H1N1 subtype is H274Y (N2 numbering), reported after *in vitro* passages as well as in clinical isolates [7, 8, 15]. During the 2008-2009 influenza season, more than 99% of the influenza A/Brisbane/59/2007-like (H1N1) strains isolated in North America and Europe carried the H274Y mutation [16]. In the A/H5N1 subtype, the H274Y substitution has also been reported in treated patients [17, 18], while *in vitro* assays have documented the NAI-resistant E119G and D198G mutants [18, 19]. More recently, the oseltamivir-resistant N294S mutation has been identified in viruses of the A/H3N2, A/H1N1 and A/H5N1 subtypes [3, 12, 18, 20]. Another naturally-occurring substitution (Q136K) has been associated with reduced sensitivity to zanamivir in A/H3N2 and A/H1N1 viruses [21, 22]. A study, performed by our group with recombinant NA proteins of the N1 subtype, has shown that the E119V mutation may confer cross-resistance to the four tested NAIs (oseltamivir, zanamivir, peramivir and A-315675) [23].

In April 2009, a novel triple reassortant swine-origin influenza A/H1N1 virus emerged to cause the first influenza pandemic of this century. By May 2010, more than 214 countries have

reported laboratory-confirmed cases of pandemic influenza A/H1N1 (pH1N1), with over 18138 deaths [24]. Although the pH1N1 virus is naturally susceptible to NAIs, about 300 cases of oseltamivir-resistant pH1N1 have been reported worldwide (1 to 1.5% of tested cases), all of them containing at least the H274Y mutation [25].

Given the fact that pH1N1 continues to be the predominant influenza virus circulating worldwide and considering the restricted number of anti-influenza drugs available, evaluation of the potential drug-resistant variants is of high priority. In the present study, we generated recombinant pH1N1 viruses using a recently-described reverse-genetics system in order to assess the impact of several NA mutations, previously reported in NAI-resistant influenza viruses, on the resistance phenotype, NA activities and *in vitro* replicative capacities.

METHODS

Generation of recombinant pH1N1 viruses. All eight genes of the first pH1N1 virus isolated in Quebec City (Canada) on May 3rd, 2009 (A/Québec/144147/09, GenBank accession numbers FN434457-FN434464) were amplified by reverse transcription-PCR and each segment was cloned into either pLLBA or pLLBG bidirectional expression/translation vectors as described by Liu et al. [26]. Seven single mutations (N2 numbering: E119G, E119V, D198G, I222V, H274Y, H294Y and S334N) were incorporated into the pLLBA plasmid containing the NA segment using appropriate primers and the QuikChangeTM Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The plasmid containing the H274Y-NA gene was used to introduce a second mutation (I222V or S334N) for the rescue of double mutants. All plasmids were sequenced to ensure the absence of undesired mutations. The eight plasmids were cotransfected into 293T human embryonic kidney cells using the LipofectamineTM 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Seventy-two h post-transfection, supernatants were collected and used to inoculate ST6GalI Madin-Darby canine kidney cells overexpressing the α2,6 sialic acid receptors (MDCK α2,6 cells kindly provided by Dr. Y. Kawaoka, Department of Pathological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison) to amplify the rescued viruses. The recombinant wild-type (WT) virus as well as each of the nine mutants were subsequently sequenced and titrated by standard plaque assays in MDCK α2,6 cells [27].

Drug-susceptibility assays. The drug resistance phenotype was determined by NA inhibition assays as described [28], with minor modifications. Briefly, recombinant viruses were standardized to a NA activity tenfold higher than that of the background and then incubated with serial three-fold dilutions of the drugs, including oseltamivir carboxylate (Hoffmann-La Roche, Basel, Switzerland), zanamivir (GlaxoSmithKline, Stevenage, UK), peramivir (BioCryst, Birmingham, AL) and A-315675 (Abbott Laboratories, North Chicago, IL). The final concentrations of the drugs ranged from 0 to 1800 nM. Methylumbelliferyl-N-acetylneuraminic acid (MUNANA, Sigma, St-Louis, MO, USA) was used as the fluorogenic substrate and the 50% inhibitory concentration (IC_{50}) was determined from the dose-response curve [29]. Mutant viruses were considered to have reduced susceptibility to NAIs if they showed a five- to ten-fold increase in their IC_{50} value when compared with WT. Viruses were considered resistant to a drug if their IC_{50} value was ten-fold greater than that of the WT [30].

***In vitro* replication assays.** Replicative capacities of the recombinant viruses were evaluated by infecting MDCK α2,6 cells with a multiplicity of infection (MOI) of 0.001 PFU/cell. Supernatants were collected at 12, 24, 36, 48, 60 and 72 h post infection and titrated by plaque assays. Sixty h post infection, the mean viral plaque area of each virus was determined from a minimum of 20 plaques with the ImageJ software (version 1.41), developed by Wayne Rasband of the National Institues of Health.

Enzymatic assays. To measure the NA enzymatic activity, fluorometric assays were conducted using MUNANA as substrate. Briefly, all recombinant viruses were standardized to an equivalent dose of $10^{5.5}$ plaque forming-units (PFU)/ml and incubated at 37°C in 50-µl reactions with different concentrations of MUNANA [18]. The final concentration of the substrate ranged from 0 to 3000 µM. Fluorescence was monitored every 90 s for 53 min (35 measures). The Michaelis constant (Km) and the maximum velocity (Vmax) were calculated with the Prism software (GraphPad, version 5), by fitting the data to the Michaelis-Menten equation using nonlinear regression.

Statistical analyses. In the replicative capacity assays, viral titers and plaque sizes of each mutant were compared with those of the WT by the use of t tests. A probability value lower than 0.05 was chosen to reject the null hypothesis.

RESULTS

The recombinant A/Québec/144147/09 pH1N1 WT virus was successfully generated by reverse genetics. Of note, phylogenetic analysis of the NA and HA genes showed that the A/Québec/144147/09 strain described in this study is closely related to the prototype A/California/07/09 pandemic strain (nucleotide identity of 99.7% and 99.6%, respectively). Using this genetic background, nine different NA mutant variants (E119G/V, D198G, I222V, H274Y, N294S, S334N, I222V-H274Y and H274Y-S334N) were also generated (Table 1). NA inhibition assays showed that the E119G mutation conferred important levels of resistance to zanamivir, peramivir and A-315675, with 832-, 51- and 123-fold increases in IC_{50} values compared with the WT virus. A multi-drug resistance phenotype to oseltamivir, zanamivir, peramivir and A-315675 (60-, 571-, 25- and 69-fold increases in IC_{50}) was observed for the E119V mutant. The D198G mutation was associated with a low level of resistance to oseltamivir as well as with a small reduction in zanamivir susceptibility (17- and 6-fold increases in IC_{50}). The oseltamivir-resistance phenotype of H274Y and N294S was confirmed with 982- and 208-fold increases in IC_{50}, while both mutants remained sensitive to zanamivir and A-315675. Resistance to peramivir (263-fold and 12-fold increases in IC_{50}) was also observed for H274Y and N294S, respectively. When compared to the single H274Y mutant, the double I222V-H274Y mutant had increased IC_{50} values for oseltamivir and peramivir (1.8- and 5-fold, respectively), whereas the H274Y-S334N mutant had decreased IC_{50} values (1.5-fold for both). The single I222V mutation was associated with a minor increase in oseltamivir IC_{50} value (6-fold increase compared with WT), whereas the single S334N mutation did not seem to contribute significantly to a resistant phenotype (Table 1).

The effect of NA mutations on viral growth was assessed *in vitro* for all recombinant viruses (Figure 1). Major differences in replication kinetics were observed among the WT and E119G/V mutant viruses. Mutant E119V had the greatest replication impairment, with viral titers that were 1.5 to 3.5 log_{10} lower than those of the WT virus at all time points. The replication of E119G was also significantly reduced compared to that of the WT by approximately 1 log_{10} during the first 48 h. While the WT virus reached its peak at 48 h post infection, the E119G mutant reached it 12 h later. However, both maximum viral titers were quite comparable, with 2.4 x 10^7 PFU/ml for the WT and 1.9 x 10^7 PFU/ml for E119G. The H274Y substitution had a reduction in viral replication of approximately 0.5 log_{10} at the 36-, 60- and 72-h time points. On the other hand, the I222V mutant showed a

significant increase in viral titers during the first 60 h. For the rest of the mutant viruses, the growth curves were not significantly different from the WT although some differences could be observed at specific time points (24 h for N294S and I222V-H274Y; 72 h for D198G and S334N). The mean plaque areas of the recombinant viruses were consistent with the viral titers observed in the yield assays (Table 2). The E119V (0.18 ± 0.10 mm^2) and E119G (0.32 ± 0.14 mm^2) mutants exhibited plaques significantly smaller than those of the WT (0.47 ± 0.25 mm^2), with no significant differences for the remaining viruses.

Kinetic analyses were performed to evaluate the impact of NA mutations on sialidase activities. Km and Vmax were the two parameters determined for each recombinant virus, the former reflecting the affinity for the substrate and the latter the activity of the enzyme (Table 2). The Vmax ratios for each mutant over the WT serve as an indicator of the relative NA activities. Except for the single S334N and double H274Y-S334N mutants, all other NA substitutions decreased the affinity of the enzyme, with higher Km values compared with the WT. On the other hand, except for E119G/V and I222V, all other mutations considerably decreased enzymatic activity, supported by lower Vmax values. A particular combination of low affinity and high NA activity was observed for the E119G and E119V viruses, with 9- and 7.1-fold increases in Km, as well as 100 and 181% Vmax ratios, respectively. Kinetic parameters were quite similar for D198G (5.1-fold increase in Km and 44% Vmax ratio) and N294S (5.2-fold increase in Km and 45% Vmax ratio). The H274Y mutant had the most affected enzymatic activity, with a 25-fold increase in Km and a Vmax ratio of only 18%. Substitution I222V resulted in a minor reduction of affinity (1.8-fold increase in Km) with an important increase in enzymatic activity (181% Vmax ratio). The double I222V-H274Y virus showed an intermediate phenotype between the two single mutants (2.4-fold increase in Km and 47% Vmax ratio). Even if a minimal effect on enzyme affinity can be attributed to the single S334N and double H274Y-S334N substitutions (0.2-fold decrease and 1.3-fold increase in Km, respectively), NA activity was significantly reduced for both mutants (61 and 23% Vmax ratios, respectively).

DISCUSSION

Although many subtype-specific NA mutations conferring resistance to NAIs in influenza A/H1N1, A/H3N2 and A/H5N1 viruses have been previously described [10, 12, 18, 19, 23, 31],

differences in viral genetic backgrounds can account for a differential effect of these mutations on antiviral resistance and/or viral fitness. Hence, it is important to assess the impact of NA substitutions using recombinant pH1N1 viruses to better evaluate the relevance of these potentially emergent drug-resistant variants. So far, only the emergence of oseltamivir- and peramivir-resistant viruses has been reported in pH1N1 influenza. In all cases, the H274Y NA substitution was detected and found responsible for the resistant phenotype, while these viruses remained susceptible to zanamivir [25, 32-36].

Here, we optimized a reverse-genetics system to produce recombinant pH1N1 viruses, expressing either the WT or nine different mutant NA proteins. The effect of five framework NA mutations (E119G/V, D198G, H274Y and N294S), previously associated with NAI resistance in different viral backgrounds, was evaluated *in vitro* with regard to drug resistance, enzymatic activity and viral replicative capacity. Two double mutants (I222V-H274Y and H274Y-S334N) described in patients with oseltamivir-resistant pH1N1 viruses [32] (and unpublished data) were also generated. The single mutants I222V and S334N, even though not yet widely reported, were included in this study to assess their contribution to resistance and/or viral fitness.

Our results confirm the oseltamivir- and peramivir-resistance phenotype conferred by the N1 subtype-specific H274Y mutation in the pH1N1 background. This is in accordance with our previous report on oseltamivir resistance in a patient infected with pH1N1 [34], as well as with the study of Memoli et al. [35]. Our results also demonstrate that, in addition to the H274Y mutation, other NA mutations are replication competent in the pH1N1 background and some can confer a specific or multi-drug resistance phenotype. However, some mutants like E119G/V replicate poorly in cell culture and are thus probably unlikely to become clinically relevant, which is in line with the fact that these mutations have not yet been detected in seasonal A/H1N1 or pH1N1 isolates. On the other hand, the I222V-H274Y mutant has the most important potential for dissemination owing to its improved replication and enzymatic kinetics.

The H274Y mutant conferred the highest level of resistance to oseltamivir and peramivir among the recombinant pH1N1 viruses with single mutation. This mutant was also the most affected in terms of NA kinetics, owing to its 25-fold reduction in affinity for the substrate and its 5-fold reduction in sialidase activity. However, such impaired enzymatic activity caused a minor impact on viral fitness, consistent with the numerous reports of this pH1N1 mutant in both immunocompromised

and immunocompetent individuals [25] as well as in limited transmission events [37]. The addition of the I222V mutation significantly increased the levels of resistance to oseltamivir and peramivir in the I222V-H274Y mutant (>1000-fold increases in IC_{50} for both NAIs compared to the WT), even though the single I222V mutant had a marginal effect on oseltamivir resistance (6-fold increase over the WT). Furthermore, the I222V substitution increased the NA activity and replicative capacity of the double mutant, restoring the fitness and partially compensating the loss of NA activity due to H274Y. This synergic effect of the I222V mutation on resistance and NA activity has been previously described in A/H3N2 [38] as well as in A/H1N1 and A/H5N1 viruses [19], and this should be a matter of careful monitoring. Interestingly, in a small outbreak where transmission of drug-resistant viruses may have occurred, the pH1N1 strains also contained both the H274Y and I222V mutations [32]. On the other hand, oseltamivir-resistant pH1N1 isolates from Canada harbouring both the H274Y and S334N NA mutations have also been identified (GenBank accession number CY060552.1). The role of S334N as a potential compensatory mutation is not as clear as for I222V. The single S334N mutant remained susceptible to the four NAIs tested in this study, while the double H274Y-S334N mutant had the same oseltamivir- and peramivir-resistance phenotype than that of the single H274Y virus, but with lower IC_{50} values. Apart from a reduction in NA activity, no effect on either the affinity of the enzyme or the replicative capacity was observed as a result of this mutation.

The oseltamivir-resistance phenotype of the N294S mutant, previously reported in A/H3N2, A/H5N1 and A/H1N1 viruses [12, 18, 20], was confirmed in the pH1N1 background. Despite the fact that this mutation also conferred resistance to peramivir in pH1N1, IC_{50} values are not high enough to disregard in advance the use of this NAI as a therapeutic alternative to oseltamivir in that context. Also, the susceptibility of the N294S mutant to zanamivir and A-315675 was not altered. Mutations in this conserved amino acid reduced the NA activity and affinity for the substrate, but not sufficiently to affect viral fitness. Yen et al. [18] described a similar effect of this mutation in A/H5N1 and A/H1N1 viruses, although Collins et al. [39] reported a higher NA activity for the N294S mutant, compared to the WT. Mutation D198G has been described only in A/H5N1 viruses *in vitro*, and was associated with low levels of resistance to oseltamivir and zanamivir, as well as a reduction in NA activity and viral fitness [19]. The even lower resistance levels to these two NAIs found in our recombinant pH1N1 virus, along with its unaffected replicative capacity, suggest that this amino acid may not play such an important role in pH1N1. Of note, the D198G mutant described by Hurt et al. [19] was obtained after many passages under zanamivir pressure, possibly leading to other mutations in the viral genome.

One of the most frequent NA mutations associated with resistance to oseltamivir in A/H3N2 viruses is the E119V, retaining susceptibility to zanamivir, peramivir and A-315675 [12, 13, 30, 40]. In line with our previous findings using recombinant proteins of the N1 subtype [3, 23], we found that this substitution also conferred resistance to all NAIs in the H1N1 background; however, this mutant had significant fitness impairment. The E119G mutant, responsible for zanamivir-resistance in A/H3N2 and A/H5N1 viruses [19, 30], also showed a peramivir- and A-315675-resistance phenotype in the pH1N1 background but remained susceptible to oseltamivir. Not surprisingly, this mutation also affected the replicative capacity of the recombinant virus [18, 19] but to a lesser extent than the E119V, eventually reaching maximum viral titers comparable to those of the WT. Interestingly, our enzymatic studies reveal a particular NA activity profile for the E119G/V mutants, not observed in the other recombinant viruses. While both substitutions caused an important reduction in the affinity for the substrate, there was no loss of enzymatic activity, which was in fact increased in the case of the E119V mutant. In an attempt to explain these observations, we should consider that the total NA enzymatic activity of the virus can be affected not only by the inherent effect of the specific amino acid substitutions, but also by differences in the levels of expression of NA proteins on the surface of the viral particles [41]. We suggest that even if a higher NA activity has been usually associated with an improvement in viral fitness, a balance between the sialidase activity (Vmax) and the affinity for the sialic acid moieties on host cell receptors (Km) may be necessary for productive virus-cell interactions. As a result, the altered balance of these two parameters observed in the E119G/V mutants may play a negative role on viral fitness, with E119V being the most seriously affected. However, further studies are needed to confirm this hypothesis.

The fact that we did not study clinically-adapted strains that may contain additional compensatory mutations may represent a limitation to this study. On the other hand, the uniform background provided by recombinant viruses makes them differ solely in the introduced NA substitutions. This strategy minimizes any possible bias in the interpretation of results as a consequence of additional mutations in the genome, enabling the description of the most comprehensive *in vitro* resistance study to NAIs in pH1N1 reported so far. Of note, some other relevant NA mutations (i.e., the zanamivir-resistant Q136K mutation [22]) have not been tested and should be considered for future analyses. Finally, animal studies should be conducted to assess the virulence and transmissibility of drug-resistant mutant strains. Such studies would be also invaluable

to address the clinical relevance of drug-resistant viruses, since increased IC_{50} values may be overcome by higher drug levels, as we recently showed for intravenous peramivir [4].

In conclusion, some NA mutations such as E119G/V can confer a multi-drug resistance phenotype in pH1N1 viruses and may represent a potential problem for immunocompromised patients. However, their potential for transmission is unlikely as suggested by poor replication kinetics. The I222V mutation increases the oseltamivir- and peramivir-resistance phenotype and also compensates for the minor reduction in viral fitness of H274Y, raising concern about the potential emergence and sustained community transmission of this double mutant virus. The results obtained in this cell culture model underscore the need for animal studies, in order to further investigate the effect of these drug-resistant mutants on virulence and especially transmission. Overall, our results highlight the importance of a continuous monitoring of antiviral resistance in clinical samples, as well as the need to develop new drugs and combination strategies.

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Table 1. Susceptibility profiles of recombinant A/Québec/144147/09 pH1N1 viruses to neuraminidase inhibitors.

NA mutants[a]	Oseltamivir IC_{50} nM ± sd (ratio)[b]	Zanamivir IC_{50} nM ± sd (ratio)[b]	Peramivir IC_{50} nM ± sd (ratio)[b]	A-315675 IC_{50} nM ± sd (ratio)[b]
WT	0.46 ± 0.01 (1)	0.15 ± 0.01 (1)	0.09 ± 0.01 (1)	0.20 ± 0.03 (1)
E119G	1.34 ± 0.12 (2.9)	124.9 ± 7.6 (832)	4.61 ± 0.97 (51.2)	24.5 ± 1.1 (123)
E119V	27.7 ± 1.4 (60.2)	85.7 ± 10.6 (571)	2.28 ± 0.08 (25.3)	13.8 ± 1.0 (69)
D198G	7.70 ± 0.72 (16.8)	0.90 ± 0.06 (6)	0.22 ± 0.01 (2.4)	0.83 ± 0.03 (4.2)
I222V	2.63 ± 0.01 (5.7)	0.35 ± 0.02 (2.3)	0.17 ± 0.00 (1.9)	0.20 ± 0.00 (1)
H274Y	451.9 ± 26.0 (982)	0.14 ± 0.01 (0.9)	26.6 ± 5.5 (263)	0.28 ± 0.01 (1.4)
N294S	95.8 ± 5.4 (208)	0.49 ± 0.02 (3.3)	1.04 ± 0.03 (12)	0.21 ± 0.01 (1.1)
S334N	0.43 ± 0.03 (0.9)	0.18 ± 0.03 (1.2)	0.08 ± 0.00 (0.9)	0.18 ± 0.01 (0.9)
I222V-H274Y	797.4 ± 51.0 (1733)	0.32 ± 0.01 (2.1)	119.8 ± 13.8 (1331)	0.39 ± 0.03 (2)
H274Y-S334N	302.8 ± 43.1 (658)	0.11 ± 0.02 (0.7)	15.99 ± 2.3 (178)	0.27 ± 0.02 (1.4)

The mean IC_{50} values of three experiments ± standard deviations (sd) are indicated.

[a]All mutations are in N2 numbering.

[b]Compared with the wild-type virus.

Table 2. Viral plaque area and neuraminidase enzymatic parameters of recombinant A/Québec/144147/09 pH1N1 viruses.

NA mutants[a]	Mean plaque area mm^2 ± sd	K_m µM ± sd (ratio)[b]	V_{max} U/sec ± sd (ratio)[b]	Vmax ratio (% of WT)
WT	0.47 ± 0.25	22.5 ± 3.1 (1)	17.5 ± 2.4 (1)	100
E119G	0.32 ± 0.14 *	202.7 ± 7.0 (9)	17.5 ± 1.9 (1)	100
E119V	0.18 ± 0.10 **	159.2 ± 11.9 (7.1)	31.6 ± 3.0 (1.8)	181
D198G	0.38 ± 0.13	115.1 ± 7.9 (5.1)	7.7 ± 0.7 (0.4)	44
I222V	0.48 ± 0.28	41.0 ± 3.2 (1.8)	31.6 ± 3.4 (1.8)	181
H274Y	0.40 ± 0.21	563.5 ± 44.2 (25)	3.1 ± 0.3 (0.2)	18
N294S	0.49 ± 0.18	117.8 ± 9.2 (5.2)	7.9 ± 0.9 (0.5)	45
S334N	0.45 ± 0.13	18.6 ± 1.5 (0.8)	10.6 ± 1.1 (0.6)	61
I222V-H274Y	0.48 ± 0.18	54.5 ± 4.3 (2.4)	8.2 ± 0.9 (0.5)	47
H274Y-S334N	0.44 ± 0.17	28.9 ± 2.3 (1.3)	4.0 ± 0.4 (0.2)	23

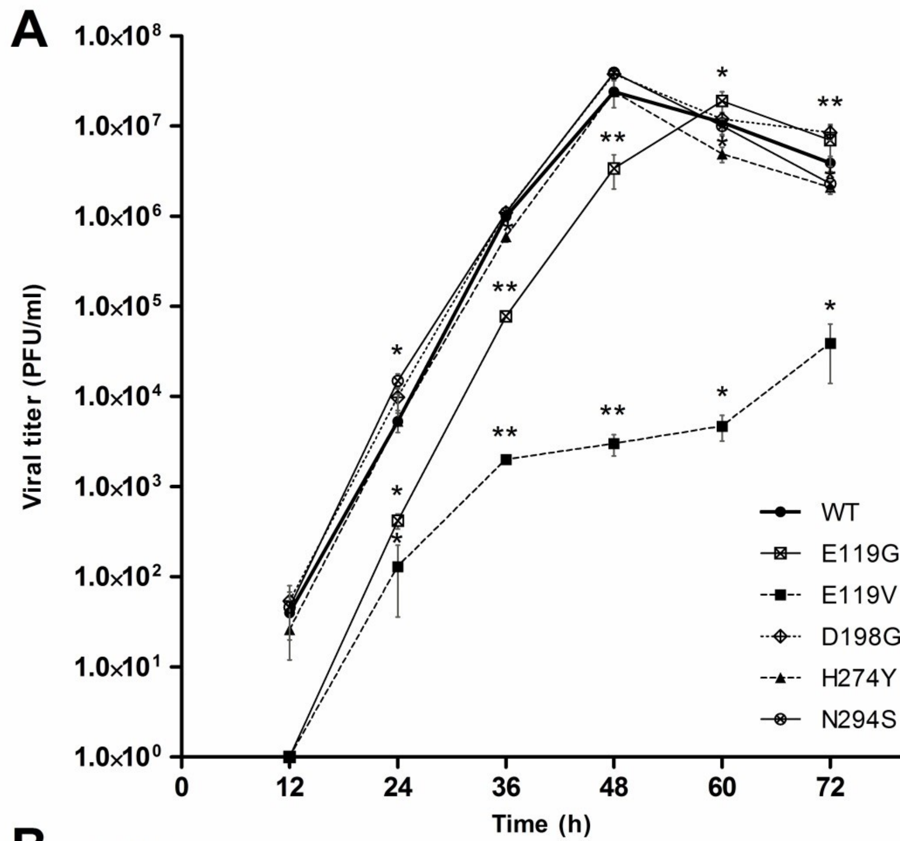
For viral plaque area, the mean values of twenty measures ± standard deviations (sd) are indicated. **P*<0.05 and ***P*<0.01 for differences in plaque area when compared with the wild-type virus by the use of the *t*-test.

For NA enzymatic parameters, the mean values of three experiments ± standard deviations (sd) are indicated.

[a]All mutations are in N2 numbering.

[b]Compared with the wild-type virus.

Figure 1. Replication kinetics of recombinant A/Québec/144147/09 pH1N1 viruses *in vitro*. Confluent MDCK α2,6 cells were infected with a MOI of 0.001 PFU/cell. Supernatants were harvested at the indicated time points and titrated by standard plaque assays. **A)** E119G, E119V, D198G, H274Y and N294S mutants. **B)** I222V, S334N, I222V-H274Y and H274Y-S334N mutants. The mean values for three experiments with standard deviations are presented. *$P<0.05$ and **$P<0.01$ for differences in viral titers when compared with the wild-type virus by the use of the *t*-test. The time points with significant differences relative to the wild-type virus are as follows: E119G (24, 36, 48, 60h), E119V (24, 36, 48, 60, 72h), D198G (72h), I222V (24, 48, 60h), H274Y (36, 60, 72h), N294S (24h), S334N (72h) and I222V-H274Y (24h).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Viral titer (PFU/ml)
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1.0×10^7
1.0×10^6
1.0×10^5
1.0×10^4
1.0×10^3
1.0×10^2
1.0×10^1
1.0×10^0
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12
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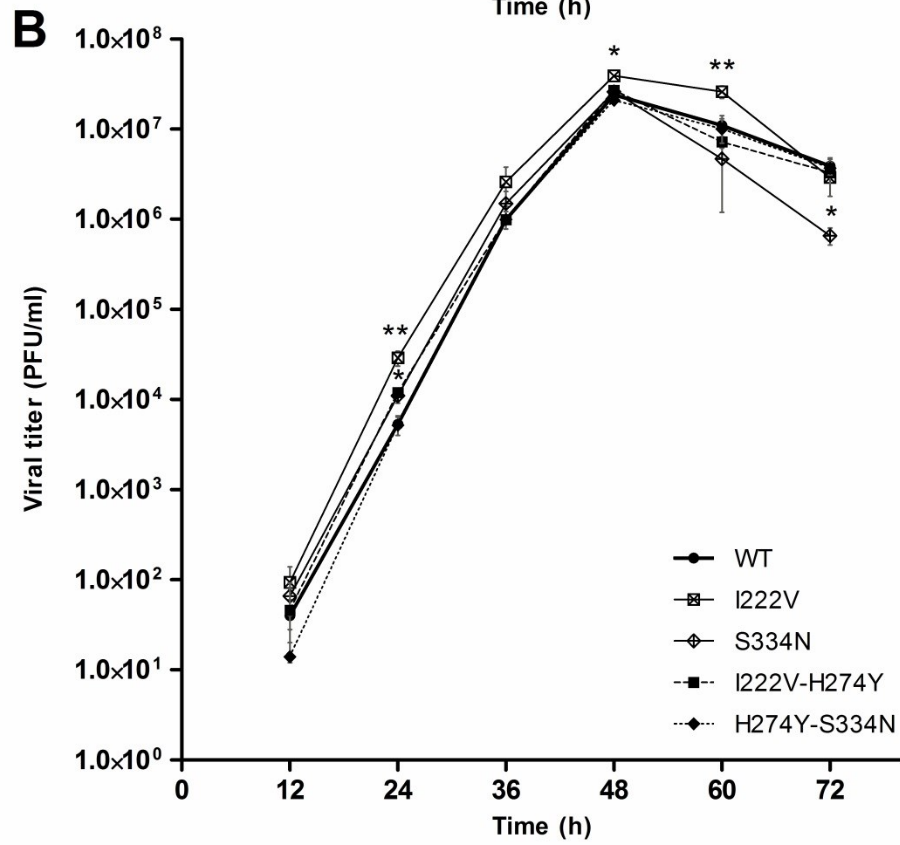
Time (h)
WT
E119G
E119V
D198G
H274Y
N294S


B
Viral titer (PFU/ml)
1.0×10^8
1.0×10^7
1.0×10^6
1.0×10^5
1.0×10^4
1.0×10^3
1.0×10^2
1.0×10^1
1.0×10^0
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12
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60
72
Time (h)
WT
I222V
S334N
I222V-H274Y
H274Y-S334N

Chapter IV: **"Impact of mutations at residue I223 of the neuraminidase protein on the resistance profile, replication level, and virulence of the 2009 pandemic influenza virus"**

4.1 Foreword

This chapter contains the text of the article **"Impact of mutations at residue I223 of the neuraminidase protein on the resistance profile, replication level, and virulence of the 2009 pandemic influenza virus"**, written by Andrés Pizzorno, Yacine Abed, Xavier Bouhy, Édith Beaulieu, Corey Mallett, Rupert Russell and Guy Boivin. This study was the first to thoroughly characterize the differential and combinatory effect of a new set of mutations of resistance at residue I222 (here referred as I223) of the NA, reported in many clinical isolates from patients with or without previous exposure to NAIs. AP, YA and GB conceived and designed the experiments; AP and XB performed the experiments; EB and CM managed the ferret experiments; RR performed the structural analysis and 3D modeling; AP, YA and GB analyzed the data; AP, YA and GB wrote the article and responded to the reviewer's comments. The article is presented in its final version accepted for publication in **Antimicrobial Agents and Chemotherapy** in 2012 (Mar;56(3):1208-14).

4.2 Article

Impact of mutations at residue I223 of the neuraminidase protein on the resistance profile, replication level and virulence of the 2009 pandemic influenza virus

Running title: I223V/R NA mutations and resistance to NA inhibitors

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ABSTRACT

Amino acid substitutions at residue I223 of the neuraminidase (NA) protein have been identified in 2009 pandemic influenza (pH1N1) variants with altered susceptibilities to NA inhibitors (NAIs). We used reverse genetics and site-directed mutagenesis to generate the recombinant A/Québec/144147/09 pH1N1 wild-type virus (WT) and five (I223R/V, H275Y, I223V- H275Y and I223R-H275Y) NA mutants. A fluorometric-based assay was used to determine IC_{50} values against oseltamivir, zanamivir and peramivir. Replicative capacity was analyzed by viral yield assays in ST6GalI-MDCK cells. Infectivity and transmission of the WT, H275Y and I223V-H275Y recombinant viruses were evaluated in ferrets. As expected, the H275Y mutation conferred resistance to oseltamivir (982-fold) and peramivir (661-fold) compared to the drug-susceptible recombinant WT. The single I223R mutant was associated with reduced susceptibility to oseltamivir (53-fold), zanamivir (7-fold) and peramivir (10-fold) whereas the I223V virus had reduced susceptibility to oseltamivir (6-fold) only. Interestingly, enhanced levels of resistance to oseltamivir and peramivir and reduced susceptibility to zanamivir (1647-, 17347- and 16-fold increases in IC_{50} values, respectively) were observed for the I223R-H275Y recombinant while the I223V-H275Y mutant exhibited 1733- 2707- and 2-fold increases in respective IC_{50} values. The I223R/V changes were associated with equivalent or higher viral titers *in vitro* as compared to the recombinant WT. Infectivity and transmissibility in ferrets were comparable between the recombinant WT and the H275Y or I223V-H275Y recombinants. In conclusion, amino acid changes at residue I223 may alter the NAI susceptibilities of pH1N1 variants without compromising fitness. Consequently, I223R/V mutations, alone or with H275Y, need to be thoroughly monitored.

Keywords. Influenza, pandemic, H1N1, fitness, resistance, NA inhibitors, recombinant viruses.

INTRODUCTION

In the last two decades, neuraminidase (NA) inhibitors (NAIs) have proved to be a valuable approach for the prevention and treatment of epidemic and pandemic influenza infections. Although many investigational compounds are currently under development, oral oseltamivir, inhaled zanamivir and, in some countries, intravenous peramivir and inhaled laninamivir are the only NAIs licensed for clinical use against influenza viruses including the 2009 pandemic (pH1N1) influenza A strain (5, 6, 9, 24). However, as for other antivirals, extensive use of NAIs may lead to the emergence and dissemination of drug-resistant variants that may compromise their clinical usefulness. Moreover, as the different NAIs target the active site of the NA enzyme with similar mechanisms of action, NA mutations conferring cross-resistance may also occur, which constitutes a serious clinical problem especially in immunocompromised individuals.

Almost 600 cases of oseltamivir-resistant pH1N1 viruses have been reported worldwide in a period of two years after the onset of the pandemic in April 2009 (37). These viruses harbor a histidine-to-tyrosine mutation at position 275 (H275Y in N1 numbering) of the NA protein, which has also been shown to confer a lesser but still moderate degree of resistance to peramivir yet remaining susceptible to zanamivir (22, 28). In addition, pH1N1 clinical isolates harbouring different amino acid substitutions at residue I223 of the NA protein have been described in patients with or without previous exposure to NAIs. In that regard, a isoleucine-to-valine (I223V) mutation was detected along with H275Y in respiratory samples of two U.S. campers receiving prophylaxis with oseltamivir (7). These oseltamivir-resistant double mutants retained susceptibility to zanamivir. In other reports, the isoleucine-to-arginine (I223R) mutation has been shown to cause a moderate degree of resistance to oseltamivir and a low level of resistance to zanamivir (11, 19, 34). Furthermore, Nguyen and colleagues reported the presence of a highly oseltamivir- and peramivir-resistant I223R-H275Y double mutant virus isolated from an immunocompromised child (26). The double I223R-H275Y mutant had also reduced susceptibility to zanamivir. In the same study, the authors commented on a clinical isolate with the isoleucine-to-lysine (I223K) mutation, leading to a resistance phenotype comparable to that of the single I223R virus.

As a framework residue of the hydrophobic pocket of the active site of the influenza NA protein, I223 is largely conserved among influenza viruses (8). Despite increased clinical reports, the impact of mutations at residue I223 alone or in combination with H275Y on viral fitness of pH1N1 viruses has not yet been thoroughly determined. To address this issue, we generated recombinant pH1N1 viruses containing I223R/V NA mutations with or without H275Y changes and we compared their NAI-resistance phenotypes and *in vitro* replicative capacities. Furthermore, we assessed virulence and transmissibility of some selected mutants in a well-validated ferret model.

MATERIALS AND METHODS

Generation of recombinant viruses. A reverse genetics system using pLLBA and pLLBG plasmids (20) was recently developed for the influenza A/Québec/144147/09 pH1N1 virus (GenBank accession numbers: FN434457.1-FN434464.1) (28). The pLLBA plasmid containing the NA gene was used for the introduction of the I223R, I223V or H275Y single mutations using appropriate primers and the QuikChangeTM Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). Secondary I223R or I223V mutations were then incorporated in the pLLBA-NA275Y mutant plasmid as described above. All recombinant plasmids were sequenced to ensure the absence of undesired mutations. The eight bidirectional plasmids were cotransfected into 293T human embryonic kidney cells using the LipofectamineTM 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Supernatants were collected 72 h post transfection and used to inoculate ST6GalI Madin-Darby canine kidney cells overexpressing the α-2,6 sialic acid receptor (ST6GalI-MDCK cells kindly provided by Dr. Y. Kawaoka, University of Wisconsin, Madison). The recombinant wild-type (WT) virus as well as the three single (I223R/V, H275Y) and two double (I223R-H275Y, I223V-H275Y) mutants were subsequently sequenced and then titrated by standard plaque assays in ST6GalI-MDCK cells (14).

NA inhibition assays. The drug resistance phenotype was determined by NA inhibition assays using the 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA, Sigma, St-Louis, MO, USA) substrate (29), with minor modifications. Briefly, recombinant viruses were standardized to a NA activity level ten-fold higher than that of the background as measured by the production of a fluorescent product from the MUNANA substrate. The drug resistance phenotype was determined by

the extent of NA inhibition after incubation with serial three-fold dilutions of the drugs (final concentrations ranging from 0 to 1800 nM), including oseltamivir carboxylate (Hoffmann-La Roche, Basel, Switzerland), zanamivir (GlaxoSmithKline, Stevenage, UK) or peramivir (BioCryst, Birmingham, AL). The 50% inhibitory concentration (IC_{50}) for each drug was calculated from the dose-response curve (4). Mutant viruses were considered to have reduced susceptibility to NAIs if they showed a 5-10-fold increase in their IC_{50} values when compared with the recombinant WT. Viruses were considered resistant to a drug if their IC_{50} value was increased by >10-fold compared to the recombinant WT (23, 25). All linear and non-linear regression tests to determine IC_{50} values were performed using GraphPad Prism software, version 5.

***In vitro* replication kinetics.** ST6GalI-MDCK cells were infected with recombinant viruses at a multiplicity of infection (MOI) of 0.001 PFU/cell and then incubated at 37 °C for 1 h in a 5% CO2 incubator. The cells were subsequently washed with phosphate-buffered saline (PBS), overlaid with Modified Eagle Medium (MEM) (GIBCO; Invitrogen, Burlington, Ontario, Canada) containing 1 µg/ml TPCK-trypsin (Sigma, Oakville, Ontario, Canada) and incubated at 37 °C with 5% CO2. Supernatants were collected at 12, 24, 36, 48, 60 and 72 h post infection (PI) and titrated by plaque assays in ST6GalI-MDCK cells. The mean viral plaque area of each virus 60 h after infection was determined from ≥20 plaques with the ImageJ software, version 1.41 (Wayne Rasband, National Institutes of Health, Bethesda, MA). The presence of the desired mutations was confirmed by sequencing of the NA gene of viruses recovered at 72 h PI.

Ferret studies. Three groups of four seronegative (900-1500 g) male ferrets (Triple F Farms, Sayre, PA) housed in individual cages were lightly anesthetised by isoflurane and received, by intranasal instillation, 250 µl of PBS containing 4.5 log TCID50/ml of the recombinant pH1N1 WT, H275Y or I223V-H275Y viruses. To evaluate direct contact transmissibility, inoculated-contact animal pairs were established by placing a naïve ferret into each cage 24 h after inoculation of the index ferret (12). To evaluate airborne transmissibility, naïve ferrets were housed in a separate special cage with a perforated plexiglass wall to prevent direct contact between animals but to allow airflow from an index ferret to its adjacent naïve neighbour (13). All ferrets were weighed daily and monitored for clinical signs of sneezing, dyspnea and level of activity. Nasal wash samples were collected from animals on days 2, 4 and 6 PI, with 5 ml of PBS containing 2% bovine serum albumin and immediately stored at -80 °C. Virus titers in nasal wash samples were determined by plaque assays

in ST6GalI-MDCK cells. The presence of the desired mutations was confirmed by sequencing of the NA gene of viruses recovered from nasal wash samples on day 6 PI. Serum samples were collected from each ferret before intranasal infection (day 0) and on day 14 PI to evaluate specific antibody levels against the A/Québec/144147/09 pH1N1 strain using standard HAI assays. Animals were sacrificed on day 14 PI. All procedures were approved by the Institutional Animal Care Committee at the Armand Frappier Institute (Laval, QC, Canada) and Laval University according to the guidelines of the Canadian Council on Animal Care.

Molecular modeling. Molecular models of the mutants were generated using the program O (18) and subsequently analyzed with the PyMOL Molecular Graphics System (Delano Scientific, Palo Alto, CA).

Statistical analyses. Viral titers and plaque areas obtained from *in vitro* replicative capacity assays as well as nasal washes of ferrets were compared by one-way ANOVA analysis of variance, with the Tukey's multiple comparison posttest.

RESULTS

Susceptibility of recombinant viruses to NAIs. In this study, a recombinant WT pH1N1 virus and its 5 (I223R/V, H275Y, I223R-H275Y and I223V-H275Y) variants were generated. All six recombinants were produced with similar viral titers ($\geq 10^6$ PFU/ml) and comparison of nucleotide sequences confirmed the absence of unintended mutations in both the HA and NA genes. Susceptibility profiles of the recombinant viruses to oseltamivir, zanamivir and peramivir are summarized on Table 1. As expected, the recombinant WT virus was sensitive to the three NAIs tested, while the H275Y mutant was resistant to oseltamivir and peramivir (982- and 661- mean fold increases in IC_{50} values, respectively) but susceptible to zanamivir. Only oseltamivir susceptibility was affected by the I223V mutation, yet to a low level (6-fold increase in IC_{50} compared to the recombinant WT). However, the combination of this substitution with H275Y in the I223V-H275Y double mutant virus increased the oseltamivir- and peramivir-resistant phenotype observed in the single H275Y virus by 2- and 4-folds, respectively, for a total increase of 1733- and 2707-folds when compared with the recombinant WT. The I223R mutation conferred moderate resistance to

oseltamivir and reduced susceptibility to zanamivir and peramivir (53-, 7- and 10-fold increases in IC_{50}, respectively, vs the recombinant WT). These IC_{50} values were further increased in the I223R-H275Y double mutant, which showed 1647-, 16- and 17347-fold higher IC_{50} values compared with the recombinant WT.

***In vitro* replicative capacity of recombinant pH1N1 viruses.** In replicative capacity experiments, the peak of replication was reached at 48 h PI by all recombinant pH1N1 viruses with viral titers ranging from 1.89×10^7 to 7.53×10^7 PFU/ml (Fig. 1). The recombinant WT and the single H275Y mutant had comparable viral titers at 48 h PI whereas significant reductions were observed for the latter at 36, 60 and 72 h PI ($P<0.05$). Viral titers obtained with the recombinant I223V were significantly higher than those of the recombinant WT at 36 ($P<0.05$), 48 ($P<0.01$) and 60 h ($P<0.05$) PI (Fig. 1A). Similarly, the I223R mutation was associated with significantly higher titers compared to the recombinant WT at 48 ($P<0.01$), 60 ($P<0.01$) and 72 h ($P<0.05$) PI (Fig. 1B). The I223R/V substitutions seemed to improve viral growth of the H275Y mutant as viral titers of both I223R-H275Y and I223V-H275Y double mutants were comparable to those of the recombinant WT at 48, 60 and 72 h PI. No significant differences in mean plaque areas were observed among any of the recombinant viruses, with values ranging from 0.350 to 0.404 mm^2 at 60 h PI.

Characteristics of recombinant pH1N1 viruses in ferrets. Following intranasal inoculation of the recombinant WT, H275Y and I223V-H275Y viruses, there were no significant differences in body weight loss among the three groups of animals at any time points and only minimal clinical signs were observed (data not shown). All index and direct contact ferrets seroconverted for A/Québec/144147/09 pH1N1 when tested 14 days after infection/contact, with HAI reciprocal geometric mean titers >1280, compared to <10 on day 0 (Fig. 2). Accordingly, pH1N1 viruses could be recovered in nasal wash samples on days 2-6 PI from all index cases and direct contact ferrets. For index cases, mean viral titers in nasal wash samples of both H275Y and I223V-H275Y groups were comparable with those of the recombinant WT group (Fig. 3A), ranging from 1.7×10^5 to 3.0×10^6 PFU/ml (day 2), 8.7×10^3 to 2.5×10^4 PFU/ml (day 4) and 1.9×10^3 to 2.7×10^3 PFU/ml (day 6 PI). Similar findings were obtained in direct contact groups (Fig. 3B), ranging from 1.5×10^1 to 3.0×10^1 PFU/ml on day 2, 1.8×10^5 to 2.3×10^6 PFU/ml on day 4, 1.9×10^4 to 3.7×10^4 PFU/ml on day 6 PI. On the other hand, when aerosol transmission was evaluated, 3 out of 4 animals in each group seroconverted (Fig. 2), with HAI reciprocal geometric mean titers >1280 in all cases (compared to

<10 on day 0), except for one ferret of the I223V-H275Y group, which had a HAI titer of 80. Interestingly, only 1 animal per group showed detectable nasal wash viral titers, reaching a maximum of 5.2 x 10^3 PFU/ml (recombinant WT), 9.6 x 10^3 PFU/ml (H275Y) and 5.0 x 10^3 PFU/ml (I223V-H275Y) on day 6.

Molecular modeling. From the computer-generated molecular model (Fig. 4) it could be inferred that the I223R mutation is likely to cause its effect through steric clashes. The extended side chain of an arginine would come very close to the hydrophobic moiety of oseltamivir, thus disrupting its binding. Peramivir has a similar bulky hydrophobic group in the same position to that of oseltamivir, and thus it is expected that the I223R and H275Y mutations would have the same detrimental effect on binding of peramivir as with oseltamivir. For zanamivir, however, the corresponding glycerol moiety sits slightly higher up in the binding site and thus will be less disrupted. In addition, any disruption of binding may be off-set by potential hydrogen bonds between the glycerol hydroxyls and the nitrogens in the arginine side chain. The I223V mutation does not significantly affect zanamivir binding but the effect on oseltamivir is more important due to the mutation reducing the hydrophobicity of the active site, hence decreasing the favourable interactions with oseltamivir. Because oseltamivir is still likely to bind in its standard way, the I223V mutation will cause a less pronounced effect than the I223R.

DISCUSSION

The active site of the influenza NA is constituted by 8 functional (R118, D151, R152, R224, E276, R292, R371 and Y406) and 11 framework (E119, R156, W178, S179, D198, I222, E227, H274, E277, N294 and E425) residues, all in N2 numbering (8). Amino acid mutations at framework residues such as I222 (I223 in N1 numbering) may interfere with the correct binding of NAIs, thus disrupting the natural susceptibility of influenza viruses to these antiviral agents (1). Previous studies demonstrated that NAI-resistant influenza variants due to framework NA substitutions usually retained susceptibility to at least one of the three commercially available NAIs (oseltamivir, zanamivir, peramivir) (27). Here, we report that mutations at residue I223, in particular the I223R change, may confer reduced susceptibility to many NAIs associated with increased replicative capacities *in vitro*. In addition, when combined with the well-known H275Y mutation, the I223R/V substitutions potentiate

the oseltamivir and peramivir resistance phenotypes and restore the viral fitness of the H275Y mutant.

The I223V mutation combined with the H275Y was previously induced by serial *in vitro* passages of the influenza A/Texas/36/91 (H1N1) virus under oseltamivir pressure (36). Some influenza A/H5N1 and B strains with different substitutions at residue I223 have also been reported (16, 31). Moreover, our group has previously described the recovery of an oseltamivir-resistant influenza A/H3N2 virus harboring the I222V (I223V in N1 numbering) mutation in combination with E119V from an immunocompromised patient under oseltamivir therapy (3). In these reports, the I223V substitution was found to alter the resistance phenotype, with a negative effect on replication kinetics of A/H5N1 viruses, but improved viral fitness in the A/H3N2 subtype (3, 16, 32, 36). The impact of amino acid changes at residue I223 has recently elicited a more serious concern in the context of the 2009 pandemic H1N1 virus. Indeed, I223R/V/K changes have been identified in pH1N1 clinical isolates with reduced susceptibility to zanamivir and/or significantly increased oseltamivir- and peramivir-resistance phenotypes, when combined with the H275Y mutation (7, 11, 19, 26, 34). In one case, pH1N1 viruses with both I223V and H275Y mutations were detected in two symptomatic adolescents who were cabin mates at a summer camp in the U.S. and were receiving oseltamivir prophylaxis (7). Potential transmission of the I223V-H275Y double mutant between the two campers or after exposure to a third unknown ill person was suggested but could not be proven.

As the previous pH1N1 mutant strains could contain additional changes in the NA gene or elsewhere in the viral genome that could influence viral fitness, we generated recombinant viruses to dissect out the role of the single mutations at residue I223, combined or not with the well-described H275Y mutation. The five recombinants generated in this study were produced in comparable viral titers (≥10^6 PFU/ml), and despite minor differences, all of them proved to be replication competent in ST6GalI-MDCK cells, excluding any major negative effects of the mutations tested on viral fitness *in vitro*. As previously reported by our group (28), the single I223V mutation conferred only a minor reduction in oseltamivir susceptibility, but its combination with H275Y significantly enhanced the oseltamivir- and peramivir-resistant phenotype of the latter mutant. The moderate degree of resistance to oseltamivir and the reduced susceptibility to zanamivir and peramivir due to the I223R substitution described in this study (53-, 7- and 10-fold increases in IC_{50}, respectively, vs the recombinant WT), are in accordance with previous findings originating from clinical isolates collected

from immunocompetent and immunocompromised patients, treated or not with NAIs (11, 19, 34, 35). Furthermore, the multi-drug reduced susceptibility phenotype obtained for the double I223R-H275Y mutant confirmed data from a recent report involving an immunocompromised girl (26).

Unlike functional NA mutations such as R292K, which severely impair viral fitness of resistant mutants, NAI-resistant influenza variants containing framework substitutions may conserve their virulence and replicative properties (15, 38). To address whether this would be the case for I223R/V changes, we assessed replicative capacities both *in vitro* in ST6GalI-MDCK cells and *in vivo* using the well-established ferret model. In contrast to a previous report that showed reduced viral titers and mean plaque sizes for the I223R mutant (19), we found that both single I223R/V mutants had improved replication *in vitro* compared to the recombinant WT virus. In fact, the addition of the I223V/R substitution to the H275Y in the double recombinant mutant restored the slightly reduced fitness of the single H275Y virus. Based on these *in vitro* data and the possible transmission of a I223V-H275Y virus between two summer campers (7), we selected the WT, H275Y and I223V-H275Y recombinants for assessing virulence and transmission in the ferret model. In line with previous reports (17, 21), ferrets infected with pH1N1 variants did not evidence symptoms of severe disease. Interestingly, we found no significant differences in either seroconversion rates or nasal wash viral titers among the three viruses tested, showing comparable viral fitness and transmission efficiency. Even if a reduction in transmissibility by respiratory droplets compared to direct contact was observed, the three different recombinant viruses tested seemed to be equally affected. Although our group has previously reported a reduction in airborne transmission of an influenza A/Québec/147365/09 pH1N1 virus with the H275Y mutation (GenBank accession numbers: FN434448.1-FN434451.1 and FN434453.1-FN434455.1) compared to a WT isolate (13), whole genome sequencing comparison between the clinical strain previously evaluated and the present recombinant A/Québec/144147/09 virus shows many substitutions in the PB1, PA, HA, M1 and NS1 protein sequences that may account for this differential effect.

The reduced number of animals per group is one major limitation of this study. As a result, we cannot completely rule out a positive or negative effect of the I223V mutation on the transmissibility of pH1N1 viruses. Furthermore, we did not evaluate viral fitness in the ferret model of the I223R and I223R-H275Y mutants associated with a multi-drug reduced susceptibility phenotype,

although a recent study has shown no marked differences in viral fitness and transmissibility between a single I223R mutant virus and the WT counterpart (35).

There is no general consensus on the definition of drug-resistance based on IC_{50} values obtained by NAI assays. As previously mentioned and based on reports by Mishin et al. (23) and the Global Neuraminidase Inhibitor Susceptibility Network (25), the criteria defined in the present study set the cut-off for resistance to a >10-fold increase in IC_{50} values compared to the recombinant WT. However, it is not known what is the significance of these fold increases in IC_{50}, as clinical success or failure will also depend on the drug concentration at the site of viral replication. In that regard, following oral administration with the standard 75 mg twice-daily treatment, the average minimum plasma concentration of oseltamivir carboxylate is approximately 330 nM, which is significantly higher than the 50% inhibitory concentration for viral NA (10). Furthermore, a trial by Shelton et al. (30) reported median pulmonary zanamivir concentrations of 326 ng/ml in healthy adults treated with the regular 10 mg inhaled dose. Altogether, these clinical data suggest that only important increases in IC_{50} values (at least 100-fold or higher) would be associated with resistance to NAIs in the clinical setting. In line with the previous statement, we and others have previously confirmed that parenteral peramivir conserves prophylactic activity against seasonal A/H1N1 variants with the H275Y mutation in mice, despite the peramivir-resistant phenotype demonstrated in NAI assays (2, 33).

In summary, I223R/V mutations may alter the susceptibility of pH1N1 viruses to one or all NAIs currently in use, without significantly compromising viral fitness. Importantly, such mutations have the potential to improve the fitness of the oseltamivir-resistant H275Y viruses and, at least in some cases, can be efficiently transmitted. Surveillance of the possible emergence and dissemination of multidrug-resistant variants in the human population due to amino acid changes at residue I223 should be increased, as well as efforts to broaden the spectrum of available anti-influenza agents.

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Table 1. Susceptibility profiles of recombinant wild-type (WT) and mutant pH1N1 viruses to neuraminidase inhibitors.

NA mutant[a]	IC_{50} nM ± sd (fold increase)[b]		
	Oseltamivir	**Zanamivir**	**Peramivir**
WT	0.46 ± 0.01 (1)	0.15 ± 0.01 (1)	0.06 ± 0.00 (1)
H275Y	451.90 ± 26.01 (982)	0.14 ± 0.01 (1)	39.66 ± 5.98 (661)
I223V	2.63 ± 0.01 (6)	0.35 ± 0.02 (2)	0.15 ± 0.01 (3)
I223R	24.48 ± 2.12 (53)	1.10 ± 0.10 (7)	0.60 ± 0.06 (10)
I223V-H275Y	797.40 ± 51.00 (1733)	0.32 ± 0.01 (2)	162.39 ± 10.24 (2707)
I223R-H275Y	757.39 ± 68.65 (1647)	2.32 ± 0.10 (16)	1040.80 ± 170.69 (17347)

[a] All mutations are in N1 numbering.

[b] Mean values ± standard deviations (sd) of three independent experiments are indicated; fold increase is relative to the wild-type (WT) virus.

Figure 1. Replication kinetics of recombinant pH1N1 viruses *in vitro*. Confluent ST6Gal1-MDCK cells were infected with recombinant viruses at a multiplicity of infection (MOI) of 0.001 PFU/cell. Supernatants were harvested at 12, 24, 36, 48, 60 and 72 h post-infection and titrated by standard plaque assays. A) Wild-type (WT), I223V, H275Y, I223V-H275Y viruses. B) WT, I223R, H275Y, I223R-H275Y viruses. The mean values for three experiments with standard deviations are presented. *P<0.05 and **P<0.01 for differences in viral titers when compared to the recombinant WT virus.

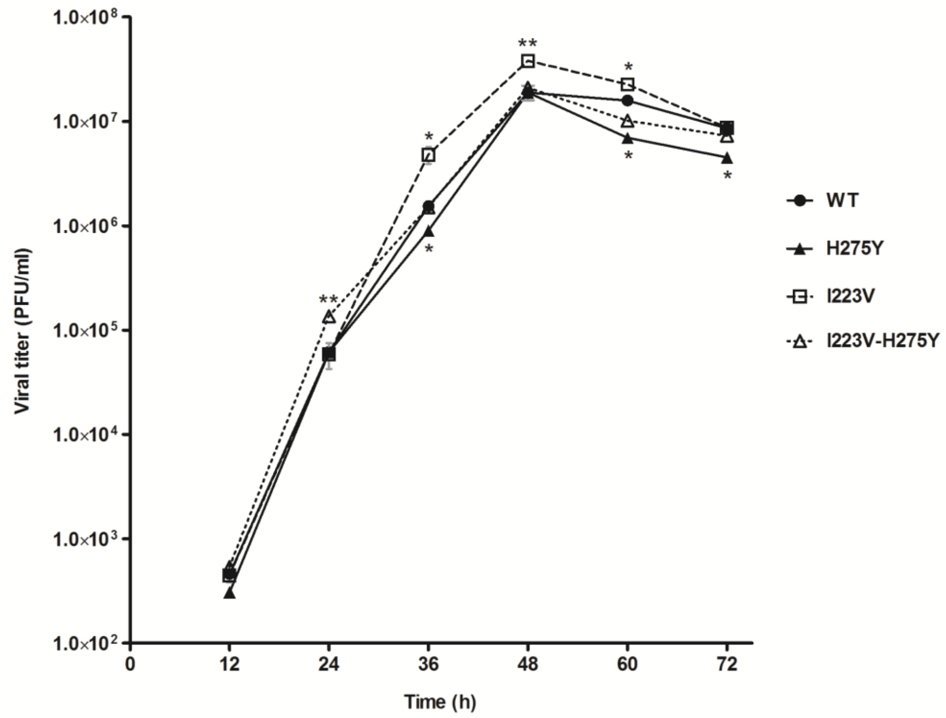
Figure 2. Seroconversion rates in ferrets infected with recombinant wild-type (WT), H275Y and I223V-H275Y pH1N1 viruses. Anti-A/Québec/144147/09 pH1N1 virus seroconversion rates of index, direct-contact and aerosol-contact ferrets were determined by hemagglutination inhibition assay (HAI). Seroconversion was defined as a >4-fold increase in hemagglutination inhibition reciprocal geometric mean titers on day 14 PI, compared to day 0 (at which time point all ferrets were seronegative).

Figure 3. Nasal wash viral titers in ferrets infected with recombinant wild-type (WT), H275Y and I223V-H275Y pH1N1 viruses. Groups of 4 ferrets were infected with 250 µl of PBS containing 4.5 log TCID50/ml of the recombinant pH1N1 WT, H275Y or I223V-H275Y variants. Inoculated-contact pairs were established 24 h post infection. Mean viral titers ± standard deviations in nasal wash samples of index (A) and direct contact (B) ferrets were determined by standard plaque assays in ST6Gal1-MDCK cells.

Figure 4. Molecular modeling of the I223R/V and H275Y NA mutations. Schematic representation of the active site of the NA with both NAIs: oseltamivir (in yellow) and zanamivir (in blue). The location of amino acids at position 223 and 275 are highlighted in stick representation, and coloured to distinguish different mutations.

Figure 1.

A



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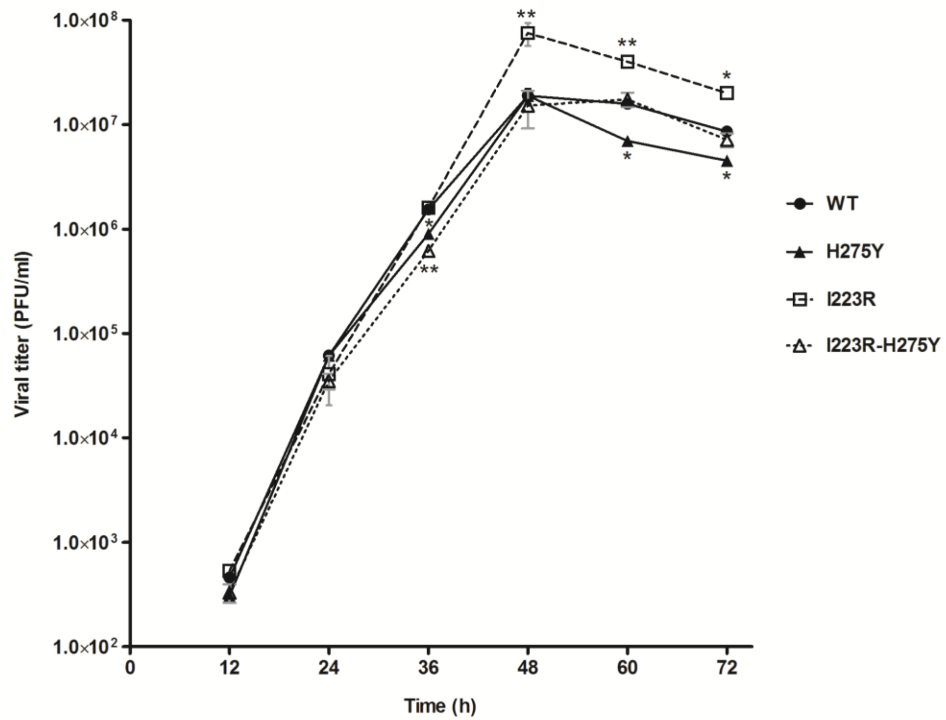


Figure 2.

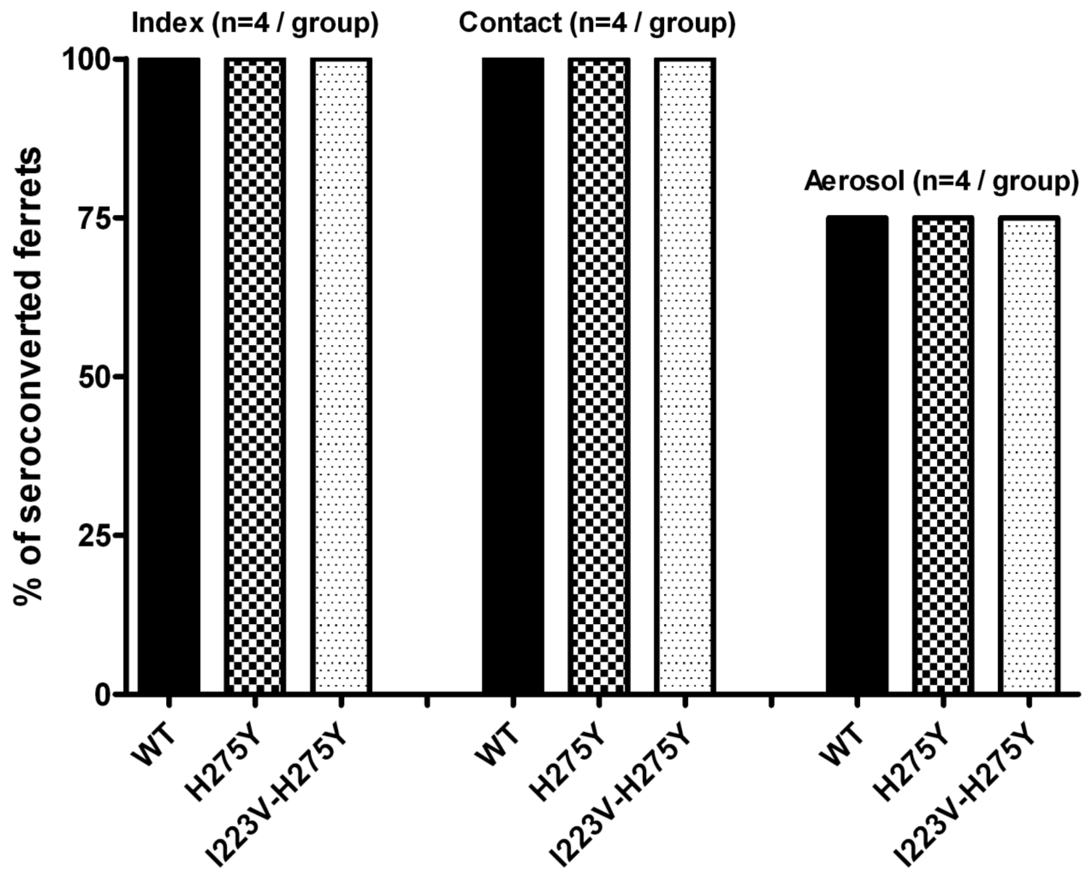
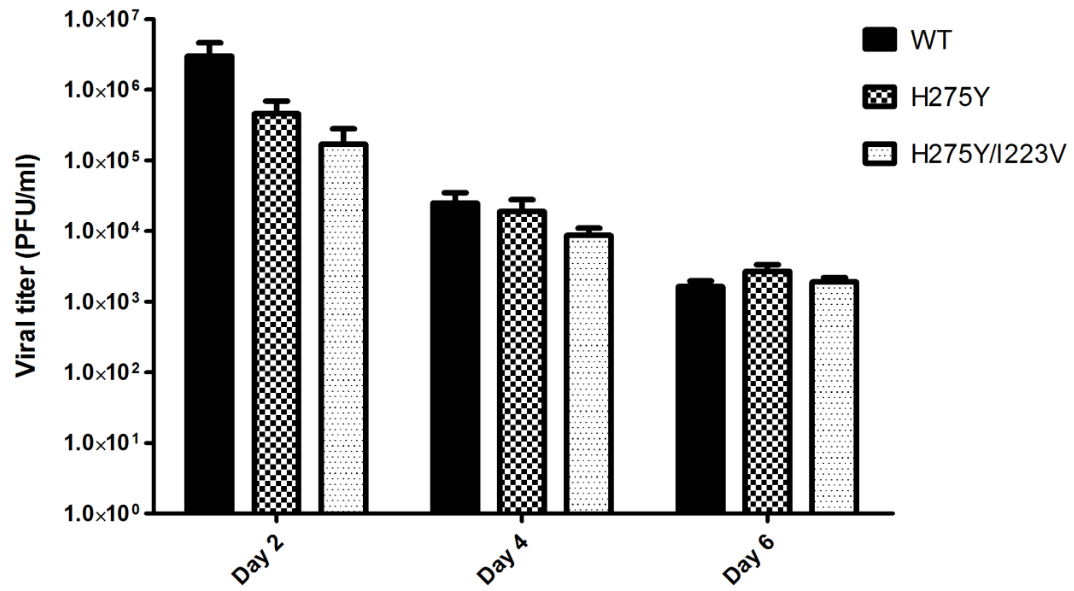


Figure 3.



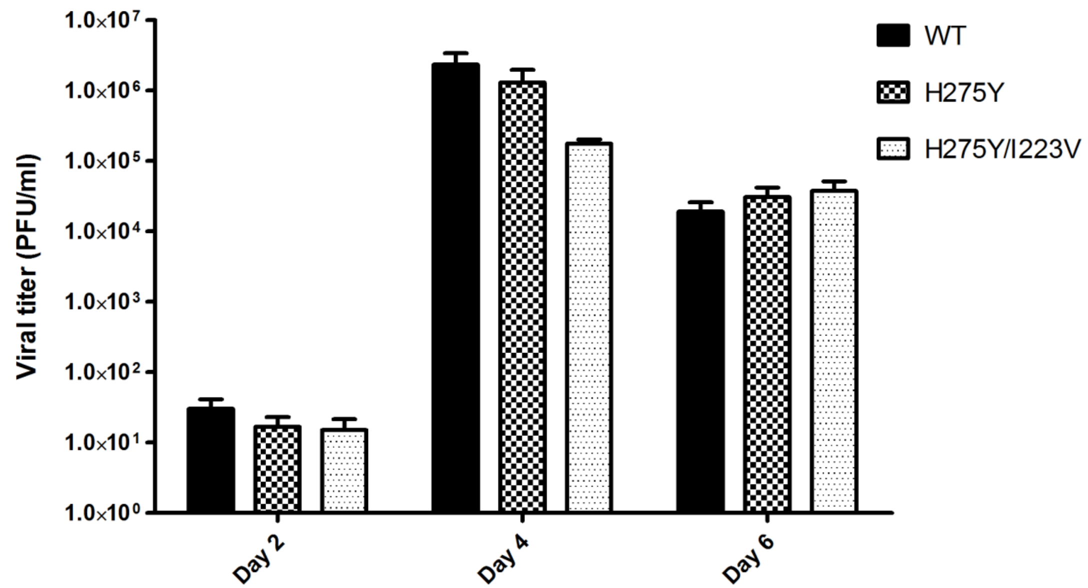
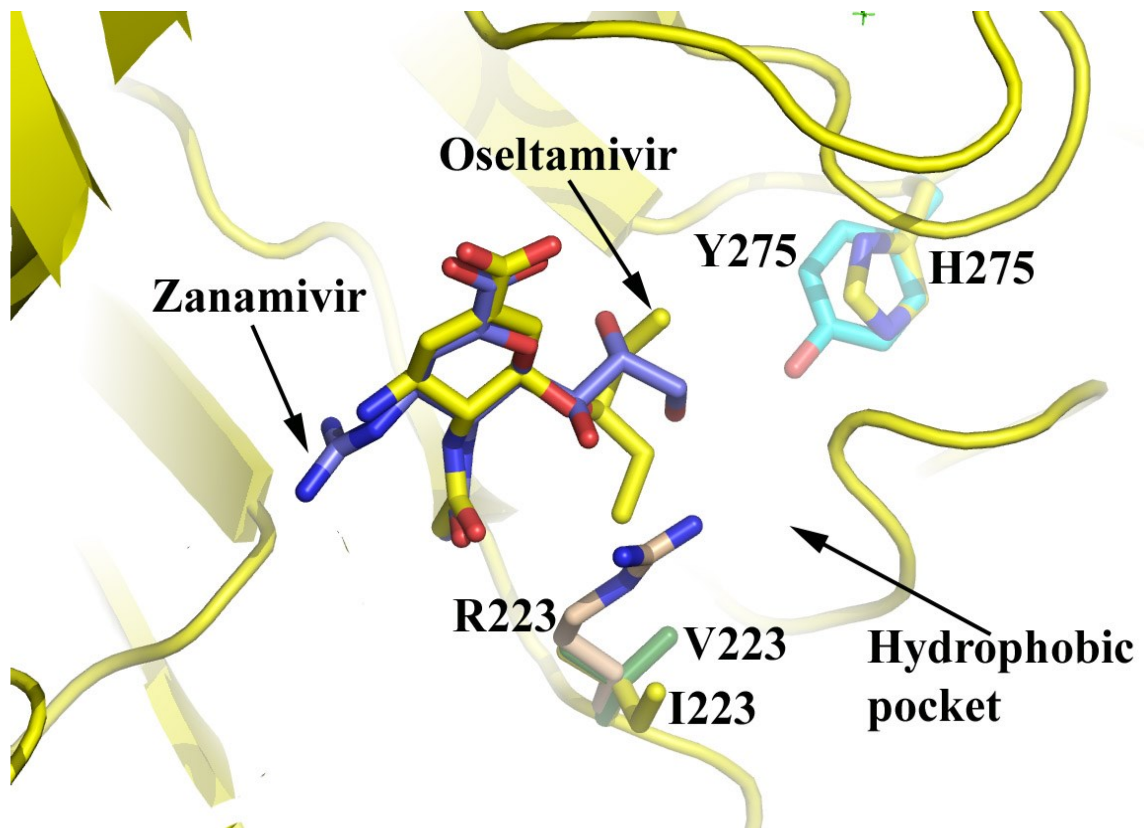


Figure 4.



Chapter V: "Evaluation of recombinant 2009 pandemic influenza A(H1N1) viruses harboring zanamivir resistance mutations in mice and ferrets"

5.1 Foreword

This chapter contains the text of the article **"Evaluation of recombinant 2009 pandemic influenza A(H1N1) viruses harboring zanamivir resistance mutations in mice and ferrets"**, written by Andrés Pizzorno, Yacine Abed, Chantal Rhéaume, Xavier Bouhy and Guy Boivin. This study aimed at assessing whether A(H1N1)pdm09 variants with reduced susceptibility to zanamivir, the first alternative to oseltamivir therapy and the only with a possibly available intravenous formulation, retained their fitness, pathogenicity and transmissibility both *in vitro* and *in vivo*. AP, YA and GB conceived and designed the experiments; AP and XB performed the *in vitro* experiments; AP and CR performed the *in vivo* experiments; AP, YA and GB analyzed the data; AP, YA and GB wrote the article and responded to the reviewer's comments. The article is presented in its final version accepted for publication in **Antimicrobial Agents and Chemotherapy** in 2013 (Apr;57(4):1784-9).

5.2 Article

Evaluation of recombinant 2009 pandemic influenza A/H1N1 viruses harbouring zanamivir-resistance mutations in mice and ferrets.

Running title: Zanamivir-resistant A(H1N1)pdm09 viruses

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ABSTRACT

Recombinant influenza A(H1N1)pdm09 wild-type (WT) and zanamivir-resistant E119G and Q136K neuraminidase mutants were generated for determining their enzymatic and replicative properties *in vitro*, as well as their infectivity and transmissibility in mice and ferrets. Viral titers of recombinant E119G and Q136K mutants were significantly lower compared to those of the WT in the first 36 h post-inoculation (p.i.) *in vitro*. The E119G and Q136K mutations were both associated with a significant reduction of total neuraminidase (NA) activity at the cell surface of 293T cells, with relative total NA activities of 14% ($P<0.01$) and 20% ($P<0.01$), respectively, compared with the WT. The E119G mutation significantly reduced the affinity (8-fold increase in Km) but not the Vmax. The Q136K mutation increased the affinity (5-fold decrease in Km) with a reduction in Vmax (8% Vmax ratio vs the WT). In mice, infection with the E119G and Q136K mutants resulted in lung viral titers that were significantly lower compared to WT on days 3 p.i. ($3.4 \pm 0.8 \times 10^6$ and $2.1 \pm 0.4 \times 10^7$ PFU/ml, respectively, vs $8.8 \pm 1.1 \times 10^7$, $P<0.05$) and 6 p.i. ($3.0 \pm 0.5 \times 10^5$ and $8.6 \pm 1.4 \times 10^5$ PFU/ml, respectively, vs $5.8 \pm 0.3 \times 10^7$, $P<0.01$). In experimentally-infected ferrets, the E119G mutation rapidly reverted to WT in donor and contact animals. The Q136K mutation was maintained in ferrets although nasal wash viral titers from the Q136K contact group were significantly lower compared to WT on days 3-5 p.i. Our results demonstrate that zanamivir-resistance E119G and Q136K mutations compromise viral fitness and transmissibility in A(H1N1)pdm09 viruses.

Keywords. Influenza, pandemic, resistance, zanamivir, neuraminidase, transmissibility, recombinant viruses.

INTRODUCTION

A novel influenza A/H1N1 virus emerged in Mexico in April 2009, and has since spread worldwide to cause the first influenza pandemic of the 21st century. Phylogenetic studies demonstrated that the A(H1N1)pdm09 virus resulted from a series of reassortment events involving human, swine and avian influenza A strains, with the neuraminidase (NA) gene originating from an avian-like Eurasian swine A/H1N1 lineage (1). Given the ancestral origin of the A(H1N1)pdm09 NA gene, the *in vitro* and *in vivo* behaviour of A(H1N1)pdm09 variants containing mutations of resistance to NAIs could be different than those of prior seasonal A/H1N1 viruses.

At present, little information regarding zanamivir resistance is available in A(H1N1)pdm09 viruses, probably related to the fact that mutations conferring highly reduced susceptibility to zanamivir have not yet emerged among clinical A(H1N1)pdm09 isolates. The aim of this study was to evaluate, in the A(H1N1)pdm09 background, the impact of certain NA mutations previously associated with reduced susceptibility to zanamivir in influenza A variants of the N1 and N2 subtypes. We were particularly interested in two NA mutations, E119G and Q136K, which were reported to confer highly reduced susceptibility to zanamivir. The E119G mutation occurred in both influenza A/Vietnam/1203/2004 and A/Chicken/Laos/26/2006 H5N1 viruses under zanamivir pressure *in vitro* and this variant showed a 1400-fold increase in zanamivir 50% inhibitory concentration (IC_{50}) but remained susceptible to oseltamivir (2). The Q136K mutation was recently described in clinical samples of influenza A/H3N2 circulating in 2007 and 2008 in Myanmar, exhibiting 30- to 50-fold increases in zanamivir IC_{50} (3). This substitution has also been reported in seasonal A/H1N1 viruses isolated between 2006 and 2008 in Australia and Southeast Asia although its clinical relevance is debatable since the mutation could not be detected in the primary clinical specimens (4).

Given the importance of determining whether the zanamivir-resistant variants retain their fitness and pathogenicity, we generated by reverse genetics a recombinant A(H1N1)pdm09 wild type (WT) virus and two (E119G and Q136K) variants and compared their enzymatic and replicative properties *in vitro*, as well as their infectivity and transmissibility in mice and ferrets.

METHODS

Generation of recombinant viruses. A recombinant influenza A(H1N1)pdm09 wild-type (WT) virus as well as two NA mutants (E119G and Q136K) were generated from the A/Québec/144147/09 virus (GenBank accession numbers FN434457-FN434464) by reverse genetics and site-directed mutagenesis as previously described (5). The recombinant viruses were amplified and subsequently titrated by standard plaque assays in ST6GalI Madin-Darby canine kidney (ST6GalI-MDCK) cells overexpressing the α2,6 sialic acid receptors, kindly provided by Dr. Y. Kawaoka from the University of Wisconsin, Madison, WI (6).

Drug susceptibility assays. The NAI susceptibilities of the three recombinant viruses were determined by NA inhibition assays as described elsewhere (7), with minor modifications. Viruses were standardized to a NA activity level ten-fold higher than that of the background as measured by the production of a fluorescent product from the 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA, Sigma, St-Louis, MO, USA) substrate. The drug susceptibility profiles were determined by the extent of NA inhibition after incubation with serial three-fold dilutions of the drugs oseltamivir carboxylate (Hoffmann-La Roche, Basel, Switzerland) or zanamivir (GlaxoSmithKline, Stevenage, UK), at final concentrations between 0 and 1800 nM. The IC_{50} values were determined from the dose-response curve.

***In vitro* replication assays.** Replicative capacities of the recombinant viruses were evaluated by infecting ST6GalI-MDCK cells at a multiplicity of infection (MOI) of 0.001 plaque forming-units (PFU)/cell in 12-well plates (8). Supernatants were collected at 12, 24, 36, 48, 60 and 72 h post infection and titrated by plaque assays. The mean viral plaque area of recombinant viruses was determined from a minimum of 20 plaques obtained after 60 h of incubation under agarose overlay using the ImageJ software (version 1.41), developed by Wayne Rasband of the National Institutes of Health as previously described (5).

Enzymatic assays. To measure the total NA enzymatic activity per infectious virus, fluorometric assays were conducted using MUNANA as substrate. Briefly, all recombinant viruses were standardized to an equivalent dose of $10^{6.5}$ PFU/ml and incubated at 37°C in 50-µl reactions with different concentrations (from 0 to 3000 µM) of MUNANA (9). Fluorescence was monitored

every 90 s for 53 min (35 measures). The Michaelis constant (Km) and the maximum velocity (Vmax) were calculated by nonlinear regression with the Prism software (GraphPad, version 5).

Cell surface NA activity. Recombinant WT and mutant (E119G and Q136K) NA proteins were expressed in 293T cells by co-transfection with the pLLBA-NA and pCAGGS-PA, -PB1, -PB2 and -NP plasmids (10). The empty pLLBA and pLLBA-HA plasmids were used as mock and negative controls, respectively. Twenty-four h post transfection, the cells were briefly treated with 0.02% EDTA in PBS and neutralized by the addition of 2% fetal bovine serum. Cells were subsequently centrifugated at 3000 RPM for 5 min, washed twice with PBS and resuspended in a non-lysing buffer (15 mM MOPS, 145 mM sodium chloride, 2.7 mM potassium chloride, 4 mM calcium chloride, adjusted to pH 7.4) to be used in an NA assay with MUNANA as substrate. Final values were normalized by the fluorescence of cells transfected with the empty pLLBA plasmid.

Mouse studies. Three groups of 12 (18-22 g) female BALB/c mice (Charles River, Lasalle, Quebec City, Canada) were housed four per cage and kept in conditions that prevented cage-to-cage infections. Mice were inoculated intranasally under isoflurane anesthesia with 10^4 PFU of the recombinant WT, E119G or Q136K viruses. Another group of mice was kept as uninfected control. All mice were monitored daily for weight loss during 12 days and four mice per group were sacrificed on day 3 and 6 p.i. for determination of lung viral titers (LVTs) by plaque assays in ST6GalI-MDCK cells.

Ferret studies. Three groups of four seronegative (800-1200 g) male ferrets (Marshall BioResources, North Rose, NY) housed in individual cages were lightly anesthetised by isoflurane and received an intranasal instillation of $10^{5.2}$ PFU of one of the recombinant viruses. Donor-contact animal pairs were established by placing a naïve ferret into each cage 24 h after inoculation of the donor ferret (11). Weight, temperature and clinical signs were monitored daily. Nasal wash samples were collected from donor animals on days 1 to 8 p.i. (24 h later from contacts), with 5 ml of PBS containing 2% bovine serum albumin and then immediately stored at -80 °C. Virus titers in nasal wash samples were determined by plaque assays in ST6GalI-MDCK cells. Serum samples were collected from each ferret before intranasal infection (day 0) and on day 14 p.i. to evaluate specific antibody levels against the A/Québec/144147/09 strain using standard HAI assays with 0.7% turkey red blood cells. Animals were sacrificed on day 14 p.i.

All animal procedures were approved by the Institutional Animal Care Committee of Laval University according to the guidelines of the Canadian Council on Animal Care.

Sequencing. NA genes of viruses recovered from *in vitro* replication assays, mouse lung homogenates and ferret nasal wash samples were amplified and sequenced using the ABI 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA) to confirm both the presence of the desired mutations and the absence of additional mutations.

Statistical analyses. Viral titers, plaque sizes, NA kinetic parameters and body weight loss were compared by one-way ANOVA analysis of variance, with the Tukey's multiple comparison posttest. The amount of NA activity at the cell surface for each mutant protein was compared to that of the WT by the use of unpaired two-tailed t tests.

RESULTS

In NA inhibition assays, both E119G and Q136K mutant viruses remained susceptible to oseltamivir but showed a highly reduced susceptibility profile to zanamivir with 821- and 749-fold increases in IC_{50} values, respectively, compared to the recombinant WT virus (Table 1). In enzymatic assays, the E119G mutation significantly reduced the affinity (8-fold increase in Km vs the WT, $P<0.01$) but not the Vmax. The Q136K mutation increased the affinity (5-fold decrease in Km vs the WT, $P<0.01$) with a reduction of Vmax (8% Vmax ratio vs the WT, $P<0.01$). Also, using recombinant NA proteins expressed in 293T cells, we observed that the E119G and Q136K mutants were both associated with a significant reduction of total NA activity at the cell surface, with relative total NA activities of 14% ($P<0.01$) and 20% ($P<0.01$), respectively, compared with the WT protein (Figure 1).

Replicative capacities in ST6GalI-MDCK cells showed that viral titers of the E119G and Q136K mutants were significantly lower compared to those of the WT in the first 36 h p.i. although similar peak titers ($2.8\pm0.5 \times 10^7$, $3.2\pm0.8 \times 10^7$ and $3.6\pm0.4 \times 10^7$ PFU/ml, respectively) were achieved for the three viruses at 48 h p.i. (Figure 2a). The E119G mutant exhibited plaques significantly smaller than those of the WT (0.33 ± 0.11 vs 0.50 ± 0.13 mm^2, $P<0.01$) but there were no significant differences between the Q136K (0.42 ± 0.14 mm^2) and the WT (Table 1). No unexpected mutations were found in viruses recovered at 36 or 72 h p.i.

No mortality was observed after intranasal inoculation of mice with 10^4 PFU of the recombinant WT, E119G or Q136K viruses. Also, no significant differences in body weight loss were observed among the three groups of infected mice, with maximal values of 7.0±1.1%, 5.9±0.8% and 5.8±0.9%, respectively, obtained on day 5 p.i. However, mean LVTs for the E119G and Q136K mutant groups were significantly lower than those of the WT group on days 3 p.i. (3.4±0.8 $x10^6$ and 2.1±0.4 $x10^7$ PFU/ml, respectively, vs 8.8±1.1 $x10^7$, P<0.05) and 6 p.i. (3.0±0.5 $x10^5$ and 8.6±1.4 $x10^5$ PFU/ml, respectively, vs 5.8±0.3 $x10^7$, P<0.001) (Figure 2b). No unexpected mutations were found in viruses recovered from lung homogenates.

Inoculation of ferrets with $10^{5.2}$ PFU of the recombinant WT, E119G or Q136K viruses yielded only mild clinical signs, and no significant differences in body weight loss (4.3±1.2%, 3.8±0.7% and 3.3±0.6%, respectively) or temperature were observed between groups. Interestingly, mean viral titers in nasal wash samples collected on days 2, 4 and 6 p.i. were comparable between the recombinant WT and E119G mutant donor groups, ranging from 1.6±0.7 $x10^6$ to 3.1±1.7 $x10^6$ PFU/ml (day 2), 4.6±2.2 $x10^5$ to 2.1±0.4 $x10^5$ PFU/ml (day 4) and 6.5±3.8 $x10^3$ to 7.8±6.1 $x10^3$ PFU/ml (day 6) (Figure 3A). In contrast, the Q136K group showed significantly reduced viral titers on day 4 (6.5±1.7 $x10^4$ PFU/ml, P<0.05) but not on days 2 (1.0±0.6 $x10^6$ PFU/ml) and 6 (7.7±6.1 $x10^3$ PFU/ml) p.i.

All contact ferrets seroconverted for A/Québec/144147/09 with HAI reciprocal geometric mean titers >1280, compared to <10 on day 0. However, mean viral titers in nasal wash samples obtained from contact ferrets of the Q136K group were significantly lower than those of the recombinant WT and E119G groups on days 3 (<10^1 vs 4.2±3.3 $x10^4$ and 2.5±1.4 $x10^4$ PFU/ml, P<0.01) and 5 (9.2±6.9 $x10^1$ vs 3.4±1.0 $x10^5$ and 4.8±0.9 $x10^5$ PFU/ml, P<0.01) p.i., respectively, but not on day 7 p.i. (1.3±0.9 $x10^4$ vs 1.6±1.5 $x10^5$ and 7.8±6.5 $x10^4$ PFU/ml) (Figure 3B). Sequence analysis of the NA gene in nasal wash samples of ferrets confirmed the presence of the Q136K mutation in the respective donor and contact groups, contrasting with the E119G mutation which reverted to the WT genotype. Indeed, mixed 119E/G viral populations with an approximate 80:20 ratio were detected in the four donor ferrets of the E119G group on day 2 p.i. At all subsequent time points, only the E119 genotype was detected in the four donor and contact ferrets.

DISCUSSION

Although the active site of the NA enzyme -which is the target of NAIs- is highly conserved, NAI-resistant influenza variants have been reported to contain different NA mutations that vary depending of the NA subtype as well as the inhibitor (12). In addition, we previously showed that the main mutation of resistance to oseltamivir (H275Y) affected differently the viral fitness of old and recent A/H1N1 viruses (13). As for seasonal A/H1N1 viruses, A(H1N1)pdm09 viruses harboring the H275Y NA mutation were found to confer resistance to oseltamivir but remained susceptible to zanamivir (5). Interestingly, we and others have reported that the replication and contact transmission of these oseltamivir-resistant A(H1N1)pdm09 H275Y variants were comparable to those of the WT virus in ferrets. However, there are contradictory data on the aerosol transmissibility of the H275Y mutant (11, 14, 15).

In contrast to oseltamivir resistance studies, little information is available on resistance to zanamivir in influenza A/H1N1 viruses in general and specifically in the A(H1N1)pdm09 background. Although mutations conferring resistance to zanamivir have not emerged among clinical A(H1N1)pdm09 isolates, we sought to anticipate such a potential event. In a previous study, we generated a series of recombinant A(H1N1)pdm09 viruses containing NA mutations previously found in other viral backgrounds, with one of them (E119G) being associated with highly reduced susceptibility to zanamivir (5). In the present work, we also generated the Q136K NA mutant and analyzed the enzymatic and *in vitro* replicative properties, as well as the infectivity and transmissibility of both recombinant E119G and Q136K variants in mice and ferrets.

Substitutions at residue E119 (G, D, A, V) were previously reported in drug-resistant variants of influenza A/H3N2 in clinic (E119V), and also in A/H1N9, A/H3N2 and B viruses after passages in the presence of zanamivir (12). Interestingly, the E119G mutation was also described in prototypic influenza A/H5N1 viruses from clades 1 and 2.3 under zanamivir pressure *in vitro*. These variants showed a zanamivir-resistant phenotype but remained susceptible to oseltamivir (2). The carboxylate of the E119 side chain interacts with the 4-guanidino group on zanamivir and the loss of this interaction in the E119G mutant results in a reduced binding of zanamivir (16). As peramivir and laninamivir also have a guanidine group, the E119G mutation would also confer cross resistance to these two NAIs but not to oseltamivir which possesses an acetamide group at that position.

Accordingly, our A(H1N1)pdm09 E119G variant was resistant to zanamivir and peramivir and remained susceptible to oseltamivir (5). Susceptibility to laninamivir could not be tested in this study.

The Q136K mutation was previously described in two influenza A/H3N2 viruses isolated in Myanmar in 2007 and 2008 (3). In that report, the presence of this mutation was also confirmed in primary clinical samples (nasopharyngeal swabs). The Q136K mutation was further identified in seasonal influenza A/H1N1 viruses isolated between 2006 and 2008 in Australia and Southeast Asia (4). In that case, however, the mutation could not be detected in primary clinical samples, suggesting either the presence of this variant at a very low proportion before cell culture amplification, or its generation as an "artifact" due to *in vitro* passages in MDCK cells. Later, pyrosequencing analysis of a set of matching clinical samples and virus isolates submitted to the WHO supported the hypothesis that propagation of human seasonal A/H1N1 viruses in MDCK cells can lead to the emergence of variants carrying mutations of resistance to NAIs, such as Q136K (17). Noteworthy, Hurt et al. (18) described the recovery of a Q136L NA mutant from nasal wash samples of a ferret infected with influenza A/Vietnam/1203/2004 (H5N1) and treated with zanamivir. This variant showed 350- and 26-fold increases in zanamivir and oseltamivir IC_{50} values, respectively. Thus, at the present time, the clinical relevance of the Q136K NA mutation is still questionable.

In the present work, the replication of the two mutant viruses with highly reduced susceptibility to zanamivir was impaired in ST6GalI-MDCK cells as well as in mice, compared to the recombinant WT virus. The reduced total NA activity at the cell surface of both mutants can account for this phenomenon, although we cannot distinguish if it was due to either a loss of NA enzymatic activity, decreased levels of folded NA protein on the cell surface, or a combination of both factors. Interestingly, a recent paper by Kaminski et al. (19) showed that the Q136K mutant had reduced numbers of NA molecules in viral particles as well as reduced intrinsic enzymatic activity compared to WT. Furthermore, the Q136K mutant showed reduced replication and delayed contact transmissibility in our ferret model contrasting to a previous report using A/Philippines/1279/2006 (H1N1) viruses (4). In that regard, A/Philippines/1279/2006 is a seasonal A/H1N1 virus, whereas the recombinant A/Québec/144147/2009 used in this study is a 2009 pandemic A/H1N1 strain. Such important structural and functional differences between the two NA proteins may explain the differential effects on enzymatic activity and viral fitness.

The Q136K mutation was maintained after its passage in ferrets unlike the E119G mutation which reverted rapidly to the WT genotype, explaining the comparable viral fitness and transmissibility of the WT and E119G groups. Therefore, the A(H1N1)pdm09 virus with the E119G NA mutation was severely compromised in the ferret model, which could be attributed to a significant reduction of NA activity expressed at the cell surface. Moreover, crystallographic structure of the NA protein of the N9 subtype revealed a salt bridge between E119 and R156 (20). It was suggested that the E119G mutation could then lead to the instability of the NA tetramers. In a previous reverse genetics study, we failed to rescue the A/WSN/33 (H1N1) E119G variant despite many attempts (21). Similarly, the E119G recombinant A/Victoria/3/75 (H3N2) variant could only be rescued in the presence of exogenous NA (22). In that study, the E119G mutant also reverted to the WT genotype after one passage in MDCK cells. In the avian A/H5N1 background, the E119G was also associated with a significant decrease of infectivity in MDCK cells (2). Finally, *in vitro* passaging of an A(H1N1)pdm09 virus in presence of zanamivir failed to select for mutations of resistance (23).

In conclusion, our study demonstrates that, in the A(H1N1)pdm09 genetic context and similarly to other genetic backgrounds, NA mutations conferring high levels of resistance to zanamivir alter enzymatic properties to a level that significantly compromises viral viability and transmissibility. This may help to explain the very low frequency of these mutations, particularly in the N1 subtype. However, the fitness of the E119G and Q136K mutants will need to be re-evaluated if they emerge in different viral backgrounds, especially when found in clinical samples.

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Table 1. Neuraminidase (NA) properties of the recombinant pandemic A/Québec/144147/09 viruses.

Recombinant	Oseltamivir IC_{50} nM (ratio)[a]	Zanamivir IC_{50} nM (ratio)[a]	K_m μM (ratio)[a]	V_{max} U/sec (ratio)[a]	Plaque area mm^2[b]
WT	0.46 ± 0.02 (1)	0.15 ± 0.01 (1)	80.9 ± 17.5 (1)	269.9 ± 4.3 (1)	0.50 ± 0.13
E119G	1.52 ± 0.13 (3.3)	123.1 ± 21.7 (821)	690.2 ± 37.3 (8.5)	238.1 ± 6.6 (0.9)	0.33 ± 0.11*
Q136K	0.08 ± 0.01 (0.2)	112.3 ± 15.8 (749)	16.6 ± 0.9 (0.2)	20.5 ± 3.5 (0.1)	0.42 ± 0.14

[a]Values indicate mean ± standard deviations from 3 experiments. Ratios compared with the recombinant wild-type (WT) virus.

[b]Values indicate mean ± standard deviations from 20 measures. *P < 0.05 when compared with the WT virus by one-way ANOVA.

Figure 1. Cell surface activity of recombinant A/Québec/144147/09 neuraminidase (NA) proteins. Recombinant WT and mutant (E119G and Q136K) NA proteins were expressed in 293T cells. The empty pLLBA and pLLBA-HA plasmids were used as mock and negative controls, respectively. Twenty-four h post transfection, the cells were briefly treated and resuspended in a non-lysing buffer to be used in an NA assay with MUNANA as substrate. Final values were normalized by the fluorescence of cells transfected with the empty pLLBA plasmid. The mean values for three experiments standard deviations are shown (**P<0.01).

Figure 2. Replicative capacities of recombinant wild-type (WT), E119G and Q136K A/Québec/144147/09 viruses *in vitro* and *in vivo*. (A) Confluent ST6Gal1-MDCK cells were infected with recombinant viruses at a multiplicity of infection (MOI) of 0.001 PFU/cell. Supernatants were harvested at 12, 24, 36, 48, 60 and 72 h post-infection and titrated by standard plaque assays. (B) Groups of 12 mice were inoculated intranasally with 10^4 PFU of the recombinant WT, E119G or Q136K viruses. Another group of mice was kept as uninfected control (not shown). Four mice per group were sacrificed on day 3 and 6 p.i. for determination of lung viral titers (LVTs) by plaque assays in ST6GalI-MDCK cells. The mean values for three experiments ± standard deviations are shown (*P<0.05, **P<0.01 and ***P<0.001).

Figure 3. Viral titers in nasal washes of ferrets infected with recombinant wild-type (WT), E119G and Q136K A/Québec/144147/09 viruses. Groups of 4 ferrets were infected with 250 µl of PBS containing $10^{5.2}$ PFU of the recombinant WT, E119G or Q136K variants. Donor-contact pairs were established 24 h post infection. Mean viral titers standard deviations in nasal wash samples of donor (A) and direct contact (B) ferrets were determined by standard plaque assays in ST6Gal1-MDCK cells (*P<0.05 and **P<0.01). Note: the E119G mutation reverted to the WT genotype in all four donor and contact ferrets as soon as day 2 p.i.

Figure 1.

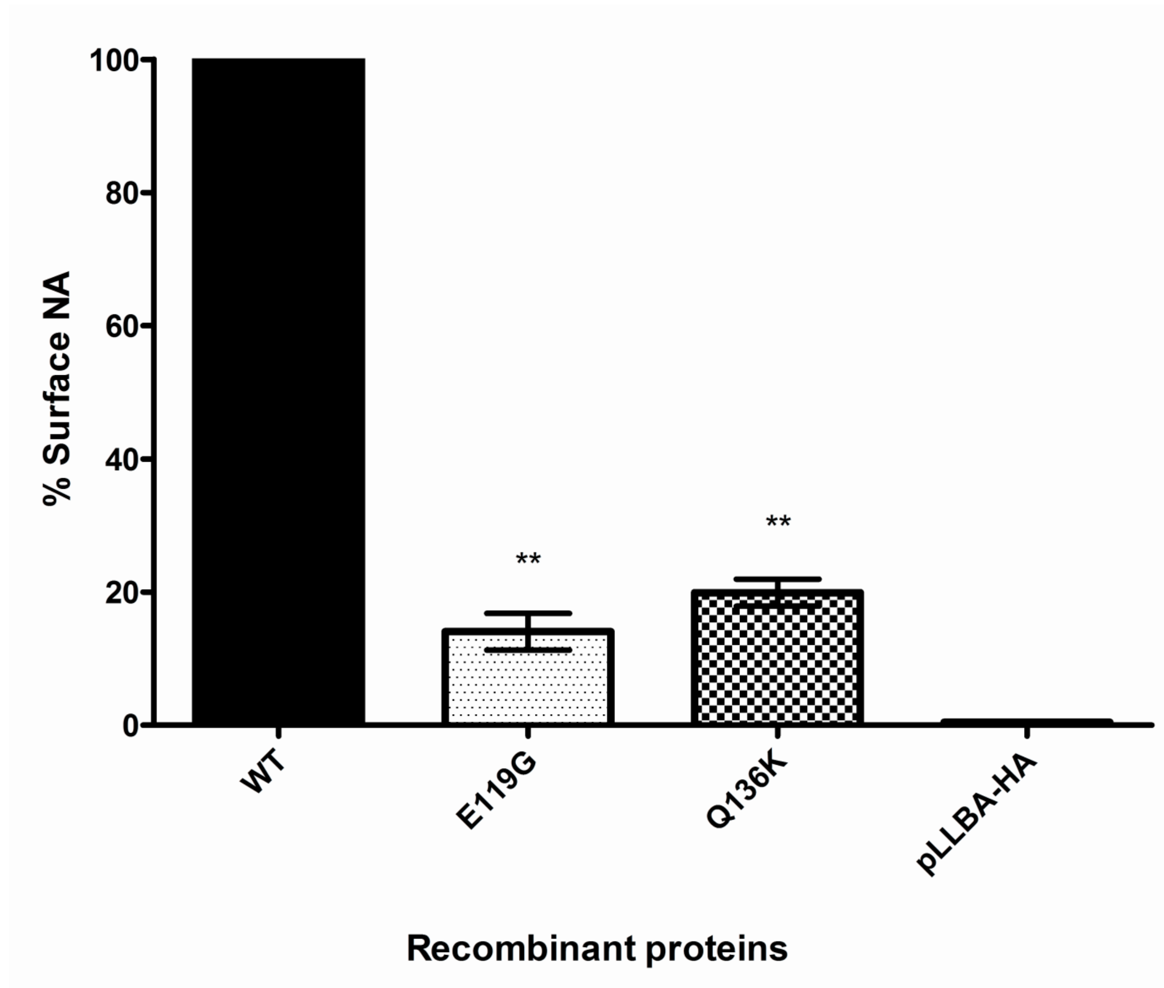


Figure 2.

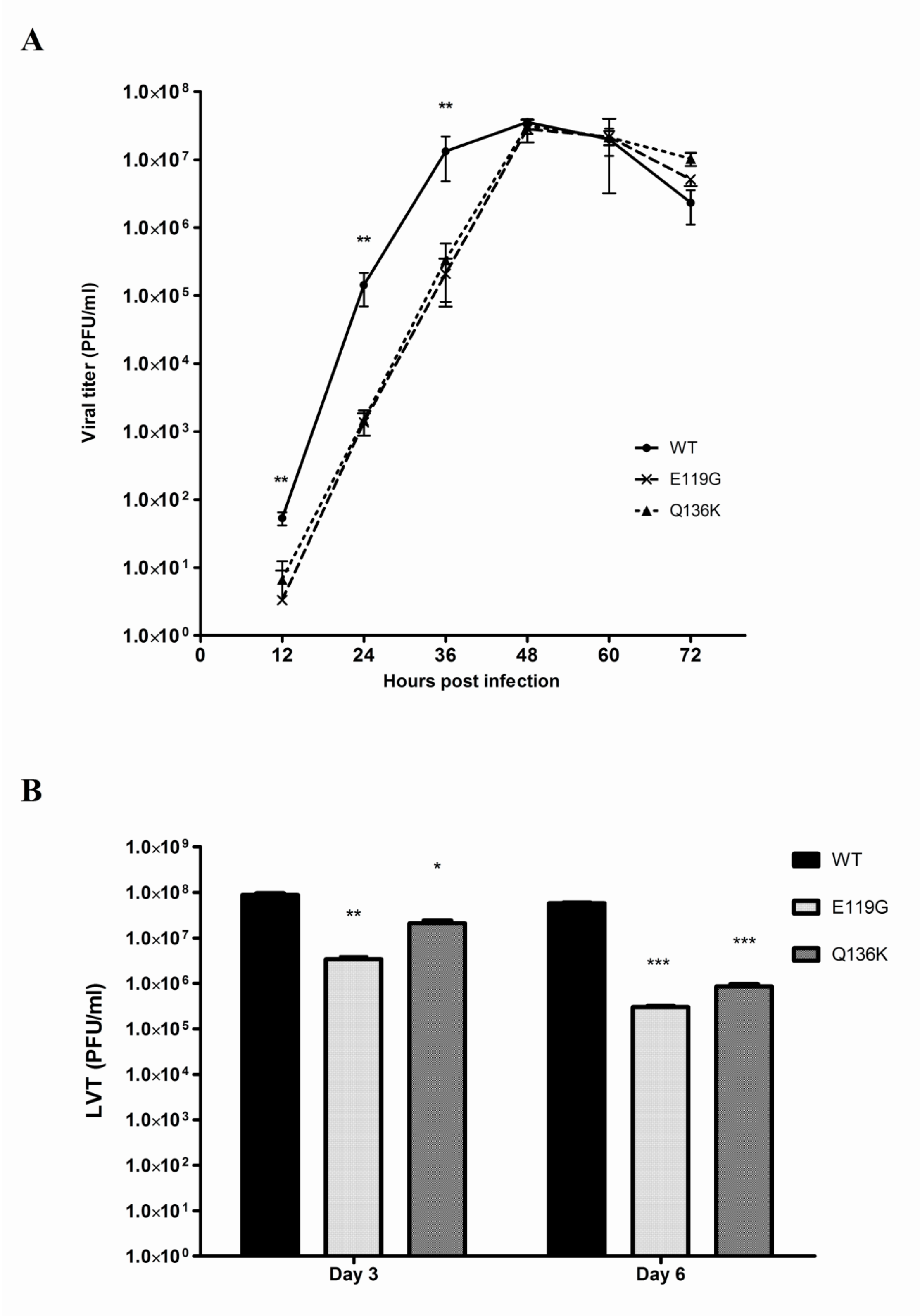
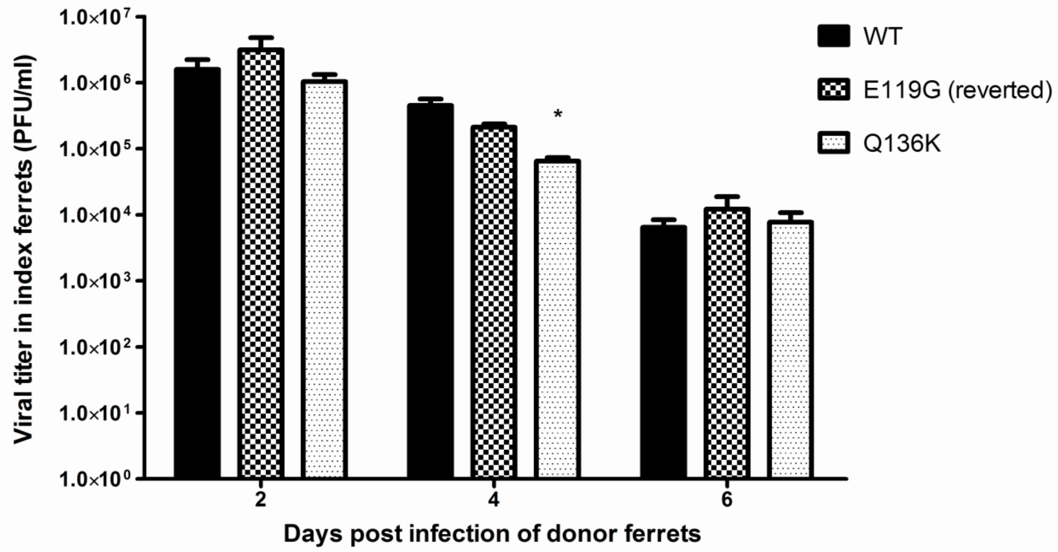
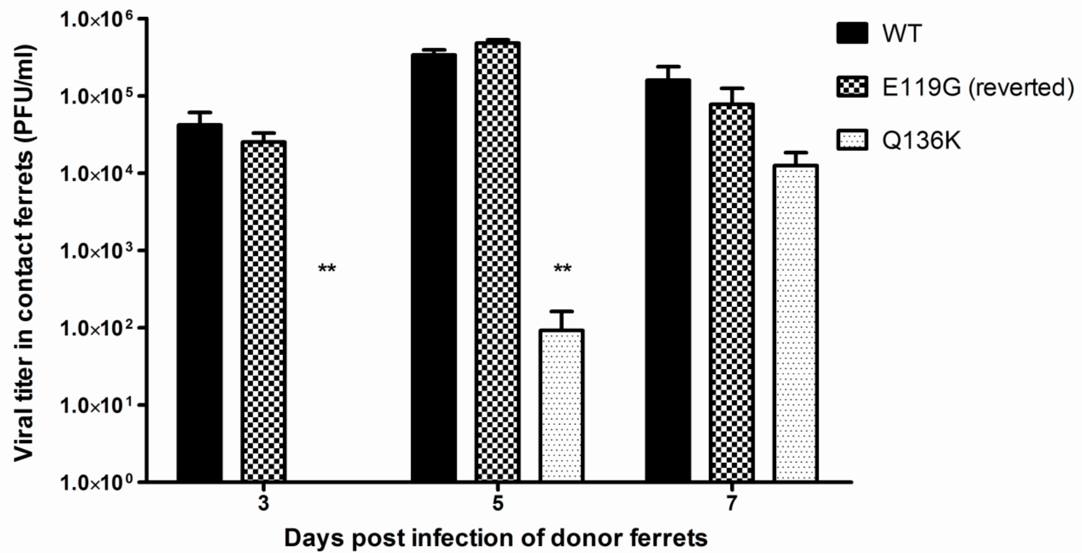


Figure 3.





Chapter VI: "Evolution of oseltamivir resistance mutations in influenza A(H1N1) and A(H3N2) viruses during selection in experimentally-infected mice"

6.1 Foreword

This chapter contains the text of the article **"Evolution of oseltamivir resistance mutations in influenza A(H1N1) and A(H3N2) viruses during selection in experimentally-infected mice"**, written by Andrés Pizzorno, Yacine Abed, Pier-Luc Plante, Julie Carbonneau, Mariana Baz, Marie-Ève Hamelin, Jacques Corbeil and Guy Boivin. In this study, we evaluated *in vivo* the effect of treatment with suboptimal doses of oseltamivir on the emergence and selection of drug-resistant viral quasispecies in mice. We also compared the sensitivity of traditional and deep sequencing methods to detect these subpopulations. AP, YA, MB and GB conceived and designed the experiments; AP performed the *in vitro* experiments; AP and MB performed the *in vivo* experiments; AP, JCa and MEH conceived and performed the deep sequencing experiments; PLP and JCo performed the bioinformatics analysis; AP, YA and GB analyzed the data; AP, YA and GB wrote the article and responded to the reviewer's comments. The article is presented in its final version accepted for publication in **Antimicrobial Agents and Chemotherapy** in 2014 (Nov;58(11):6398-405).

6.2 Article

Evolution of oseltamivir resistance mutations in influenza A(H1N1) and A(H3N2) viruses during selection in experimentally-infected mice

Running title: *In vivo* selection of oseltamivir resistance.

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ABSTRACT

The evolution of oseltamivir resistance mutations during selection through serial passages in animals is still poorly described. Herein, we assessed the evolution of neuraminidase (NA) and hemagglutinin (HA) genes of influenza A/WSN/33 (H1N1) and A/Victoria/3/75 (H3N2) viruses recovered from lungs of experimentally-infected BALB/c mice receiving suboptimal doses (0.05 and 1 mg/kg/day) of oseltamivir over two generations. Traditional phenotypic and genotypic methods as well as deep sequencing analysis were used to characterize potential selection of mutations and population dynamics of oseltamivir-resistant variants. No oseltamivir-resistance NA or HA changes were detected in the recovered A/WSN/33 viruses. However, we observed a positive selection of the I222T NA substitution in the recovered A/Victoria/3/75 viruses, with a frequency increasing over time and with oseltamivir concentration from 4% in the initial pre-therapy inoculum up to 28% after two lung passages. Although the presence of mixed I222T viral populations in mouse lungs only led to a minimal increase in oseltamivir IC_{50} values (by a mean of 5.7 fold) compared to that of the baseline virus, the expressed recombinant A/Victoria/3/75 I222T NA protein displayed a 16-fold increase in oseltamivir IC_{50} level when compared to the recombinant WT. In conclusion, the combination of serial *in vivo* passages under NAI pressure and temporal deep sequencing analysis enabled, for the first time, the identification and selection of the oseltamivir-resistant I222T NA mutation in an influenza H3N2 virus. Additional *in vivo* selection experiments with other antivirals and drug combinations would provide important information on the evolution of antiviral resistance in influenza viruses.

Keywords: influenza, neuraminidase, oseltamivir, resistance, deep sequencing, mice.

INTRODUCTION

Influenza viruses are major human respiratory pathogens, responsible for seasonal epidemics in temperate countries and occasional but devastating pandemics. Influenza A viruses of H1N1 and/or H3N2 subtypes have been most frequently associated with annual epidemics during the past forty years. Neuraminidase inhibitors (NAIs) such as oseltamivir, the most widely used NAI, and zanamivir target the active site of the influenza neuraminidase (NA) molecule, which is constituted by 8 catalytic (R118, D151, R152, R224, E276, R292, R371, and Y406; N2 numbering is used here and throughout the text) and 11 framework (E119, R156, W178, S179, D198, I222, E227, H274, E277, N294, and E425) residues that are largely conserved among influenza A and B viruses (1). Accordingly, these antivirals are active against all influenza strains and are thus recommended for the treatment of severe infections and also in high-risk individuals. However, as for other antivirals, extensive use of NAIs could lead to the emergence and transmission of drug-resistant variants that may compromise their clinical usefulness.

The generation and characterization of drug-resistant influenza variants through *in vitro* passages under NAI pressure can improve our understanding of the mechanisms of resistance to these anti-influenza agents. Indeed, the main NA mutations conferring resistance to oseltamivir in humans such as the H274Y change in the N1 subtype, and the E119V and R292K changes in the N2 subtype were previously predicted by *in vitro* studies (2-4). However, *in vitro* studies do not mimic completely the distribution and configuration of sialic acid receptors in the respiratory tract, nor they account for the role of the immune response. In addition to these oseltamivir resistance NA mutations, clinical influenza A(H1N1) and A(H3N2) variants occasionally contain other substitutions in the NA and/or hemagglutinin (HA) genes (5-7), although the contribution of these secondary mutations to the phenotype of resistance and to viral replicative capacity is not completely understood. Moreover, only a few studies have investigated the *in vivo* (animal) evolution of NA and HA genes from influenza viruses exposed to NAI pressure (8, 9).

The advent of next-generation deep sequencing has considerably expanded the limits of more traditional detection techniques such as RT-PCR and automated Sanger sequencing in terms of increased sensitivity and multiplexing. This technology enabled the detection and identification of rare low-frequency variants occurring in a given viral population (10). In this study, we selected oseltamivir-resistant variants by performing sequential passages of influenza A/WSN/33 (H1N1) and

A/Victoria/3/75 (H3N2) strains in mice receiving suboptimal doses of oseltamivir. We used traditional phenotypic and genotypic methods to characterize the recovered viruses, as well as next-generation deep sequencing analysis to assess genomic diversity and population dynamics of oseltamivir-resistant variants.

MATERIALS AND METHODS

Ethics statement. All animal procedures were approved by the Institutional Animal Care Committee of Laval University according to guidelines of the Canadian Council of Animal Care.

Viral stocks. Two mouse-adapted strains were selected for this study. The recombinant influenza A/WSN/33 (H1N1) wild-type (WT) virus was previously generated using a reverse genetics system that includes eight influenza virus RNA-transcription plasmids (pPOLI-PA, -PB1, -PB2, -NP, -HA, -NA, -M, and -NS) and polymerase and nucleoprotein expression plasmids (pCAGGS-PA, -PB1, -PB2, -NP) kindly provided by Dr. Peter Palese (Mount Sinai School of Medicine, New York, NY) (11). One-µg aliquots from each of the twelve plasmids were co-transfected into 293T human embryonic kidney cells using the LipofectamineTM 2000 reagent (Life Technologies Corporation, Grand Island, NY) as previously described (11). Supernatants were collected 72 h post-transfection and used to inoculate Madin-Darby canine kidney (MDCK) cells for viral amplification. The mouse-adapted influenza A/Victoria/3/75 (H3N2) virus (a gift from Dr. Donald Smee, Utah State University, Logan, UT) was passaged twice in MDCK cells in the absence of NAIs before animal infection. Both viruses were sequenced using the automated ABI 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA) and titrated by standard plaque assays in MDCK cells.

Mouse studies. i) First passage (P1). In a single experiment, groups of sixteen 6- to 8-week old female BALB/c mice (Charles River, ON, Canada) were infected by intranasal instillation of 2 x10^3 plaque forming units (PFU) of A/WSN/33 (H1N1) or A/Victoria/3/75 (H3N2) viruses in 30 µl of saline. Oral treatments (by gavage) with either saline or suboptimal doses of oseltamivir (0.05 and 1 mg/kg) were started 4 h before viral infection (day 0) and were continued once daily until day 5 post-infection (p.i.) to favor the induction of drug-resistant strains. The low concentration of oseltamivir was chosen to rapidly select for drug resistance based on clinical reports when low-dose prophylaxis or treatment

was used (12, 13). Animals were weighed daily for 14 days and monitored for clinical signs and mortality. On days 4 (expected maximum weight loss) and 6 (first day after the end of oseltamivir treatment) p.i., four mice per group were sacrificed and lungs were removed aseptically. For determination of viral titers, harvested lung tissues were homogenized in 1 ml of Dulbecco's Modified Eagle Medium (Life Technologies Corporation) with antibiotics using the Tissue Lyser bead mill homogenizer (Qiagen, Toronto, ON, Canada). Cells were pelleted by centrifugation (2,000 X g, 5 min) and supernatants were collected. A 100-µl aliquot of each lung supernatant was stored at -80 °C for phenotypic testing (viral isolation and NA inhibition assay), and the remaining ~900 µl were pooled by treatment group (4 lungs per pool) and stored at -80 °C for subsequent RNA extraction and viral titration by plaque assays in MDCK cells.

ii) Second passage (P2). Groups of sixteen 6- to 8-week old female BALB/c mice were infected intranasally with 1 $x10^3$ PFU of viruses originating from pooled supernatants of lung homogenates recovered on day 6 p.i. of the first passage (P1). Treatments were carried out in the same way as for P1, with the exception that the two groups treated with oseltamivir in P1 (0.05 and 1 mg/kg) were both treated with oseltamivir 1 mg/kg during the second passage (P2). The group treated with saline during P1 ("P1-untreated") also received saline during P2. Mortality, weight loss, and clinical signs were monitored for 14 days. Lungs were also removed from 4 mice per group on days 4 and 6 p.i. for NA inhibition assays, then pooled for subsequent RNA extraction and viral titration.

RT-PCR amplification and automated sequencing. RNA was isolated from the pooled supernatants of lung homogenates using the QIAamp Viral RNA Mini Kit (Qiagen, Toronto, ON, Canada), then was resuspended in a volume of 50 µl. Complementary DNA (cDNA) was synthesized using specific A/WSN/33 and A/Victoria/3/75 NA and HA primers and the SuperScript II reverse transcriptase enzyme (Life Technologies Corporation). Full-length viral NA and HA cDNAs were amplified by PCR using the Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Whitby, ON, Canada) and specific primers (available upon request) in standard conditions. Nucleotide sequences of PCR products were determined using the ABI 3730 DNA Analyzer and the heights of the chromatogram peaks were analyzed using BioEdit, version 7.0.5.

Cloning of PCR products. Complete PCR-amplified NA genes from pooled lung homogenates collected on day 6 p.i. during P2 were purified and then cloned by blunt-end ligation

into the pJET 1.2 vector using the CloneJET PCR Cloning Kit (Fisher Scientific, Ottawa, ON, Canada) according to the manufacturer's instructions. At least 18 NA-positive recombinant plasmids per group were randomly selected and subjected to sequence analysis with the ABI 3730 DNA Analyzer.

Deep sequencing. DNA libraries were prepared using 0.5 ng of each of the full-length viral NA and HA PCR products obtained from the viral inoculums and from pooled lung homogenates recovered on days 4 and 6 p.i. of both P1 and P2, using the Nextera® XT Sample Preparation kit (Illumina, San Diego, CA), according to the manufacturer's instructions. Previously-cloned A/Quebec/144147/09 (H1N1)pdm09 NA and HA genes (14) were amplified in the same conditions as the samples and included in the preparation of DNA libraries as controls. A purification step with AMPure XP beads (Beckman Coulter, Mississauga, ON, Canada) to remove very short library fragments was performed prior to library normalization. The libraries were then multiplexed, clustered, and finally sequenced using the Nextera® XT kit (Illumina) as described by the manufacturer, except that 10% of phiX Control (Illumina) was added to the library pool. The paired-end sequencing (2 x 250 nt) was performed on a MiSeq system (Illumina) and reads were demultiplexed using CASAVA 1.8.2 (Illumina), allowing no mismatch in the bar codes. After sequencing, reads were filtered for quality using FASTX Toolkit 0.0.13 quality trimmer and only reads passing the filter (quality score >30 and read length >150 nt) were kept for the following analysis.

Bioinformatics analysis. The reads obtained from the A/WSN/33 (H1N1) and A/Victoria/3/75 (H3N2) inoculums (viral stocks) were assembled de novo using Ray 2.2.0 (15, 16) and the consensus sequence of the two NA and HA genes were extracted from the contig files based on their similarity to reference sequences (GenBank accession numbers J02177, HE802059, HM641200 and V01098). The extremities of each gene were verified and completed manually based on the reference sequences to facilitate downstream analyses. Reads were aligned to the full-length assembled gene sequences to ascertain the absence of assembly errors. Single nucleotide polymorphisms (SNPs) were searched with GATK 2.7.4 (17) and no mismatches between the reads and the assembled contigs were detected. These curated and validated sequences were considered at this point as the reference sequences for assessing the emergence of mutations. The reads from each sample were aligned to each corresponding reference sequence using BWA 0.6.1 (18) and the nucleotide depth for each position was assessed with GATK. To correct for potential erroneous

calling of SNPs due to the intrinsic error rate of the RT-PCR amplification and sequencing reactions, we used the A(H1N1)pdm09 NA and HA control amplicons to calculate the proportion of nucleotides not matching the known reference sequences and obtained a mismatch frequency of 0.13±0.10%. Therefore, we established a 1% cut-off value, over which all positions with a nucleotide different from the reference sequence were considered significant. These sequences were selected using a simple Python script (available at https://github.com/plpla/SNP_finder).

Evolution of A/Victoria/3/75 (H3N2) I222T NA genotype during *in vitro* passages. The A/Victoria/3/75 (H3N2) inoculum (stock) virus and the virus recovered on day 6 p.i. from the P2-oseltamivir/P1-oseltamivir 1 mg/kg group were subjected to 4 serial passages in MDCK cells infected at a multiplicity of infection (MOI) of 0.001 in the absence of drug pressure. Passages in the presence of increasing concentrations of oseltamivir (from 10 nM at passage 1 to 80 nM at passage 4) were concomitantly performed. At each passage, viral RNA was isolated from supernatants of infected cell cultures and complete NA genes were amplified by RT-PCR. PCR products were purified and sequenced with the ABI 3730 DNA Analyzer and the heights of the chromatogram peaks were analyzed using BioEdit, version 7.0.5.

Expression of recombinant NA proteins. The pPOLI transcription plasmids containing the WT NA gene of either A/WSN/33 (H1N1) or A/Victoria/3/75 (H3N2) viruses were used for the introduction of single mutations (F149S and D434V in A/WSN/33, and I222T in A/Victoria/3/75) using appropriate primers and the QuikChangeTM Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The resulting plasmids were sequenced to ensure the absence of undesired mutations. The expression and analysis of recombinant NA proteins were performed as previously described (19). Briefly, 293T cells were co-transfected with 1 µg of each of the 4 expression plasmids (pCAGGS-PA, -PB1, -PB2, -NP) of A/WSN/33 and one of the respective pPOLI-NA transcription plasmid of A/WSN/33 or A/Victoria/3/75. At 48 h post-transfection, cells were treated with 0.02% ethylenediaminetetraacetic acid (EDTA) in PBS and harvested. After one wash with PBS, the cells were resuspended in PBS containing 3.5 mM CaCl2 and used in NA inhibition assays to determine the susceptibility of expressed proteins to oseltamivir.

Drug susceptibility assays. The drug susceptibility phenotype was determined by a fluorometric NA inhibition assay as described elsewhere (20). Stock viruses, viruses isolated from mouse lungs before pooling on days 4 and 6 p.i. and passaged once in MDCK cells, or recombinant

proteins were standardized to a NA activity level ten-fold higher than that of the background as measured by the production of a fluorescent product from the 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA, Sigma, St-Louis, MO, USA) substrate. Drug susceptibility profiles were determined by the extent of NA inhibition after incubation with serial three-fold dilutions of oseltamivir carboxylate (Hoffmann-La Roche, Basel, Switzerland) at final concentrations ranging between 0 and 1800 nM. The 50% enzyme-inhibitory concentration (IC_{50}) values were determined from the dose-response curves.

Statistical analysis. Weight loss, lung viral titers (LVT) and mutation frequencies from *in vitro* passages were compared by one-way analysis of variance (ANOVA) with Tukey's multiple-comparison post-test using GraphPad, version 5.

RESULTS

Effect of oseltamivir treatment on A/WSN/33 (H1N1) and A/Victoria/3/75 (H3N2) virus infections in mice. As shown in Table 1, i.n. inoculation of mice with 2 $x10^3$ PFU of A/WSN/33 or A/Victoria/3/75 viruses (P1) resulted in mortality rates of 87.5% and 25%, and mean maximum weight losses on day 4 of 19.9±1.5% and 15.3±1.8%, respectively, in both untreated groups. No mortality or significant weight loss were observed in any of the groups infected and treated with oseltamivir during P1. In line with these observations, mean LVT on day 6 p.i. were higher for the untreated groups, compared to those treated with 0.05 mg/kg and 1 mg/kg of oseltamivir, although these differences were significant only in the case of A/WSN/33 (8.6±0.3 $x10^6$ versus 3.5±0.1 $x10^6$ and 0.59±0.03 $x10^6$ PFU/lung, respectively, $P<0.05$), but not in the case of A/Victoria/3/75 (2.1±0.2 $x10^6$ vs 1.7±0.1 $x10^6$ and 1.3±0.3 $x10^6$ PFU/lung, respectively). Notably, mean LVT in untreated groups were significantly higher in mice infected with A/WSN/33 than in those infected with A/Victoria/3/75 on day 6 of P1 (8.6±0.3 $x10^6$ vs 2.1±0.2 $x10^6$ PFU/lung, respectively, $P<0.05$) but not on P2.

For the second passage (P2), mice were infected with 1 $x10^3$ PFU of viruses originating from pooled supernatants from the lung homogenates recovered on day 6 p.i. of P1, and the oseltamivir pressure was maintained at the highest concentration used in P1 (1 mg/kg). No mortality was observed in any of the groups, and only small mean weight losses on day 4 p.i. were observed in

both P2-untreated/P1-untreated groups (5.3±1.2% in A/WSN/33 and 4.8±0.9% in A/Victoria/3/75) (Table 1). Significantly higher LVT on day 6 p.i were observed in the A/WSN/33 P2-untreated/P1-untreated ($3.2±0.4 \times 10^6$ PFU/lung) group, when compared to the P2-oseltamivir/P1-oseltamivir 0.05 mg/kg ($5.9±0.4 \times 10^3$ PFU/lung) and P2-oseltamivir/P1-oseltamivir 1 mg/kg ($5.4±0.3 \times 10^3$ PFU/lung) groups ($P<0.001$). Conversely, for A/Victoria/3/75, mean LVT on day 6 p.i. were comparable between the P2-untreated/P1-untreated ($3.1±0.3 \times 10^6$ PFU/lung), P2-oseltamivir/P1-oseltamivir 0.05 mg/kg ($2.4±0.3 \times 10^6$ PFU/lung) and P2-oseltamivir/P1-oseltamivir 1 mg/kg ($3.0±0.4 \times 10^6$ PFU/lung) groups.

Characterization of viruses recovered from mouse lungs. Both pre-therapy inoculum viruses were susceptible to oseltamivir, with IC_{50} values of 0.75±0.04 nM for A/WSN/33 and 0.26±0.04 nM for A/Victoria/3/75. Since it is difficult to perform NAI assays directly from lung homogenates, 100-µl aliquots from each individual lung (4 per group) recovered at the four time points of the study were taken prior to pooling and then propagated once in MDCK cells for phenotypic analysis (Table 2). The WHO guidelines on the determination of NAI resistance for influenza A virus isolates define “reduced inhibition” by a 10-100-fold increase in IC_{50} values, and “highly reduced inhibition” by a >100-fold increase in IC_{50} values (21). According to this criterion, none of the viruses recovered from mouse lungs had reduced or highly-reduced inhibition to oseltamivir when compared to the respective pre-therapy inoculum viruses. For A/WSN/33 (H1N1) viruses, the maximum increase in oseltamivir IC_{50} (mean of 2.7-fold; range: 1.9 to 3.5) was observed in isolates obtained from the P1-oseltamivir 0.05 mg/kg goup on day 4 p.i. Of note, the low NA activity displayed by P1-oseltamivir 1 mg/kg and P2-oseltamivir/P1-oseltamivir 0.05 mg/kg A/WSN/33 viruses precluded NA inhibition testing. On the other hand, IC_{50} values seemed to slightly increase with oseltamivir pressure in A/Victoria/3/75 (H3N2) viruses, with the maximum increase (mean of 5.7-fold; range: 5.5 to 5.8) observed for the P2-oseltamivir/P1-oseltamivir 1 mg/kg goup on day 6 p.i.

To detect the possible emergence and/or selection of NA variants due to oseltamivir pressure, RNA from pooled lung homogenates recovered on days 4 and 6 p.i. of P2 was amplified by specific RT-PCR and subjected to automated conventional sequencing (Table 3). Interestingly, chromatogram analysis of the NA gene from A/Victoria/3/75 revealed the presence of mixed I222T viral populations with an approximate 80:20 (WT:mutant) ratio in the P2-oseltamivir/P1-oseltamivir 1 mg/kg group, on both days 4 (not shown) and 6 p.i. In addition, no other mutations or mixed viral populations were identified in the remaining A/Victoria/3/75 groups or in any of the A/WSN/33 groups.

To further characterize the genotypic profile of recovered viruses, amplicons obtained from pooled lung homogenates recovered on day 6 p.i. of P2 were cloned and at least 18 NA-positive cDNA clones were sequenced (Table 3). Analysis of A/WSN/33 clones revealed that 20/21 clones were identical to the WT sequence in the P2-untreated/P1-untreated group, while the remaining clone had a deletion of 1 nt at position 351 that resulted in a frame shift. This frame shift generated a stop codon, and the resulting formation of a truncated 118 aa NA protein. In the P2-oseltamivir/P1-oseltamivir 0.05 mg/kg group, 18/20 clones were identical to the WT sequence. The remaining two clones showed single M389T or D434E mutations. A deletion of 28 nt at position 400 that resulted in a truncated 134 aa NA protein was observed in 1/23 clones of the P2-oseltamivir/P1-oseltamivir 1 mg/kg group. The remaining 22/23 clones were identical to the WT sequence. For the A/Victoria/3/75 virus, 14/18 clones of the P2-untreated/P1-untreated group were identical to the WT sequence, 3 clones presented a single A46P, I73T or Q131H mutation, and 1 clone presented both Q131H and V398I mutations. In the P2-oseltamivir/P1-oseltamivir 0.05 mg/kg group, 13/19 clones were identical to the WT sequence, whereas the remaining presented the single N200D (2 clones), I222T (3 clones) or V398I (1 clone) substitutions. In the P2-oseltamivir/P1-oseltamivir 1 mg/kg group, 4/18 clones presented the I222T mutation and 2 other clones had either a single Q131H or V398I substitution. The remaining 12/18 clones were identical to the WT sequence.

Characterization of genomic diversity and population dynamics of oseltamivir-resistant variants by deep sequencing. The frequencies of SNPs found in viruses recovered from lung homogenates on day 6 p.i. of P2 by conventional sequencing and clone analysis were compared with those found by deep sequencing on Table 3. In general, SNPs found by molecular cloning correlated well with the frequencies found at these positions by deep sequencing. We also analyzed the changes in the frequency of individual SNPs as a function of oseltamivir concentration, day of specimen collection and passage number. None of the hallmark NA mutations associated with resistance to oseltamivir in N1 (I222V, H274Y and N294S) or N2 (E119V and R292K) viruses were observed. Furthermore, no NA or HA mutations found in any of the groups infected with the A/WSN/33 virus showed frequency variations that could be considered as indicators of a positive selection due to passage or oseltamivir pressure when compared to the initial inoculum. The only exceptions were the F149S and D434V NA substitutions that, despite being undetectable by molecular cloning, emerged by deep sequencing in the P2-oseltamivir/P1-oseltamivir 1 mg/kg group

at the last time point of the study (day 6 p.i. of P2) with frequencies of 22.9% and 7.1%, respectively (not shown).

Conversely, in the A/Victoria/3/75 virus, the temporary evolution of the I222T NA substitution revealed a pattern of positive selection by oseltamivir pressure, with an increase in frequencies that correlated with both drug concentration and days of treatment, reaching a peak of 28.2% for the P2-oseltamivir/P1-oseltamivir 1 mg/kg group on day 6 p.i. (Figure 1). This oseltamivir-dependent selection was further confirmed by *in vitro* passages of the inoculum (stock) virus as well as the virus recovered on day 6 p.i. from the P2-oseltamivir/P1-oseltamivir 1 mg/kg group. Chromatogram analysis showed no changes in the frequencies of the T222 NA viral populations after 4 passages in the absence of oseltamivir, remaining undetectable in the inoculum virus and at a mean of 19.9±0.9% in the P2-oseltamivir/P1-oseltamivir 1 mg/kg virus. On the other hand, after the fourth passage under increasing concentrations of oseltamivir (final concentration of 80 nM), the frequencies of the T222 NA viral populations shifted from undetectable levels to a mean of 9.6±0.8% in the inoculum virus and from 19.9±0.9% to 29.1±0.9% ($P<0.001$) in the P2-oseltamivir/P1-oseltamivir 1 mg/kg virus. Interestingly, deep sequencing analysis revealed that the I222T substitution was already present in the initial inoculum (stock) virus, albeit at a low frequency (4.1%) that had no impact on the susceptibility to oseltamivir of the whole viral population and precluding its detection by conventional sequencing methods. Finally, a positive selection of the N500D HA substitution was observed in the A/Victoria/3/75 virus (not shown), resulting in the loss of a potential N-linked glycosylation site. This mutation evolved in a passage-dependent yet drug-independent manner, increasing its frequency from 8.4% in the initial inoculum to 30.9%, 26.1% and 35.5% on day 6 p.i. for the P2-untreated/P1-untreated, P2-oseltamivir/P1-oseltamivir 0.05 mg/kg and P2-oseltamivir/P1-oseltamivir 1 mg/kg groups, respectively. The complete results with all minor NA and HA variants found by deep sequencing are presented in the Supplementary File.

Susceptibility profiles of recombinant proteins. To assess the effects of unknown NA mutations on oseltamivir susceptibility, WT and mutant recombinant NA proteins were expressed and subjected to NA inhibition assays. While oseltamivir susceptibility levels of recombinant A/WSN/33 F149S and D434V NA proteins were comparable to that of the WT (IC_{50} values of 0.47±0.05 nM, 0.41±0.04 nM, and 0.48±0.03 nM, respectively), the I222T A/Victoria/3/75 (H3N2) mutant protein

showed a “reduced inhibition” phenotype, with a 16-fold increase in IC_{50} value compared to the WT (4.69±0.37 nM vs 0.29±0.01 nM, respectively).

DISCUSSION

Development of resistance to oseltamivir, the most widely used NAI, remains an important concern, not only among immunocompromised patients (22, 23) but also in the general population. This is especially worrisome given the conserved viral fitness observed for the previously disseminated seasonal A/Brisbane/59/2007 (H1N1) H274Y variant (24, 25) as well as recent reports on community outbreaks of A(H1N1)pdm09 H274Y strains (26, 27). Moreover, resistance to oseltamivir conferred by the H274Y and N294S mutations as well as the R292K mutation have been already detected in patients infected with A(H5N1) strains and the novel A(H7N9) virus, respectively (28, 29). Previous studies demonstrated that NA mutations conferring resistance to oseltamivir in the clinical setting could be predicted to some extent by performing *in vitro* serial passages of influenza viruses under oseltamivir pressure (3). However, very limited information is available on the selection of oseltamivir-resistant variants in mice, as well as on the temporal evolution of viral quasi-species under selective NAI pressure.

In the present study, we selected two mouse-adapted influenza viruses, A/WSN/33 (H1N1) and A/Victoria/3/75 (H3N2), to perform two serial passages in mice receiving suboptimal doses of oseltamivir and analyzed the phenotypic and genotypic characteristics of the recovered viruses. Although no NA or HA substitutions associated with oseltamivir resistance were detected in the A/WSN/33 background, we observed a positive selection of the I222T NA substitution in the A/Victoria/3/75 virus. Indeed, this mutation was selected in a dose-dependent manner, increasing its frequency with *in vivo* passages, which also correlated with an increase in the T/I ratio during *in vitro* passages under oseltamivir pressure. Of interest, the T222 genotype was associated with a 16-fold increase in oseltamivir IC_{50} level shown by the expressed recombinant mutant NA protein when compared to the recombinant WT. Such phenotype was not strain specific as the recombinant A/Hong Kong/1/68 (H3N2) NA protein harboring the I222T change also showed reduced susceptibility to oseltamivir (data not shown).

Many factors may account for our inability to select any mutations of resistance to oseltamivir in the A/WSN/33 background under the conditions used in our experimental design. The 2.7-fold maximum increase in oseltamivir IC_{50} value, along with the low NA activity of some recovered viruses and the significant reduction in lung viral titers (LVT) observed in both treated groups suggest that the initial viral inoculum was too low, even if low dose oseltamivir regimens were used (0.05 and 1 mg/kg/day as compared to more regular treatment of 20 mg/kg/day). As a result, the low amounts of virus recovered on day 6 p.i. of the first passage led to a reduced viral inoculum for the second passage thus enhancing the excessive pressure imposed by oseltamivir treatment. In that regard, a higher viral inoculum coupled to lower oseltamivir concentrations and/or increased serial passages would be interesting to test in future experiments. In addition, the late selection of two NA mutations (F149S and D434V) that do not affect susceptibility to oseltamivir is worth mentioning. Since both residues are located relatively far from the active site, they should not affect the enzymatic properties of the NA, but recent reports of permissive mutations at least in influenza viruses of the N1 subtype (24, 25, 30) warrant the need for experiments to assess their potential role in viral fitness.

The selection of the I222T NA substitution in the A/Victoria/3/75 (H3N2) virus is the major finding of our study. Amino acid I222 is a well conserved framework residue among several influenza A subtypes and influenza B viruses (1). Together with W178, I222 forms a hydrophobic pocket within the NA active site in which lie both the methyl group of the C_4 acetamide of sialic acid and oseltamivir (31). Due to its interaction with the pentoxyl group of oseltamivir, several substitutions at the I222 residue have been identified in different influenza backgrounds both *in vitro* and in clinical studies (22, 32-34). Recently, the addition of the I222T mutation to the H274Y in recombinant influenza A/California/04/2009 (H1N1pdm09) viruses was shown to increase the oseltamivir IC_{50} value from 574-fold in the single H274Y mutant to 1377-fold in the double I222T/H274Y mutant, when compared to the recombinant WT (35). In that case, the presence of a threonine at position 222 favored the formation of hydrogen bonds between this residue and its neighboring R152. As a result, R152 translocated to a position closer to T222 causing steric hindrance to the binding of the pentoxyl group of oseltamivir. Moreover, the single I222T mutant remained susceptible to oseltamivir (6.4-fold increase in IC_{50} compared to the WT) and this variant produced viral titers comparable to those of the WT virus in replicative capacity experiments. Also, when introduced in the recombinant influenza B/Yamanashi/166/1998 background, the I222T NA mutation reduced susceptibility to oseltamivir and peramivir (13- and 15-fold increases in IC_{50} values, respectively, compared to the WT) (36) with no

alteration of viral fitness *in vitro*. In line with available data on the mild effect of the I222T NA mutation on oseltamivir susceptibility, the I222T variant described in the present study derived form an A/Victoria/3/75 (H3N2) NA protein conferred a “reduced inhibition” phenotype, with 16-fold increase in oseltamivir IC_{50} value. Furthermore, reports on the isolation of influenza B viruses harboring the I222T mutation from untreated patients indicate that this variant possibly occur naturally without NAI selective pressure (37). These results are in agreement with previous reports suggesting that framework NA mutations decreasing susceptibility to NAIs generally do not alter the replicative capabilities (38). As a result, it is likely that the A/Victoria/3/75 (H3N2) I222T variant will retain viral fitness, yet this hypothesis and the potential contribution of the loss of a N-linked glycosylation site in the HA due to the N500D mutation on viral replication kinetics and antigenicity remain to be tested.

Our study also highlights the power of deep sequencing in terms of versatility and sensitivity for the detection of expected as well as unexpected minor variants within the viral population. In that regard, we were able not only to detect differences in the range of 1% in the time-course population dynamics but, also importantly, the presence of a 4% oseltamivir-resistant variant in the initial inoculum, both of which would have remained undetected by Sanger sequencing approaches. We were not able to determine at what time the A/Victoria/3/75 (H3N2) virus acquired the I222T mutation, but its presence in the initial inoculum certainly biased its subsequent selection. As recently shown for A(H1N1)pdm09 and A(H7N9) viruses, early detection of minor viral populations could be of major importance for the rapid and accurate implementation of antiviral therapy in some patients, since the resistance variant may be initially masked in the clinical samples containing mixed populations but rapidly favored and transmitted upon treatment (10, 39). In fact, we reported the rapid emergence of an oseltamivir-resistant variant in the household contact of an index patient infected with an oseltamivir-susceptible A(H1N1)pdm09 virus containing a minor sub-population of ~2% of H274Y mutant only detectable by deep sequencing (10). In this case, the rapid selection of the H274Y virus in the contact patient was favored by the use of a suboptimal (prophylactic) dose of oseltamivir.

In conclusion, the combination of serial *in vivo* passages under NAI pressure and temporal deep sequencing analysis used in this study has allowed the selection and identification of the I222T NA mutation in an influenza H3N2 virus, conferring reduced inhibition to oseltamivir. To our knowledge, this is the first report of this substitution in the N2 background although the clinical significance of this mutation remains to be assessed. Additional *in vivo* selection experiments with

other antivirals and/or drug combinations would provide important information on the evolution of antiviral resistance in influenza viruses, particularly in the case of new avian viruses infecting humans.

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Table 1. Effect of oseltamivir therapy in mice infected with A/WSN/33 (H1N1) and A/Victoria/3/75 (H3N2) viruses.

Virus / Regimen[a]	Mean % weight loss on day 4 p.i.[b] ± sd (N = 8)	% Mortality on day 14 p.i. (N = 8)	Mean LVT[c] on day 6 p.i. ± sd (N = 4)
A/WSN/33 (H1N1):			
P1-untreated	19.9 ± 1.5	87.5	$8.6 \pm 0.3 \times 10^{6}$
P1-oseltamivir 0.05 mg/kg	< 1	0	$3.5 \pm 0.1 \times 10^{6}$ *
P1-oseltamivir 1 mg/kg	< 1	0	$0.59 \pm 0.03 \times 10^{6}$ *
P2-unt/P1-untreated	5.3 ± 1.2	0	$3.2 \pm 0.4 \times 10^{6}$
P2-ose/P1-ose 0.05 mg/kg	< 1	0	$5.9 \pm 0.4 \times 10^{3}$ ***
P2-ose/P1-ose 1 mg/kg	< 1	0	$5.4 \pm 0.3 \times 10^{3}$ ***

A/Victoria/3/75 (H3N2):			
P1-untreated	15.3 ± 1.8	25	$2.1 \pm 0.2 \times 10^6$
P1-oseltamivir 0.05 mg/kg	< 1	0	$1.7 \pm 0.1 \times 10^6$
P1-oseltamivir 1 mg/kg	< 1	0	$1.3 \pm 0.3 \times 10^6$
P2-unt/P1-untreated	4.8 ± 0.9	0	$3.1 \pm 0.3 \times 10^6$
P2-ose/P1-ose 0.05 mg/kg	< 1	0	$2.4 \pm 0.3 \times 10^6$
P2-ose/P1-ose 1 mg/kg	< 1	0	$3.0 \pm 0.4 \times 10^6$

Note: the viral inoculum was 2 $\times 10^3$ PFU in P1 and 1 $\times 10^3$ PFU in P2.

[a]P1; passage 1, P2; passage 2, unt; untreated, ose; oseltamivir.

[b]p.i.; post-infection.

[c]LVT; lung viral titers in plaque forming units (PFU)/lung. Mean values and standard deviations (sd) from a single experiment performed in triplicate are presented. $^{*}P$ <0.05, $^{***}P$ <0.001 compared to the corresponding untreated group of each passage.

Table 2. Oseltamivir susceptibility of viruses recovered from individual lungs of mice infected with A/WSN/33 (H1N1) and A/Victoria/3/75 (H3N2) viruses.

Virus / Regimen[a]	Mean oseltamivir IC_{50}[b] in nM (range) on day 4 p.i.[c] (N = 4)	Fold[d]	Mean oseltamivir IC_{50} in nM (range) on day 6 p.i. (N = 4)	Fold
A/WSN/33 (H1N1):				
P1-untreated	1.36 (1.09 - 1.87)	1.8	1.07 (0.94 - 1.20)	1.4
P1-oseltamivir 0.05 mg/kg	2.06 (1.46 - 2.65)	2.7	1.27 (1.04 - 1.47)	1.7
P1-oseltamivir 1 mg/kg	low activity[e]	-	low activity	-
P2-unt/P1-untreated	0.61 (0.46 - 0.81)	0.8	0.36 (0.34 - 0.37)	0.5
P2-ose/P1-ose 0.05 mg/kg	low activity	-	low activity	-
P2-ose/P1-ose 1 mg/kg	0.75 (0.70 - 0.80)	1.0	0.68 (0.65 - 0.72)	0.9

A/Victoria/3/75 (H3N2):				
P1-untreated	0.37 (0.26 - 0.49)	1.4	0.45 (0.39 - 0.55)	1.7
P1-oseltamivir 0.05 mg/kg	0.78 (0.48 - 1.14)	3.0	0.57 (0.56 - 0.58)	2.2
P1-oseltamivir 1 mg/kg	1.24 (0.88 - 1.85)	4.8	1.02 (0.98 - 1.07)	3.9
P2-unt/P1-untreated	0.73 (0.50 - 1.06)	2.8	0.21 (0.19 - 0.22)	0.8
P2-ose/P1-ose 0.05 mg/kg	0.63 (0.42 - 0.94)	2.4	0.72 (0.68 - 0.74)	2.8
P2-ose/P1-ose 1 mg/kg	1.30 (1.17 - 1.48)	5.0	1.47 (1.42 - 1.52)	5.7

[a]P1; passage 1, P2; passage 2, unt; untreated, ose; oseltamivir.

[b]IC_{50}; 50% enzyme-inhibitory concentration. Mean values from a single experiment performed in duplicate are presented.

[c]p.i.; post-infection.

[d]Fold increase vs oseltamivir IC_{50} values of the pre-therapy inoculum viruses (A/WSN/33 = 0.75 ± 0.04 nM, A/Victoria/3/75 = 0.26 ± 0.04 nM).

[e]low activity; NA activity was detected but not high enough to determine IC_{50}.

Table 3. Detection of single nucleotide polymorphisms (SNPs) in the NA of viruses recovered from lungs of mice (day 6 p.i. of passage 2) infected with A/WSN/33 (H1N1) and A/Victoria/3/75 (H3N2) viruses by automated (Sanger) sequencing, colony screening (cloning) and deep sequencing.

Virus / Regimen[a]	NA position[b]	Reference[c]	Sanger (%)	Cloning (%)	Deep sequencing (%)	Depth[d]
A/WSN/33 (H1N1)						
P2-ose/P1-ose 0.05 mg/kg	389	M	M	M/T (95:5)	M/T (99:1)	124898
	434	D	D	D/E (95:5)	D/E[e] (98:2)	77999
A/Victoria/3/75 (H3N2):						
P2-unt/P1-untreated	46	A	A	A/P (94:6)	A/P (94:6)	57911
	73	I	I	I/T (94:6)	I/T (96:4)	72738
	131	Q	Q	Q/H (88:12)	Q/H (96:4)	78897

	398	V	V	V/I (94:6)	V/I (93:7)	66338
P2-ose/P1-ose 0.05 mg/kg	200	N	N	N/D (89:11)	N/D (88:12)	81069
	222	I	I	I/**T** (84:**16**)	I/**T** (85:**15**)	78541
	398	V	V	V/I (95:5)	V/I (87:13)	56232
P2-ose/P1-ose 1 mg/kg	131	Q	Q	Q/H (94:6)	Q/H (98:2)	128656
	222	I	I/**T** (80/**20**)	I/**T** (78/**22**)	I/**T** (72/**28**)	167234
	398	V	V	V/I (94:6)	V/I (95:5)	104690

Note: data corresponds to viruses recovered from pooled lung homogenates on day 6 p.i. of the second passage (P2). Only treatment groups in which SNPs were detected are shown (deletions are not shown). The residue in **bold** confers reduced susceptibility to oseltamivir.

[a]P1; passage 1, P2; passage 2, unt; untreated, ose; oseltamivir.

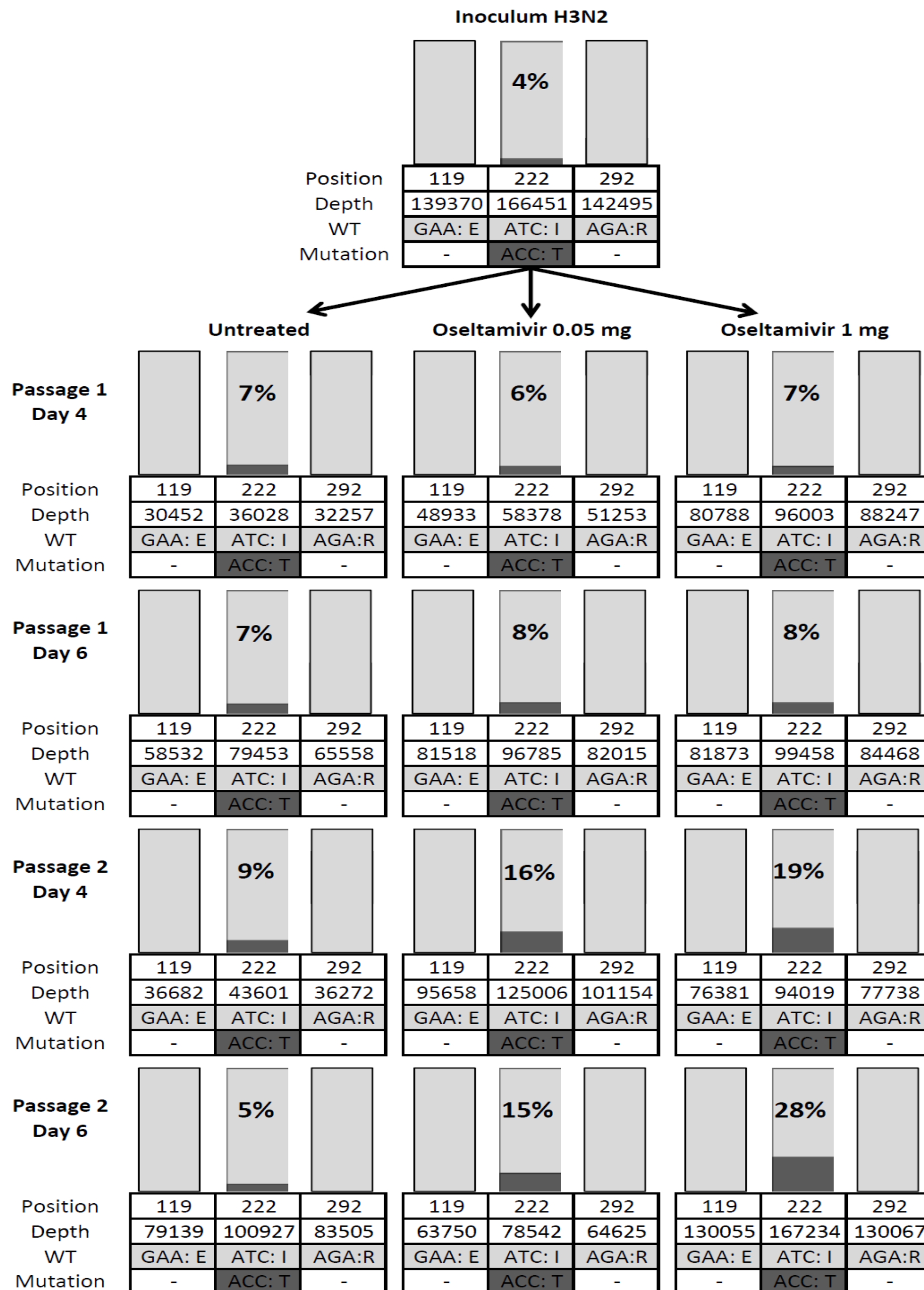
[b]N2 numbering.

[c]Reference; predominant residue found in these positions in the reference sequences.

[d]Mean number of reads that cover that position in the deep sequencing analysis.

[e]A D/V (93:7) substitution was also found at this position by deep sequencing but not by cloning in the P2-ose/P1-ose 1 mg/kg group.

Figure 1. Longitudinal analysis of viral population diversity by deep sequencing. Ratios of major and minor codons are represented for the hallmark NA positions associated with resistance to oseltamivir in N2 viruses (E119 and R292) as well as for residues with sustained significant (>1%) frequency variation over time (I222). Codons and single-letter amino acid codes are indicated below the position number.



Chapter VII: "Role of permissive neuraminidase mutations in influenza A/Brisbane/59/2007-like (H1N1) viruses"

7.1 Foreword

This chapter contains the text of the article **"Role of permissive neuraminidase mutations in influenza A/Brisbane/59/2007-like (H1N1) viruses"** written by Yacine Abed, Andrés Pizzorno, Xavier Bouhy, and Guy Boivin. In this article we sought to test the hypothesis whether the unexpected emergence and worldwide dissemination of the oseltamivir-resistant A/Brisbane/59/2007-like seasonal (H1N1) virus in 2008-09 was due to the presence of permissive NA mutations that did not affect NAI susceptibility but improved viral fitness of the H274Y (here referred as H275Y) mutant instead. YA, AP and GB conceived and designed the experiments; YA, AP and XB performed the experiments; AP and XB contributed reagents/materials/analysis tools; YA, AP and GB analyzed the data; YA, AP and GB wrote the article and responded to the reviewer's comments. The article is presented in its final version accepted for publication in **PLoS Pathogens** in 2011 (Dec;7(12):e1002431).

7.2 Article

Role of permissive neuraminidase mutations in influenza A/Brisbane/59/2007-like (H1N1) viruses.

Running title: Permissive NA mutations in NAI-resistant viruses

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ABSTRACT

Neuraminidase (NA) mutations conferring resistance to NA inhibitors were believed to compromise influenza virus fitness. Unexpectedly, an oseltamivir-resistant A/Brisbane/59/2007 (Bris07)-like H1N1 H275Y NA variant emerged in 2007 and completely replaced the wild-type (WT) strain in 2008-2009. The NA of such variant contained additional NA changes (R222Q, V234M and D344N) that potentially counteracted the detrimental effect of the H275Y mutation on viral fitness. Here, we rescued a recombinant Bris07-like WT virus and 4 NA mutants/revertants (H275Y, H275Y/Q222R, H275Y/M234V and H275Y/N344D) and characterized them *in vitro* and in ferrets. A fluorometric-based NA assay was used to determine Vmax and Km values. Replicative capacities were evaluated by yield assays in ST6Gal1-MDCK cells. Recombinant NA proteins were expressed in 293T cells and surface NA activity was determined. Infectivity and contact transmission experiments were evaluated for the WT, H275Y and H275Y/Q222R recombinants in ferrets. The H275Y mutation did not significantly alter Km and Vmax values compared to WT. The H275Y/N344D mutant had a reduced affinity (Km of 50 vs 12 µM) whereas the H275Y/M234V mutant had a reduced activity (22 vs 28 U/sec). In contrast, the H275Y/Q222R mutant showed a significant decrease of both affinity (40 µM) and activity (7 U/sec). The WT, H275Y, H275Y/M234V and H275Y/N344D recombinants had comparable replicative capacities contrasting with H275Y/Q222R mutant whose viral titers were significantly reduced. All studied mutations reduced the cell surface NA activity compared to WT with the maximum reduction being obtained for the H275Y/Q222R mutant. Comparable infectivity and transmissibility were seen between the WT and the H275Y mutant in ferrets whereas the H275Y/Q222R mutant was associated with significantly lower lung viral titers. In conclusion, the Q222R reversion mutation compromised Bris07-like H1N1 virus *in vitro* and *in vivo*. Thus, the R222Q NA mutation present in the WT virus may have facilitated the emergence of NAI-resistant Bris07 variants.

INTRODUCTION

Influenza viruses are respiratory pathogens associated with significant public health consequences. Each year, influenza epidemics can be responsible for significant morbidity in the general population and excess mortality in elderly patients and individuals with chronic underlying conditions. Influenza A viruses of the H1N1 subtype have been associated with seasonal influenza epidemics for many decades and, in presence of immunological pressure, such viruses continue to evolve through genetic variability which is mainly confined to virus segments encoding surface glycoproteins i.e., the hemagglutinin (HA) and neuraminidase (NA) [1]. Consequently, viral strains to be used in annual influenza vaccines should be regularly updated to ensure optimal protection. Besides vaccines, neuraminidase inhibitors (NAI) including inhaled zanamivir, oral oseltamivir and intravenous peramivir provide an important additional measure for the control of influenza infections [2]. These antivirals target the active center of the influenza NA molecule, which is constituted by 8 functional (R-118, D-151, R-152, R-224, E-276, R-292, R-371, and Y-406; N2 numbering) and 11 framework (E-119, R-156, W-178, S-179, D-198, I-222, E-227, H-274, E-277, N-294, and E-425; N2 numbering) residues that are largely conserved among influenza A and B viruses [3]. However, the emergence of NAI-resistant viruses, as a result of drug use or due to circulation of natural variants, may compromise the clinical utility of this class of anti-influenza agents.

The H275Y (H274Y in N2 numbering) NA mutation conferring resistance to oseltamivir and peramivir has been detected with increasing frequency in seasonal A/H1N1 viruses since 2007 to the extent that almost all characterized A/Brisbane/59/2007-like (Bris07) (H1N1) influenza strains that circulated worldwide during the 2008-09 season were H275Y variants [4,5]. Interestingly, this drug-resistant strain seemed to have emerged independently of NAI use [6,7]. The rapid dissemination of the H275Y Bris07 variants in the absence of antiviral pressure suggests that the H275Y NA mutation may not compromise viral fitness and transmissibility in this recent H1N1 viral background. This contrasts with previous studies that analyzed the role of the H275Y mutation using older (A/Texas/36/91 [8] and A/New Caledonia/99/01 [9]) drug-selected H1N1 variants. Recent reports by our group and others have confirmed the differential impact of the H275Y mutation on viral fitness and enzymatic properties in the context of old and recent influenza H1N1 isolates [10,11]. In an attempt to provide a molecular explanation for this observation, previous authors suggested that secondary NA mutations such as D344N that emerged in H1N1 variants isolated after the 2006-07

season were associated with higher NA activity and affinity and could have facilitated the emergence of the H275Y mutation [11,12]. Such drug-resistant mutants may have a better HA-NA balance than the susceptible viruses and indeed completely replaced them in a short period of time. In addition, Bloom and colleagues recently described two other secondary NA mutations at codons 222 and 234 that may have counteracted the compromising impact of the H275Y mutation [13]. In that study, the V234M and R222Q mutations were shown to restore the viral fitness of an A/New Caledonia/20/99 H1N1 variant containing the H275Y mutation [13].

To further investigate which secondary NA mutations may have facilitated the introduction of the H275Y mutation in contemporarily seasonal H1N1 viruses and allowed their dissemination, we developed a reverse genetics system using a clinical Bris07 (H1N1) isolate as genetic background and evaluated the impact of the H275Y oseltamivir resistance mutation as well as several potential compensatory NA mutations on enzyme activity, viral fitness and transmissibility.

RESULTS

In the present study, five recombinant Bris07 influenza viruses were generated i.e., the WT virus (containing the putative permissive mutations) that briefly circulated during the 2007-08 season, the single H275Y oseltamivir-resistant variant and three double mutants containing the H275Y mutation as well as reversion of potential permissive mutations (H275Y/Q222R, H275Y/M234V and H275Y/N344D). NA enzymatic properties using equivalent titers of recombinants were first analyzed with determination of relative NA enzymatic activity (Vmax values), which reflects the total NA activity per virion, and Km values, which reflect the affinity for the substrate. As shown in Table 1, the single H275Y mutation had no significant impact on NA affinity and activity compared to the WT virus in the context of the Bris07 background. By contrast, the double H275Y/Q222R mutation was associated with a significant reduction of both NA affinity (Km of 40.31 vs 11.95 µM, $P<0.001$) and relative NA activity (7.01 vs 28.19 U/sec, $P<0.001$) compared to the WT (Table 1 and Fig. 1). The H275Y/M234V mutant had a Km value comparable to that of the WT, whereas its relative NA activity was significantly reduced (Vmax of 21.89 vs 28.19 U/sec, $P<0.05$). The H275Y/N344D mutant showed a significantly reduced affinity (Km of 50.77 vs 11.95 µM, $P<0.001$) with no change in NA activity compared to the WT. When comparing the double mutants to the single H275Y mutant, the Km

values were significantly increased for the H275Y/Q222R and H275Y/N344D mutants (P<0.001) whereas only the double H275Y/Q222R mutant had a significantly lower relative NA activity (P<0.001).

Using recombinant NA proteins expressed in 293T cells, we further investigated the impact of NA mutations on the amount of NA activity at the cell surface. As shown in Fig. 2, all studied mutations were associated with a significant reduction of total surface NA activity compared to the WT with relative total surface activities of 66% (P<0.01), 9.72% (P<0.001), 32.07% (P<0.001) and 54.89% (P<0.01) for the H275Y, H275Y/Q222R, H275Y/M234V and H275Y/N344D mutant proteins, respectively. When compared to the single H275Y mutant, H275Y/Q222R (P<0.001), H275Y/M234V (P<0.001) and H275Y/N344D (P<0.05) double mutants also had significantly reduced surface NA activities. The differences observed in total surface NA activity between the different recombinant NA proteins may be due to a decreased number of NA molecules that reached the cell surface or to less activity per enzyme.

We next determined the phenotype of resistance to NAIs for the 5 recombinant viruses. As expected, the presence of the H275Y mutation was associated with resistance to oseltamivir (mean fold increase of 2627 in IC_{50} values) and peramivir (mean fold increase of 998) with no impact on zanamivir susceptibility (Table 2). Interestingly, comparison of the levels of resistance for the double recombinant mutants versus the single H275Y mutant revealed a significant reduction in the level of resistance to peramivir for the double H275Y/Q222R mutant (IC_{50} of 35.25 nM vs 59.85 nM, P<0.01). A similar trend was observed for oseltamivir (IC_{50} of 651.86 nM vs 1024.54 nM) although, in this case, the difference between IC_{50} values was not statistically significant.

Viral fitness of recombinant A/Brisbane/59/2007-like viruses was assessed *in vitro* using ST6Gal1-MDCK cells. The double H275Y/Q222R mutant produced viral plaques with a significantly reduced area compared to the recombinant WT (0.13 mm^2 vs 0.53 mm^2, P<0.001) whereas the remaining recombinants generated plaques of comparable sizes (Table 1). Of note, the reduction in plaque size for the H275Y/Q222R mutant was also significant compared to that of the single H275Y mutant (P<0.001). In replication kinetics experiments, the peak viral titers for all recombinants were obtained at 36 h post-infection (PI) with viral titers ranging from 5.6 x 10^6 PFU/ml (H275Y/Q222R) to 5.3 x 10^7 PFU/ml (WT) (Fig. 3). The WT, the single (H275Y) and the double (H275Y/N344D) mutants had comparable viral titers at all time points. By contrast, and in accordance with plaque size data,

the double H275Y/Q222R mutant was associated with a significant reduction in viral titers at 36 h ($P<0.001$) and 48 h ($P<0.05$) PI compared to the WT (Fig. 3). There was also a significant reduction in the viral titer obtained at 36 h PI for the double H275Y/M234V mutant compared to the WT ($P<0.001$). When compared to the single (H275Y) mutant, viral titers of the double H275Y/Q222R and H275Y/M234V mutants were significantly lower at 36 h ($P<0.001$).

Intranasal inoculation of ferrets with the WT and two mutant (H275Y and H275Y/Q222R) Bris07 recombinant viruses resulted in a febrile response that peaked on day 2 PI (Fig. 4A). The area under the curve (AUC) of temperatures between days 0 and 6 PI was similar for the 3 groups of ferrets i.e. 6.81 ± 1.19 for the WT virus, 5.99 ± 1.9 for the H275Y/Q222R mutant and 7.26 ± 0.55 for the H275Y mutant. There was no significant difference in body weight between the three groups of animals at any time points (data not shown). As shown in Fig. 5A, mean viral titers in nasal wash samples collected on day 2 PI from ferrets infected with the recombinant WT and the single H275Y mutant were comparable ($4 \times 10^5 \pm 2.9 \times 10^4$ PFU/ml for the WT and $2.6 \times 10^5 \pm 8.7 \times 10^4$ PFU/ml for the H275Y mutant) whereas the H275Y/Q222R mutant had a reduced mean viral titer ($4.6 \times 10^4 \pm 4.2 \times 10^3$ PFU/ml; $P<0.05$ vs WT). Similarly, mean viral titers in nasal wash samples of ferrets infected with the H275Y/Q222R were significantly lower than those of the H275Y mutant ($P<0.05$) and WT virus ($P<0.01$) on day 4 PI ($3.4 \times 10^3 \pm 1.7 \times 10^3$, $1.1 \times 10^4 \pm 6.7 \times 10^3$ and $1.5 \times 10^4 \pm 9.6 \times 10^2$ PFU/ml, respectively). On the other hand, the three recombinants were associated with comparable mean viral titers on day 6 PI ($2 \times 10^2 \pm 4.6 \times 10^1$ PFU/ml for the WT, $1.1 \times 10^2 \pm 5.8 \times 10^1$ PFU/ml for the H275Y/Q222R and $1.3 \times 10^2 \pm 8.1 \times 10^1$ PFU/ml for the H275Y).

All contact ferrets seroconverted for A/Brisbane/59/2007 when tested 14 days after contact, with geometrical mean hemagglutination inhibition (HAI) titers of 160 ± 33, 145 ± 119 and 95 ± 55 for the WT, H275Y and H275Y/Q222R recombinant viruses, respectively. A febrile response could be observed on days 4 and 5 in the WT and the H275Y groups, respectively, but not in the H275Y/Q222R group (Fig. 4B). The AUC of temperatures between days 2 and 6 PI was similar between groups of ferrets infected with the recombinant WT (5.29 ± 0.34) and its H275Y variant (4.54 ± 0.19) whereas the AUC of the H275Y/Q222R group was significantly lower than that of the WT group (4.09 ± 0.96; $P<0.05$). Viral titers in nasal wash samples collected on days 2, 4 and 6 PI are shown in Fig. 5B. Only the WT virus was detected on day 2 PI. Mean viral titers were comparable for the H275Y mutant and the WT virus on days 4 and 6 PI. In contrast, the

H275Y/Q222R mutant was associated with significantly lower mean viral titers compared to WT on both day 4 ($2.7 \times 10^2 \pm 1.2 \times 10^2$ vs $1.2 \times 10^4 \pm 3.5 \times 10^3$ PFU/ml, $P<0.01$) and day 6 PI ($3.8 \times 10^3 \pm 2.1 \times 10^3$ vs $1.2 \times 10^4 \pm 2.5 \times 10^3$ PFU/ml, $P<0.01$).

DISCUSSION

In this study, we used recombinant viruses derived from a clinical WT Bris07 strain to demonstrate using both *in vitro* and ferret experiments that the R222Q NA mutation was the main but possibly not the only permissive mutation that allowed the widespread dissemination of the oseltamivir-resistant H275Y mutant during the 2007-09 influenza seasons. Although such mutant seems to have disappeared since the emergence of the pandemic H1N1 virus in April 2009, understanding the mechanisms leading to the transmission of this unique virus is of great importance and could have an impact on the future use of NAIs.

The influenza NA protein plays a major role during the viral replication cycle. Its sialidase activity promotes virion release by removing sialic residues from viral glycoproteins and infected cells [14]. The NA enzyme also mediates virus penetration in the mucin layer of the respiratory tract, facilitating virus spread [15]. Importantly, the catalytic site of the NA enzyme has been shown to be conserved in all influenza A subtypes and influenza B viruses [3]. Therefore, the influenza NA protein has been considered as a suitable target for designing anti-influenza agents for both prophylactic and therapeutic purposes. Besides its functional role, the NA protein is a major structural surface glycoprotein that is exposed to the host immune pressure [14]. The NA gene, like the HA one, is therefore subject to more genetic variations than the rest of the influenza genome. Consequently, some amino acid (a.a.) changes, part of antigenic sites of the NA protein, may significantly contribute to the emergence of drifted variants, whereas certain substitutions located in or near the catalytic site may also affect the NA enzyme properties. For instance, Hensley and colleagues have recently identified NA mutations conferring resistance to zanamivir in variants of an influenza A/Puerto Rico/8/1934 H1N1 virus that was subjected to anti-HA monoclonal antibodies pressure [16].

In this study, we focused on a.a. changes that occurred in the NA protein during the evolution of recent seasonal influenza H1N1 viruses and that may have been involved in the development and dissemination of resistance to NAIs. These changes included the well-known framework H275Y mutation, responsible for the resistance phenotype to oseltamivir and peramivir, as well as other substitutions (V234M, R222Q and D344N) that may have contributed to the emergence and dissemination of resistance by acting as permissive/compensatory mutations.

Phylogenetic analyses previously demonstrated that the V234M mutation was already present in oseltamivir-susceptible A/Solomon Islands/3/2006 (SI06) viruses [13]. In another report, NA enzyme properties of SI06 viruses were found to be similar to those of older oseltamivir-susceptible strains such as A/New Caledonia/99/2001 in terms of relative NA activity (Vmax) and affinity (Km) [11]. By contrast, the appearance of the R222Q and D344N mutations in H1N1 viruses isolated after 2007 was associated with a significant increase in NA affinity (decreased Km values) in both 275H and 275Y strains [11]. In accordance with these observations, we demonstrated a sharp impact for the Q222R and N344D reversion mutations on Km values using our Bris07 recombinants (Table 1). Besides its effect on NA affinity, the Q222R reversion mutation was also associated with a significant decrease in relative NA activity (Table 1 and Fig. 1) and total NA activity that was expressed on the cell surface (Fig. 2), in line with previously-reported results in another viral background [13]. As a result, the H275Y/Q222R mutant virus was significantly compromised *in vitro* based on plaque size and replication kinetics patterns. Such decreased viral replication of the H275Y/Q222R mutant was also evident *in vivo*, resulting in lower viral titers in nasal wash samples and an absence of febrile response in contact ferrets. However, the H275Y/Q222R mutant was transmitted to all naïve ferrets by direct contact meaning that the combination of several permissive NA mutations and/or mutations elsewhere in the viral genome may be necessary to recapitulate the epidemiological observations showing increased transmission of the oseltamivir-resistant Bris07 virus. Also, it should be noted that naïve (non-immune) ferrets may not completely capture the fitness of Bris07 in humans with pre-existing immunity. Alternatively, the Q222R mutation could affect airborne transmission which has not been evaluated in our study. Of note, possibly due to the lower affinity of Q222R for MUNANA, less NAIs were required for competitive inhibition of the H275Y/Q222R mutant compared to the H275Y mutant. Residue 222 is located in the vicinity of the catalytic site of the N1 enzyme based on 3-D structure analysis [17]. Thus, substitution of a charged (R) by an uncharged (Q) a.a. at codon 222 may be the main change that dramatically altered the NA

enzyme properties of recent seasonal H1N1 viruses. Of interest, only one NA substitution (R194G) was sufficient to restore the viral fitness of an influenza A/WSN/33 (H1N1) virus containing the compromising H275Y NA mutation [13].

In addition to the R222Q mutation, a permissive role was also suggested for V234M and D344N substitutions [11,13]. Interestingly, in a recent report on the evolution of influenza NA genes, positive epistasis (i.e. combination of mutations that are substantially more beneficial than single mutations alone) was detected in pairs of codons within the NA gene of the N1 subtype including 275-222, 275-234, and 275-344 [18]. In our study, although the M234V and N344D reversions were associated with decreased relative NA activity and affinity, respectively (Table 1 and Fig. 1), none of these mutations significantly altered the viral fitness *in vitro*. Nevertheless, a possible synergy between these mutations and Q222R cannot be completely excluded.

Our study revealed that the H275Y NA mutation was not deleterious to fitness in the Bris07 genetic context in contrast to older H1N1 strains. However, this mutant did not have a replicative advantage compared to the WT as suggested by epidemiological studies. Indeed, the recombinant WT virus and its H275Y variant demonstrated similar replication kinetics during *in vitro* experiments. In addition, these recombinants had comparable infectivity and contact transmissibility in ferrets. Thus, the presence of the permissive mutations (R222Q, V234M and D344N) in the NA protein of our WT strain was apparently not sufficient to alter the viral fitness to the level that a compensatory change, such as the H275Y mutation, would be necessary. Therefore, we believe that changes in the NA gene alone may not provide a complete explanation for the emergence and spread of the oseltamivir-resistant H275Y Bris07 variant. Other changes in the genome might have been involved in this event. For instance, Yang and colleagues recently demonstrated that the dominant H275Y variant that emerged in Taiwan in 2007-2008 was a result of intra-subtypic reassortments between HA, NA, PB2 and PA genes from one clade (clade 2B) and the remaining 4 genes from another one (clade 1) [19]. Furthermore, the H275Y NA substitution and other changes in NA, HA, PB1 and PB2 proteins occurred in that background [19]. Thus, it would be also interesting to assess the effect of HA and particularly polymerase mutations that differed between WT and H275Y mutant clinical Bris07 isolates on replicative capacities and transmissibility.

Despite the fact that the secondary mutations described here were not investigated individually but in conjunction with H275Y, our study provides a comprehensive analysis of relevant

permissive NA mutations in the contemporarily seasonal H1N1 background. This included *in vitro* characterization, assessment of viral fitness and contact transmission in ferrets as well as NA enzyme properties of recombinant mutants. In particular, our investigation clearly demonstrated the positive impact of one specific NA substitution (i.e. R222Q) in conjunction with the oseltamivir resistance H275Y mutation on enzymatic properties and viral fitness of the Bris07 H1N1 strain. Noteworthy, our results suggest that total NA activity was more likely predictive of *in vitro* and *in vivo* viral fitness than the enzyme affinity (Km) parameter. Whether the Q222R mutation is also deleterious in the absence of H275Y was not investigated here; however, in a previous work, influenza A/Paris/497/2007 (222Q/275H) and A/Solomon Islands/3/2006 (222R/275H) seasonal H1N1 isolates grew to comparable titers in *in vitro* kinetics experiments [11]. Although clinical 2009 pandemic H1N1 variants containing such permissive mutations have not been reported, a computational approach had recently led to the identification of R257K and T289M as potential secondary mutations in that context [20]. Thus, monitoring for resistance in influenza viruses should take into consideration not only NA resistance-mutations themselves but also permissive/secondary ones as the latter may significantly affect the clinical and epidemiological impacts of seasonal or pandemic influenza viruses.

MATERIALS AND METHODS

Ethics Statement. All procedures were approved by the Institutional Animal Care Committee at Laval University according to the guidelines of the Canadian Council on Animal Care.

Rescue of recombinant viruses. Reverse transcription-PCR using universal influenza primers [21] was used to amplify the eight genomic segments of an oseltamivir-susceptible A/Quebec/15230/08 (H1N1) isolate whose HA and NA genes shared respectively 99.53% and 99.71% nucleotide identity with those of the influenza A/Brisbane/59/2007 vaccine strain [10]. All segments were cloned into the pJET plasmid (Fermentas, Burlington, ON, Canada) and sequenced. Sequence analysis confirmed the presence of histidine (H), glutamine (Q), methionine (M) and asparagine (N) residues at residues 275, 222, 234 and 344 (N1 numbering), respectively, of the NA protein. The PB1, PB2 and PA segments were sub-cloned into pLLBG whereas the HA, NA, NP, M1/M2 and NS1/NS2 segments were sub-cloned into pLLBA bidirectional expression/translation

vectors as described [22]. The pLLBA plasmid containing the NA gene was used for the introduction of the H275Y mutation using appropriate primers and the QuikChangeTM Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The resulting pLLB-NA275Y mutant plasmid was then used for reverting potential compensatory mutations (Q222R, M234V or N344D) as described above. All recombinant plasmids were sequenced to confirm the absence of undesired mutations. The eight bidirectional plasmids were cotransfected into 293T human embryonic kidney cells using the LipofectamineTM 2000 reagent (Invitrogen, Carlsbad, CA) as previously described [23]. Supernatants were collected 72 h post-transfection and used to inoculate ST6Gal1-MDCK cells kindly provided by Dr. Y. Kawaoka, University of Wisconsin, Madison, WI). The recombinant wild-type (WT) and H275Y, H275Y/Q222R, H275Y/M234V and H275Y/N344D mutant viruses were subsequently sequenced and titrated by standard plaque assays in ST6Gal1-MDCK cells.

NA enzyme kinetics assays. A fluorometric based assay using MUNANA (Methylumbelliferyl-N-acetylneuraminic acid) (Sigma, St-Louis, MO) as substrate was performed to determine total NA enzymatic activity per infectious virus [24]. Briefly, recombinant viruses were standardized to an equivalent dose of 106 plaque forming-units (PFU)/ml and incubated at 37°C in 50-µl reactions with different concentrations of MUNANA. The final concentration of the substrate ranged from 0 to 3000 µM. Fluorescence was monitored every 90 s for 53 min (35 measures). The Michaelis-Menten constant (Km) and the relative NA activity (Vmax) were calculated with the Prism software (GraphPad, version 5), by fitting the data to the Michaelis-Menten equation using nonlinear regression [25].

Cell surface NA activity. Recombinant NA plasmids and pCAGGS-PA, -PB1, -PB2 and -NP plasmids were used to co-transfect 293T cells in order to express recombinant NA enzymes [26]. Twenty-four hours after transfection, the cells were briefly treated with trypsin-EDTA and neutralized by the addition of serum followed by centrifugation at 3000 RPM for 5 min. After washing twice with PBS, the cells were resuspended in a non-lysing buffer (15 mM MOPS, 145 mM sodium chloride, 2.7 mM potassium chloride and 4 mM calcium chloride, adjusted to pH 7.4) and used in an NA assay using the MUNANA substrate [13].

NA inhibition assays. The drug resistance phenotype was determined by NA inhibition assays using the MUNANA substrate as previously described [26], with minor modifications. Briefly, recombinant viruses were standardized to a NA activity ten-fold higher than that of the background

and then incubated with serial three-fold dilutions of the drugs (final concentrations ranging from 0 to 1800 nM), including oseltamivir carboxylate (Hoffmann-La Roche, Basel, Switzerland), zanamivir (GlaxoSmithKline, Stevenage, UK) and peramivir (BioCryst, Birmingham, AL). The 50% inhibitory concentration (IC_{50}) was determined from the dose-response curve.

***In vitro* replication kinetics experiments.** Replicative capacities of the recombinant viruses were evaluated by infecting ST6Gal1-MDCK cells with a multiplicity of infection (MOI) of 0.001 plaque-forming units (PFUs)/cell. Supernatants were collected every 12 h until 60 h PI and titrated by plaque assays. The mean viral plaque area of recombinant viruses was determined from a minimum of 16 plaques obtained after 60 h of incubation under agarose overlay using the ImageJ software (version 1.41), developed by Wayne Rasband of the National Institutes of Health as previously described [25].

Ferret studies. Groups of 4 seronegative (900-1500 g) male ferrets (Triple F Farms, Sayre, PA) were lightly anesthetised by isoflurane and received an intranasal instillation of 1.25×10^5 PFUs of the recombinant Bris07-like WT, H275Y or H275Y/Q222R variants. Temperature of ferrets was measured by rectal thermometers every day until day 10 PI. Ferrets were weighed daily and nasal wash samples were collected from animals on days 2, 4 and 6 PI. Virus titers from nasal wash samples were determined by plaque assays using ST6Gal1-MDCK cells. Serum samples were collected from each ferret before intranasal infection and on day 14 PI to evaluate specific antibody levels against the seasonal Bris07 strain using standard HAI assays. To evaluate contact-transmissibility, inoculated-contact animal pairs were established by placing a naïve ferret into each cage 24 h after inoculation of the index ferret [27]. Contact animals were monitored for clinical signs and nasal wash and serum samples were collected as described above for determination of viral titers and serological status, respectively.

Statistical analyses. NA kinetic parameters (Km and Vmax values), NAI IC_{50} values and viral titers *in vitro* and in nasal washes of ferrets were compared by one-way ANOVA analysis of variance, with the Tukey's multiple comparison post test. The amount of NA activity on the cell surface and plaque sizes of the recombinants were compared to those of the WT virus and/or the H275Y mutant by the use of unpaired two-tailed t tests.

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Table 1. Neuraminidase enzymatic properties and plaque areas of recombinant A/Brisbane/59/2007-like (H1N1) viruses.

Recombinants	Km[a] (μM)	Relative NA activity (Vmax)[a] (U/sec)	Vmax ratio vs WT	Plaque area (mm^2)[b]
WT	11.95 ± 2.4	28.19 ± 0.74	1.00	0.53 ± 0.17
H275Y	16.92 ± 2.25	23.81 ± 1.95	0.84	0.50 ± 0.16
H275Y/Q222R	40.31 ± 5.5 ***	7.01 ± 0.11***	0.25	0.13 ± 0.06 ***
H275Y/M234V	18.18 ± 1.2	21.89 ±1.24*	0.78	0.49 ± 0.13
H275Y/N344D	50.77 ± 1.0 ***	24.96 ± 2.48	0.89	0.50 ± 0.15

[a]Values indicate mean *Km* and relative NA activity (*Vmax)* values of a representative experiment performed in triplicate ± standard deviations (SD).

[b]Values indicate mean plaque area (N = 16) ± SD. *P<0.05, ***P<0.001 compared to WT.

Table 2. Susceptibility profiles of recombinant A/Brisbane/59/2007-like (H1N1) viruses against neuraminidase (NA) inhibitors as assessed by MUNANA NA inhibition assays.

Recombinants	Oseltamivir[a] (nM)	Zanamivir[a] (nM)	Peramivir[a] (nM)
WT	0.39 ± 0.02	0.18 ± 0.03	0.06 ± 0.01
H275Y	1024.54 ± 114.10	0.27 ± 0.06	59.85 ± 2.42
H275Y/Q222R	651.86 ± 116.88	0.18 ± 0.04	35.25 ± 2.11**
H275Y/M234V	1038.56 ± 116.09	0.20 ± 0.01	47.87 ± 1.33
H275Y/N344D	735.07 ± 67.91	0.28 ± 0.03	47.15 ± 3.76

[a] Values indicated mean IC_{50} values of three experiments ± standard deviations.

***$P<0.01$* compared to the single H275Y mutant.

Figure 1.

Neuraminidase (NA) enzyme kinetics of recombinant A/Brisbane/59/2007-like (H1N1) viruses. The rate of substrate conversion velocity (V_0) by NA enzymes from a standardized dose of 10^6 PFU/ml of recombinant virus was determined. The fluorogenic substrate (MUNANA) was used at final concentrations of 0 to 3000 µM. Fluorescence was measured every 90 sec for 53 min at 37 °C using excitation and emission wavelengths of 355 and 460 nm, respectively. The data of one representative experiment performed in triplicate is shown.

Figure 2.

Surface activity of recombinant A/Brisbane/59/2007-like (H1N1) neuraminidase proteins. 293T cells were transfected with pCAGGS-PA, -PB1, -PB2, and -NP plasmids in addition to plasmids expressing the WT or mutant A/Brisbane/59/2007-like neuraminidases (NA) proteins. At 24 h post-transfection, cells were treated with a non-lysing buffer and surface NA activity was measured by using the fluorogenic substrate (MUNANA). Percent surface NA activities were determined in triplicate experiments ± standard deviations. **$P<0.01$ and ***$P<0.001$ compared to the WT surface NA activity.

Figure 3.

Replication kinetics of recombinant A/Brisbane/59/2007-like viruses *in vitro*. Confluent ST6Gal1-MDCK cells were infected with recombinant viruses at a multiplicity of infection (MOI) of 0.001 PFU/cell. Supernatants were harvested at 12 h, 24 h, 36 h, 48 h and 60 h post-infection and titrated by standard plaque assays. The mean values for three experiments with standard deviations are presented. *$P<0.05$ and ***$P<0.001$ for differences in viral titers when compared to the recombinant WT virus.

Figure 4.

Body temperatures of infected and contact ferrets. Body temperatures were recorded by rectal thermometer during 10 days post-inoculation in groups of 4 index ferrets infected with 1.25 x 10^5 PFU of recombinant A/Brisbane/59/2007-like wild-type (WT) virus as well as H275Y and H275Y/Q222R mutants (A) and in groups of 4 naïve ferrets that were placed in direct contact with index ferrets 24 h later (B).

Figure 5.

Mean viral titers in nasal wash samples of infected and contact ferrets. Mean viral titers ± standard deviations were determined in nasal washes by using standard plaque assays in groups of 4 index ferrets infected with 1.25 x 10^5 PFU of recombinant A/Brisbane/59/2007-like wild-type (WT) virus as well as H275Y and H275Y/Q222R mutants (A) and in groups of 4 naïve ferrets that were placed in direct contact with index ferrets 24 h later (B).*$P<0.05$ and **$P<0.01$ for differences in viral titers when compared to the recombinant WT virus.

Figure 1.

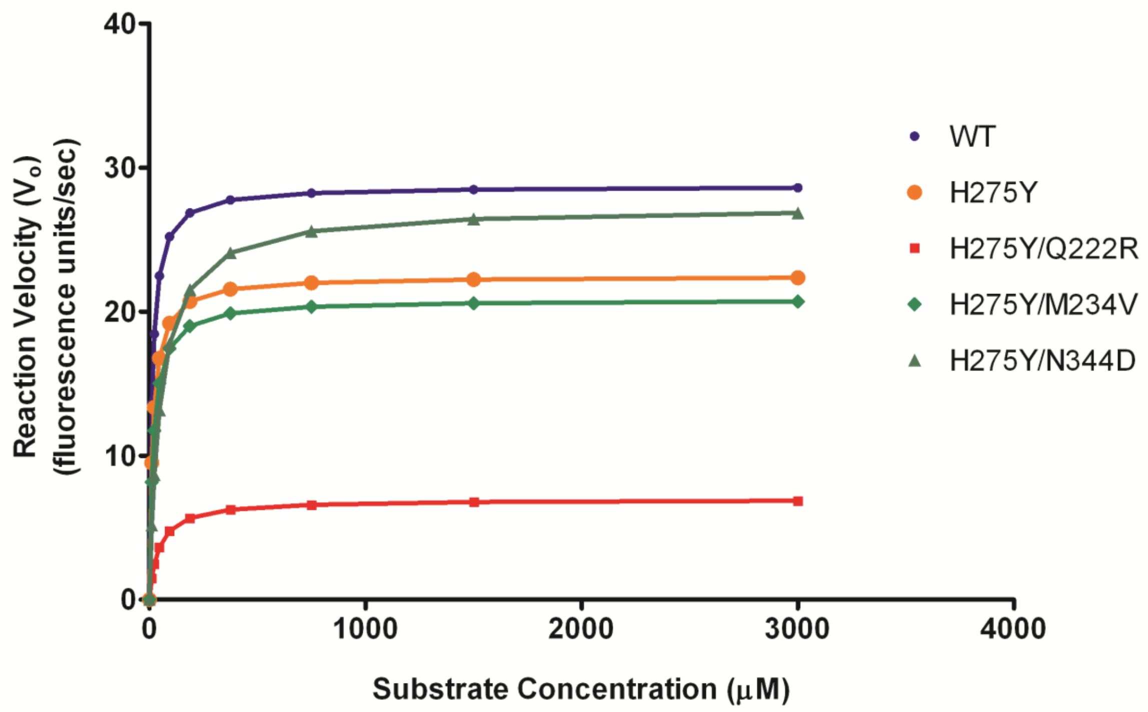
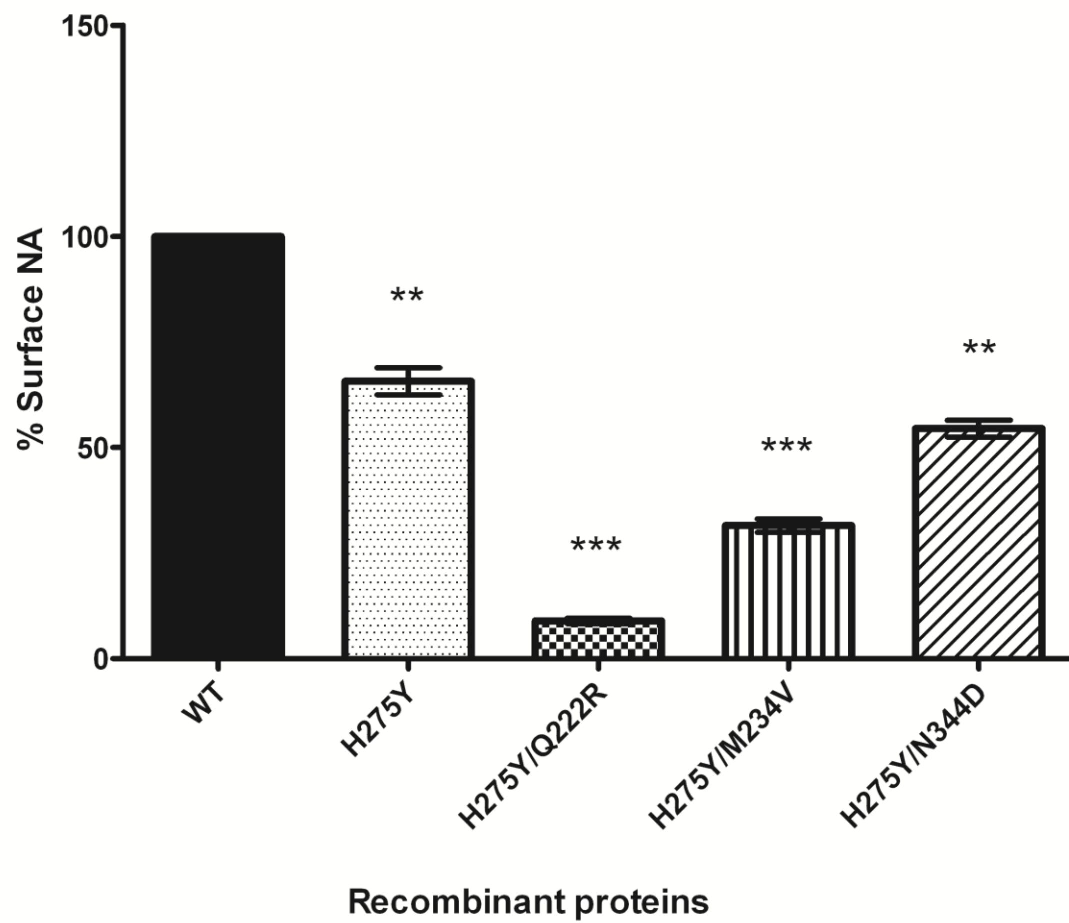


Figure 2.



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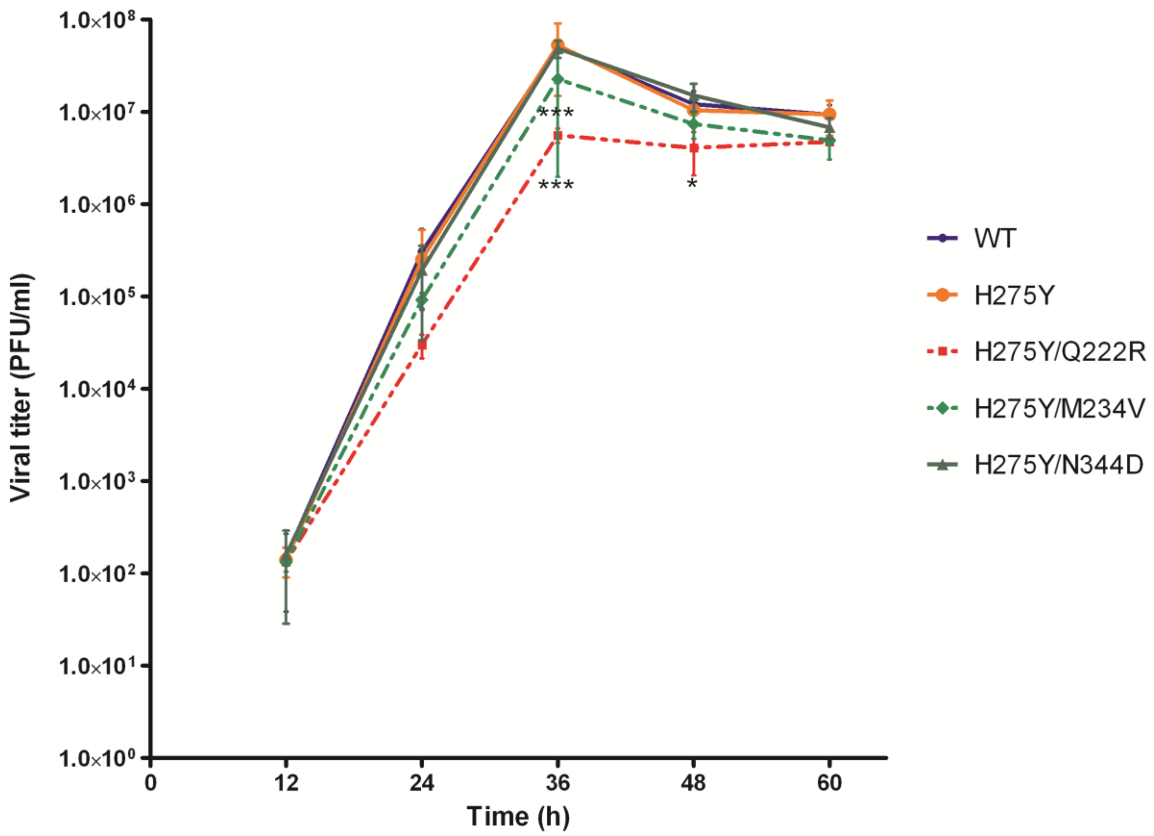

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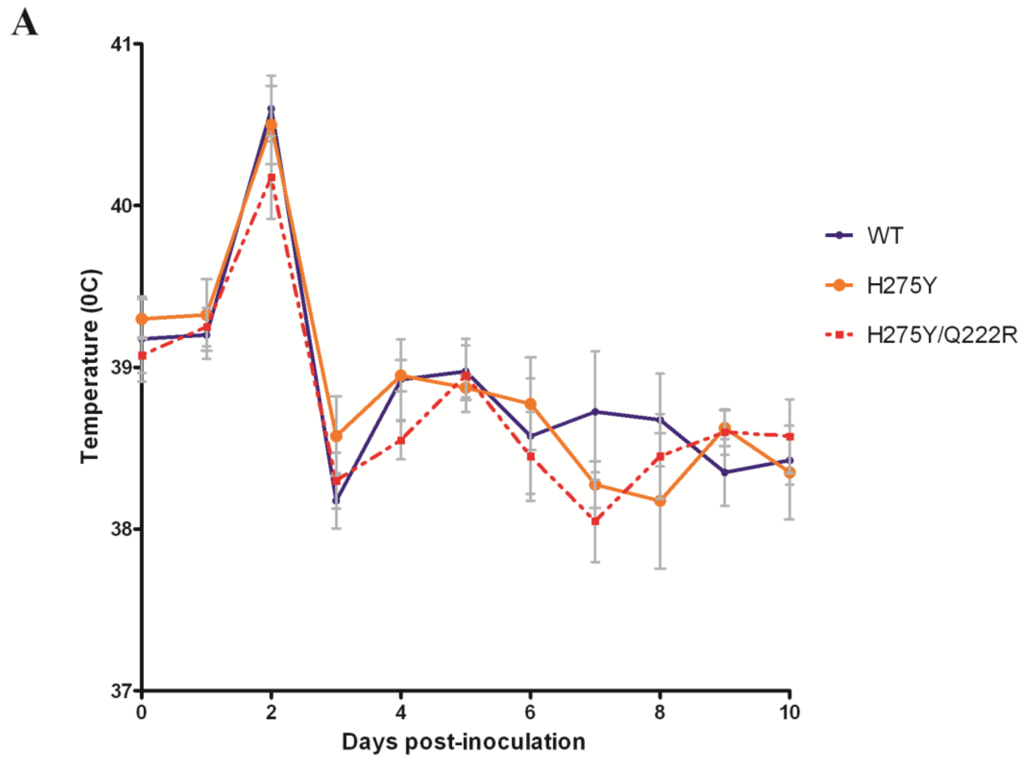


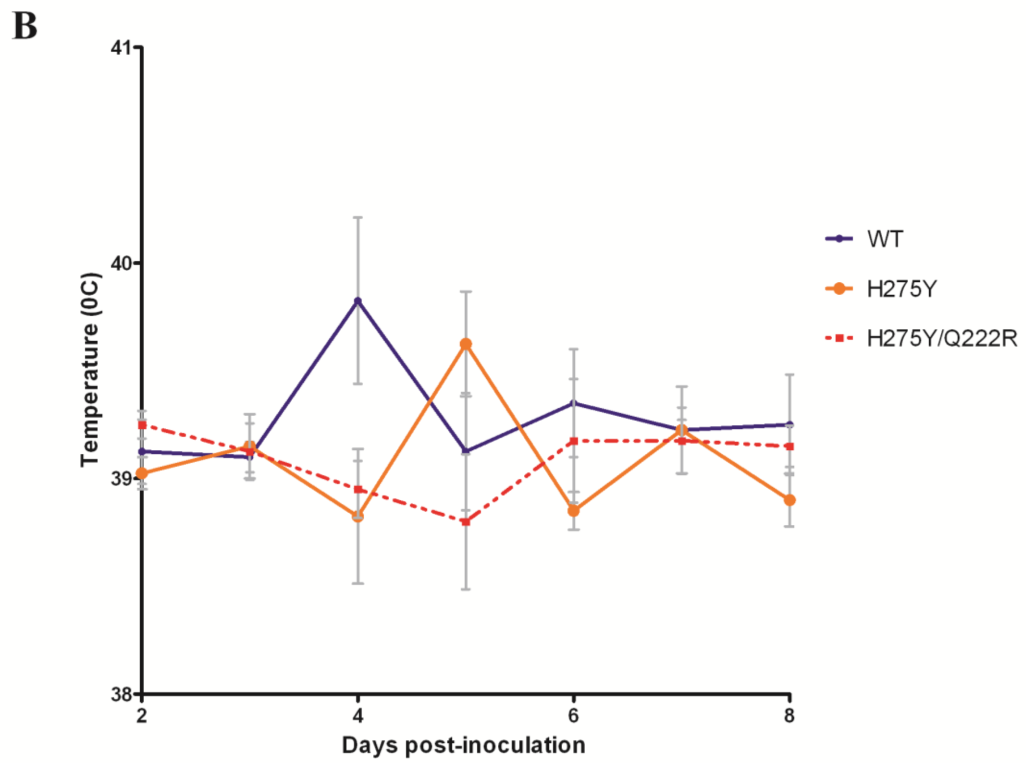
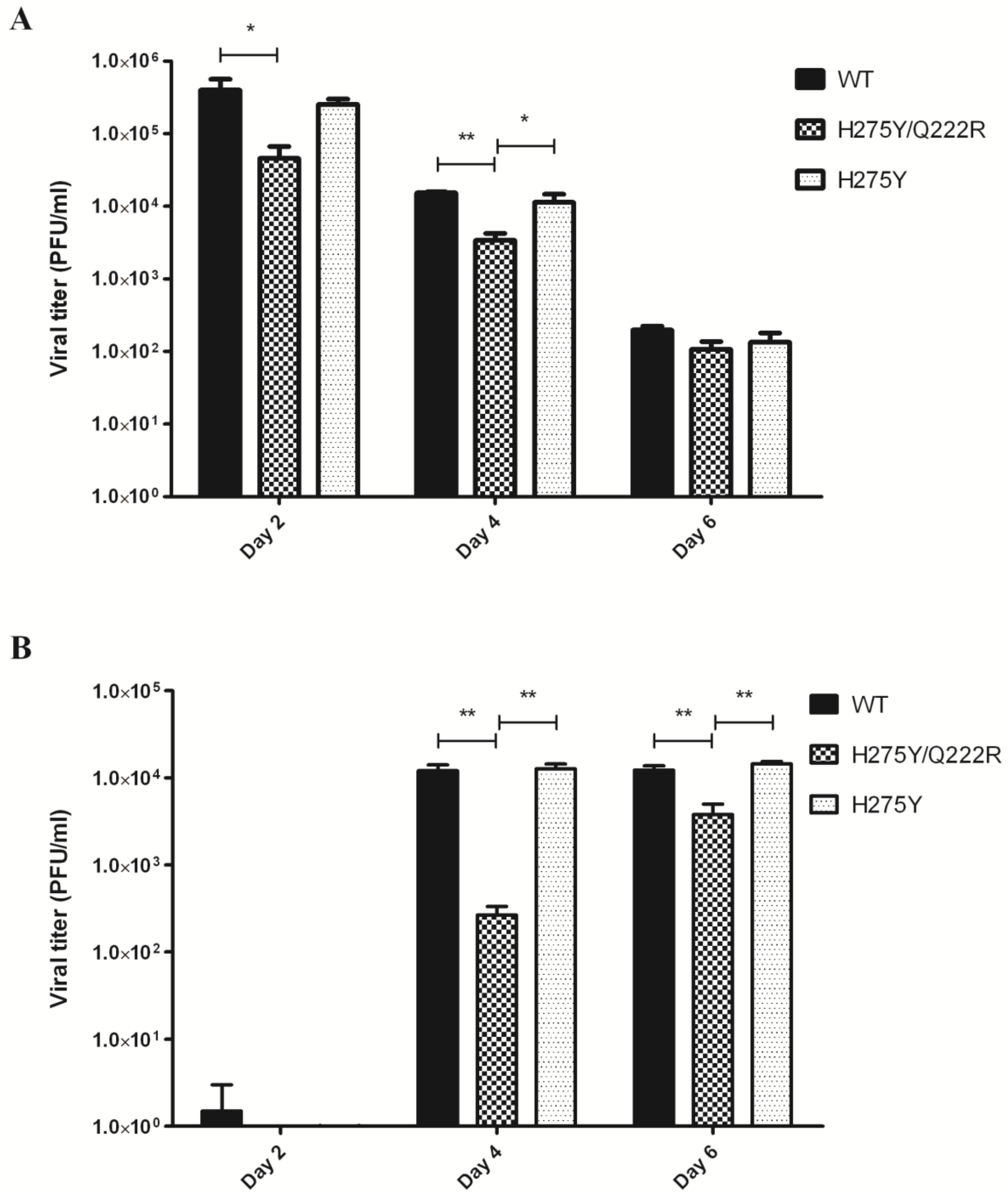


Figure 5.



Chapter VIII: "Impact of potential permissive neuraminidase mutations on viral fitness of the H275Y oseltamivir-resistant influenza A(H1N1)pdm09 virus *in vitro*, in mice and in ferrets"

8.1 Foreword

This chapter contains the text of the article **"Impact of potential permissive neuraminidase mutations on viral fitness of the H275Y oseltamivir-resistant influenza A(H1N1)pdm09 virus *in vitro*, in mice and in ferrets"** written by Yacine Abed, Andrés Pizzorno, Xavier Bouhy, Chantal Rhéaume and Guy Boivin. The objective of this study was to determine if the presence of permissive mutations was a phenomenon restricted to A/Brisbane/59/2007-like (H1N1) viruses or if it could also occur in A(H1N1)pdm09 viruses. We therefore evaluated *in vitro* and *in vivo* the effect on viral fitness and transmissibility of several candidate mutations in combination with the oseltamivir-resistant H274Y (here referred as H275Y), as an indicator of their potential emergence and spread in the community. YA, AP and GB conceived and designed the experiments; YA, AP and XB performed the *in vitro* experiments; AP and CR performed the *in vivo* experiments; AP and XB contributed reagents/materials/analysis tools; YA, AP and GB analyzed the data; YA, AP and GB wrote the article and responded to the reviewer's comments. The article is presented in its final version accepted for publication in the **Journal of Virology** in 2014 (Feb;88(3):1652-8).

8.2 Article

Impact of potential permissive neuraminidase mutations on viral fitness of the H275Y oseltamivir-resistant influenza A(H1N1)pdm09 virus *in vitro*, in mice and in ferrets.

Running title: Permissive NA mutations in A(H1N1)pdm09 viruses.

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ABSTRACT

Neuraminidase (NA) mutations conferring resistance to NA inhibitors (NAIs) generally compromise the fitness of influenza viruses. The only NAI-resistant virus that widely spread in the population, the A/Brisbane/59/2007 (H1N1) strain, contained permissive mutations that restored the detrimental effect caused by the H275Y change. Computational analysis predicted other permissive NA mutations for A(H1N1)pdm09 viruses. Herein, we investigated the effect of T289M and N369K mutations on the viral fitness of the A(H1N1)pdm09 H275Y variant. Recombinant A(H1N1)pdm09 wild-type (WT), H275Y, H275Y/T289M, H275Y/N369K and H275Y/V241I/N369K (a natural variant) NA mutants were generated by reverse genetics. Replication kinetics were performed using ST6GalI-MDCK cells. Virulence was assessed in C57BL/6 mice and contact transmission was evaluated in ferrets. The H275Y mutation significantly reduced viral titers during the first 12-36 h post-infection (PI) *in vitro*. Nevertheless, the WT and H275Y viruses induced comparable mortality rates, weight loss and lung titers in mice. The T289M mutation eliminated the detrimental effect caused by the H275Y change *in vitro* while causing greater weight loss and mortality in mice, with significantly higher lung viral titers on days 3 and 6 PI than the H275Y mutant. In index ferrets, the WT, H275Y, H275Y/T289M and H275Y/V241I/N369K recombinants induced comparable fever, weight loss and nasal wash viral titers (NWVTs). All tested viruses were transmitted at comparable rates in contact ferrets with the H275Y/V241I/N369K recombinant demonstrating higher NWVTs than the H275Y mutant. Permissive mutations may enhance the fitness of A(H1N1)pdm09 H275Y viruses *in vitro* and *in vivo*. The emergence of such variants should be carefully monitored.

Keywords: influenza, H1N1, oseltamivir, neuraminidase, mutation, resistance.

INTRODUCTION

Influenza viruses are responsible for respiratory tract infections of significant public health importance. Influenza A viruses of the H1N1 subtype have been associated with seasonal influenza epidemics from the 1918-19 pandemic (1) until 1957, with a resurgence in 1977. Those seasonal A/H1N1 viruses then consistently circulated until 2009, at which time they were replaced by the triple-reassortant influenza A/H1N1 pandemic virus [A(H1N1)pdm09] of swine origin (2). As the M gene of this pandemic virus contained the S31N mutation conferring resistance to adamantanes (amantadine and rimantadine), only neuraminidase inhibitors (NAIs) (i.e., oral oseltamivir, inhaled zanamivir and, to a lower extent, intravenous peramivir) were considered for the pharmacological control of A(H1N1)pdm09 infections (3). During the 2009-10 pandemic and thereafter, approximately 0.4-1.0% of all tested A(H1N1)pdm09 viruses were found to be highly resistant to oseltamivir (4, 5) and these strains were initially mainly identified in immunocompromised patients who had received prolonged treatment or as a result of antiviral prophylaxis (6, 7). As was the case for human A/H1N1 viruses that circulated before the 2009 pandemic, the majority of drug-resistant A(H1N1)pdm09 viruses harboured the H275Y (N1 numbering) NA mutation. Since its introduction in humans in April 2009, the transmission of the A(H1N1)pdm09 H275Y variant in the community has remained limited (8). Such low level of drug resistance contrasts with the situation observed with the seasonal influenza A/Brisbane/59/2007 (H1N1) H275Y variant which began to circulate in Europe and USA in 2007-08 before spreading worldwide during the subsequent season (9). The efficient fitness and transmissibility of oseltamivir-resistant A/Brisbane/59/2007 H275Y viruses were attributed to additional “permissive” NA mutations (R222Q, V234M, D334N) which counteracted the detrimental impact of the H275Y substitution on enzymatic function and replicative properties (10, 11). The possible permissive role for T82K, K141E and R189K mutations within the hemagglutinin gene were also proposed (12). Despite no HA changes were involved in the enhanced transmissibility observed for an oseltamivir-resistant A/Brisbane/59/07-like isolate compared to a closely related drug-susceptible (274H) isolate when investigated in the guinea pigs model (13). Using the same computational analysis that previously led to the successful identification of permissive mutations in the A/Brisbane/59/2007 (H1N1) background, Bloom and colleagues have recently proposed a set of potential permissive NA mutations including T289M and N369K for influenza A(H1N1)pdm09 H275Y viruses (14). The latter mutations have been shown to enhance surface expression of an oseltamivir-resistant influenza NA protein but their role in replicative capacity and virulence has not been

demonstrated. Interestingly, the compensatory role of the N369K mutation and that of another NA mutation (V241I) was also recently suggested in the context of large community outbreaks of oseltamivir-resistant influenza A(H1N1)pdm09 viruses implicating transmission events reported in Australia (15) and Spain (16).

In this study, we generated several recombinant influenza A(H1N1)pdm09 H275Y variants in order to assess the impact of N369K, T289M, and V241I permissive mutations on *in vitro* replicative capacity, as well as on animal virulence and transmissibility.

MATERIALS AND METHODS

Generation of recombinant A(H1N1)pdm09 viruses. The recombinant wild-type (WT) influenza A/Quebec/144147/09, an A/California/07/2009-like A(H1N1)pdm09 virus, was generated using bidirectional pLLBA/G plasmids as previously described (17). The pLLBA plasmid containing the NA gene was used for the introduction of single (H275Y, N369K and T289M), double (H275Y/N369K and H275Y/T289M) and triple (H275Y/V241I/N369K) mutations (N1 numbering) using appropriate primers and the QuikChangeTM Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The resulting pLLB-NAWT, pLLB-NA274Y, pLLB-NA289M, pLLB-NA369K, pLLB-NA274Y/289M, pLLB-NA274Y/369K and pLLB-NA274Y/241I/369K plasmids were sequenced to ensure the absence of undesired mutations. pLLB-NA plasmids and the remaining 7 (pLLB-HA, -M, -NS, -NP, -PA, -PB1 and -PB2) plasmids from A/Quebec/1441417/09 were cotransfected into 293T human embryonic kidney cells using the LipofectamineTM 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Supernatants were collected 72 h post-transfection and used to inoculate Madin-Darby canine kidney cells overexpressing the α2,6 sialic acid receptors (ST6GalI-MDCK cells), kindly provided by Dr. Y. Kawaoka from the University of Wisconsin, Madison, WI. The resulting recombinant WT and NA mutant viruses were subsequently sequenced and titrated by plaque assays in ST6GalI-MDCK cells.

NA enzyme kinetics assays. A fluorometric-based assay using MUNANA (Methylumbelliferyl-N-acetylneuraminic acid) (Sigma, St-Louis, MO) as substrate was performed to determine NA enzymatic parameters. Briefly, recombinant viruses were standardized to an equivalent

dose of 10^6 plaque forming-units (PFU)/ml and incubated at 37°C in 50-µl reactions with different concentrations of MUNANA in a 23 mM N-Morpholino-ethanesulphonic acid, pH 6.5 and 3 mM CaCl2 (18). The final concentration of the substrate ranged from 0 to 3000 µM. Fluorescence was monitored every 90 s for 53 min (35 measures). The Michaelis-Menten constant (Km) and the maximum velocity (Vmax) normalized to PFU were calculated with the Prism software (GraphPad, version 5), by fitting the data to the Michaelis-Menten equation using nonlinear regression.

Cell surface NA activity. Recombinant NA plasmids and pCAGGS-PA, -PB1, -PB2 and -NP plasmids (1 µg each) were used to co-transfect 10^6 293T cells in order to express recombinant NA enzymes (19). At 24 h post-transfection, cells were briefly treated with trypsin-EDTA and neutralized by the addition of serum before the collection of cells by centrifugation at 3000 RPM for 5 min. After two washes with PBS, the cells were resuspended in a non-lysing buffer (15 mM MOPS, 145 mM sodium chloride, 2.7 mM potassium chloride and 4 mM calcium chloride, adjusted to pH 7.4) and used in an NA assay using the MUNANA substrate (11).

***In vitro* replication kinetics experiments.** Replicative capacities of the recombinant viruses were evaluated by infecting ST6GalI-MDCK cells with a multiplicity of infection (MOI) of 0.001 plaque-forming units (PFUs)/cell. Supernatants were collected every 12 h until 72 h post-inoculation (PI) and titrated by plaque assays using ST6GalI-MDCK cells.

Mouse studies. Groups of sixteen 6- to 8-week old female C57BL/6 mice (Charles River, ON, Canada) were infected by intranasal inoculation of 10^5 PFUs of recombinant WT or NA mutant A(H1N1)pdm09 viruses. Animals were weighed daily for 12 days and monitored for clinical signs. Four mice per group were sacrificed on days 3 and 6 PI and lungs were removed aseptically. For determination of viral titers, harvested lung tissues were homogenized in Dulbecco's Modified Eagle Medium (Life Technologies Corporation, Grand Island, NY) with antibiotics using a bead mill homogenizer (Tissue Lyser, Qiagen). Cells were pelleted by centrifugation (2,000 g, 5 min) and supernatants were titrated by plaque assays in ST6GalI-MDCK cells.

Ferret studies. Groups of 4 seronegative (800-1200 g) male ferrets (Marshall BioResources, North Rose, NY) housed in individual cages were lightly anesthetised by isoflurane and received an intranasal instillation of 5 x 10^4 PFUs of the recombinant A(H1N1)pdm09 WT or one of the three selected NA mutants (H275Y, H275Y/T289M, and H275Y/V241I/N369K). Ferrets were weighed daily

and body temperature was measured by rectal thermometers every day until day 10 PI. Nasal wash samples were collected from animals on a daily basis until day 8 PI and viral titers were determined every other day by plaque assays in ST6GalI-MDCK cells. Serum samples were collected from each ferret before intranasal infection (day 0) and on day 14 PI to evaluate specific antibody levels against the A/California/07/2009 A(H1N1)pdm09 and A/Wisconsin/15/2009 (H3N2) viral strains using standard hemagglutination inhibition (HAI) assays with 0.7% turkey red blood cells. To evaluate contact transmissibility, inoculated-contact animal pairs were established by placing a naïve ferret into each cage 24 h after inoculation of the donor ferret. Contact animals were monitored for clinical signs and nasal wash and serum samples were collected as described above for determination of viral titers and serological status, respectively. All animal procedures were approved by the Institutional Animal Care Committee of Laval University according to the guidelines of the Canadian Council on Animal Care.

Sequencing. NA genes of viruses recovered from *in vitro* replication assays, mouse lung homogenates and ferret nasal wash samples were amplified and sequenced using the ABI 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA) to confirm both the presence of the desired mutations and the absence of additional mutations.

Statistical analyses. All data were expressed as the mean of 3 replicates. NA kinetic parameters, surface NA activity, viral titers and body weight loss were compared by one-way analysis of variance (ANOVA) with Tukey's multiple-comparison post-test. Areas under the curve of temperature values over 10 days were compared by unpaired two-tailed t tests with GraphPad, version 5.

RESULTS

Characterization of permissive NA mutations *in vitro*. The effects of the different mutations selected for this study on NA enzyme kinetics parameters for viruses normalized to 10^6 PFU are summarized in Table 1. Compared to the affinity of the single H275Y mutant (Km of 80.84±7.93 μM), an increase in NA affinity was observed for the WT, T289M, N369K, H274/T289M and H275Y/I241V/N369K variants, supported by significantly lower Km values. Of note, the Km

values of the H275Y/T289M (36.02±3.38 μM) and the WT (26.21±1.74 μM) viruses were not significantly different. The WT and T289M were the only two NA variants which also exhibited significant differences in Vmax compared to the H275Y (11.39±0.39 and 14.87±0.68 vs 2.21±0.09 U/sec, respectively, $P<0.01$). The Vmax ratio of the H275Y/T289M was 3.5-fold higher than that of the H275Y (0.74 vs 0.19). Moreover, using recombinant NA proteins expressed in 293T cells, the H275Y mutation was shown to be associated with a significant reduction in NA activity at the cell surface compared to the WT protein, with a relative total surface activity of 19.73% ($P<0.001$) (Figure 1). In all cases, the addition of the potentially permissive mutations to the H275Y variant partially compensated for the loss of NA activity, with relative total surface activities of 43.63% ($P<0.001$), 27.45% ($P<0.05$) and 74.49% ($P<0.001$) for the H275Y/T289M, H275Y/N369K and H275Y/V241I/N369K, respectively. Of interest, the cell-surface activity of the triple H275Y/V241I/N369K mutant was significantly higher than that of the double (H275Y/N369K) mutant ($P<0.001$).

The impact of NA mutations on viral fitness of recombinant viruses was assessed in ST6GalI-MDCK cells (Figure 2). The H275Y mutant showed significantly reduced viral titers compared to the rest of the recombinant viruses in the first 12 h PI and also compared to the WT and the H275Y/T289M at 24 h and 36 h PI. The H275Y/T289M double mutant still displayed viral titers significantly higher than those of the H275Y at 48 h PI ($2.9±0.9 \times 10^8$ vs $7.6±1.8 \times 10^7$ PFU/ml, $P<0.001$). The growth curves of the remaining mutant viruses were not significantly different from that of the H275Y virus beyond 12 h PI. Sequence analysis of viral samples from infected cell culture supernatants did not show mutations other than expected ones in viruses recovered at 72 h PI.

Effect of permissive NA mutations in mice. As shown in Table 2, intranasal inoculation of mice with 10^5 PFU of the recombinant viruses resulted in mortality rates of 50% for the WT and H275Y/T289M, 37.5% for the H275Y, 12.5% for the H275Y/N369K and 0% for the H275Y/V241I/N369K groups. Interestingly, the H275Y/T289M group showed significantly greater mean weight loss compared to the H275Y group on day 4 PI (13.2±1.5% vs 6.2±1.8%, respectively, ($P<0.01$) (Figure 3) as well as a shorter mean number of days to death (4.75 vs 6.33 days, $P<0.05$) (Table 2). In line with the previous observations, lung viral titers (LVT) were significantly higher for the H275Y/T289M group compared to those of the H275Y group, on both days 3 ($7.8±0.3 \times 10^6$ vs $3.9±0.1 \times 10^6$ PFU/ml, respectively, $P<0.05$) and 6 PI ($3.5±0.3 \times 10^6$ vs $0.7±0.1 \times 10^6$ PFU/ml,

respectively, $P<0.05$). Conversely, no significant differences in weight loss, mean number of days to death, or LVT were observed between any of the other 3 groups (WT, H275Y/N369K and H275Y/V241I/N369K) and the H275Y group (Table 2). The LVT of the H275Y/T289M group were significantly higher than those of the WT group on day 3 PI ($7.8±0.3 \times 10^6$ vs $1.8±0.2 \times 10^6$ PFU/ml, respectively, $P<0.05$). Sequence analysis of the NA gene from lung samples of mice confirmed the presence of the desired mutations and no other mutations or mixed viral populations.

Effect of permissive NA mutations in ferrets. Intranasal inoculation of ferrets with 5×10^4 PFU of the recombinant WT, H275Y, H275Y/T289M or H275Y/V241I/N369K viruses yielded mild clinical signs, with febrile responses that peaked on days 2 and 4 PI for the donor and contact animals, respectively, in all groups (data not shown). Although the WT and H275Y/T289M groups exhibited the highest peak temperature values, no significant differences in the area under curve (AUC) of temperatures from day 0 to 10 PI were observed among any of the four groups. Also there were no significant differences in body weight loss (maximum of 3.6%) at any time points among groups (data not shown).

Mean viral titers in nasal wash samples collected from donor animals on days 2 and 4 PI were comparable among the four groups, ranging from $2.0±0.6 \times 10^3$ to $1.4±0.9 \times 10^4$ PFU/ml and $4.5±0.2 \times 10^2$ to $1.9±1.4 \times 10^3$ PFU/ml, respectively (Figure 4A). Nasal wash viral titers were undetectable on day 6 PI, except for one ferret in each of the H275Y/T289M and H275Y/V241I/N369K groups. All contact animals seroconverted for A/California/07/2009 (H1N1) with reciprocal geometric mean HAI titers between 180 and >1280 on day 14 PI, compared to <10 on day 0. For A/Wisconsin/15/2009 (H3N2), reciprocal geometric mean HAI titers were <10 on both days 0 and 14 PI. As shown in Figure 4B, mean viral titers in nasal wash samples obtained from contact ferrets of the H275Y/V241I/N369K group were significantly higher than those of the H275Y group on days 3 ($1.2±0.8 \times 10^4$ vs $1.9±0.5 \times 10^2$ PFU/ml, respectively, $P<0.05$) and 5 ($2.7±1.3 \times 10^4$ vs $5.9±3.2 \times 10^3$, respectively, $P<0.05$) PI, but not on day 7 PI ($1.4±0.7 \times 10^3$ vs $8.9±5.7 \times 10^2$ PFU/ml, respectively). Mean nasal wash viral titers of contact ferrets of the WT group were also significantly higher than those of the H275Y group on day 5 PI ($1.9±0.9 \times 10^4$ vs $5.9±3.2 \times 10^3$ PFU/ml, respectively, $P<0.05$). On the other hand, mean viral titers were comparable for contact ferrets of the H275Y/T289M and H275Y groups at all 3 time points. Sequence analysis of the NA gene in nasal wash samples of ferrets confirmed the presence of the desired mutations in each of the four animals

of the respective donor and contact groups, with no other mutations or mixed viral populations detected.

DISCUSSION

In this study, we found that the T289M NA mutation in particular, as well as the N369K substitution, partially restore the replicative capacity and virulence of the H275Y A(H1N1)pdm09 variant *in vitro* and in mice. Moreover, direct contact transmission experiments in ferrets have shown that all tested viruses were successfully transmitted at comparable rates, with the H275Y/V241I/N369K recombinant demonstrating higher nasal wash viral titers than the H275Y mutant in contact animals.

Given widespread adamantane resistance, NAIs constitute the most clinically relevant class of anti-influenza agents that have been used in many countries as therapeutic and prophylactic modalities for seasonal or pandemic influenza infections. Since their introduction in the late '90s, some concerns have been raised with regard to the emergence and spread of NAI-resistant variants, which may compromise the clinical utility of these antiviral agents. Previous reports have shown that NAI-resistant influenza viruses may emerge under NAI pressure *in vitro* or in the clinic as a result of NA mutations which occur in a subtype- and drug-specific manner (20, 21). In the N1 subtype, the resistance phenotype to oseltamivir is usually mediated by the framework H275Y NA mutation (22). As NAIs target the active site of a key viral enzyme, resistance mutations occurring within the NA gene were expected to compromise the viral fitness of these variants. In fact, when present in older A/H1N1 strains including A/WSN/33 (19), A/Texas/36/91 (23), A/New Caledonia/20/99 (24) and A/Mississipi/3/01 (25), the H275Y NA mutation was found to be deleterious both *in vitro* and in animal models. However, more recent seasonal A/H1N1 mutant strains such as A/Brisbane/59/2007-like isolates were fully virulent and transmissible (9). Bloom and colleagues demonstrated that the H275Y mutation was associated with a significant decrease in the amount of the NA enzyme which is expressed on the cell surface (11). These authors suggested that, as the H275Y mutation apparently altered the NA protein folding, additional (so called "permissive") NA mutations occurring at distant sites in the 3D-structure could restore an adequate NA folding (11). Using a computational approach based on potentially important phylogenetic sites (PIPS), the R194G NA mutation was predicted to

correct the deficiency caused by the H275Y mutation in the A/WSN/33 background and, as expected, the double R194G/H275Y mutation restored the viral fitness of this strain *in vitro* (11). This method was also successfully used to predict the ability of R222Q and V234M mutations to correct the deficiency caused by the H275Y change in the A/Texas/36/91 virus (11). Our group further demonstrated the compensatory nature of these mutations in recombinant viruses by reverting the latter two substitutions (i.e., Q222R and M234V) in the NA of the oseltamivir-resistant A/Brisbane/59/2007 (H1N1) H275Y variant (10). As predicted, these substitutions significantly altered the *in vitro* viral fitness and contact transmissibility of the drug-resistant virus in ferrets.

A similar computational approach was recently performed by Bloom and colleagues (14) to predict permissive NA mutations in the A(H1N1)pdm09 virus background harboring the oseltamivir-resistant H275Y mutation. A rationale exists for using this approach since, like it was observed in older seasonal A/H1N1 viruses (11), the H275Y mutation also induced a significant decrease of total NA protein and cell-surface enzymatic activity in the 2009 pandemic virus. Of note, the compromising impact of the H275Y substitution in the context of the A(H1N1)pdm09 background has been controversial. While some studies reported a statistically significant impairment in viral growth *in vitro* (17, 26), other groups did not detect significant differences in term of replicative capacity between the A(H1N1)pdm09 WT virus and its H275Y variant (27, 28). In studies based on the ferret model, the A(H1N1)pdm09 H275Y variant demonstrated comparable contact transmission efficiency compared to the WT (29, 30) whereas the mutant appeared to compromise airborne transmission (which is probably more biologically relevant) in some but not all investigations (26, 27, 31, 32). Nevertheless, the fact that the oseltamivir-resistant A(H1N1)pdm09 virus has not replaced the WT counterpart indicates some fitness alteration due to the H275Y mutation. It is thus worthwhile to assess the future impact of potential permissive NA mutations on the viral fitness of drug-resistant influenza A(H1N1)pdm09 viruses as these strains have been circulating worldwide since 2009. Recent outbreaks implicating transmission of oseltamivir-resistant A(H1N1)pdm09 strains in the absence of antiviral treatment also justify this investigation. In those cases, a set of permissive NA mutations (such as N369K and V241I) may have facilitated the emergence of the H275Y resistance mutation and improved virus transmissibility (15, 16). Of importance, during the 2010-11 influenza season in US, up to 74% of oseltamivir resistance cases were not associated with drug exposure (33).

As part of our study, we generated recombinant viruses and proteins in order to characterize the two best candidates from a list of 12 potentially permissive NA mutations proposed for A(H1N1)pdm09 viruses (14), namely the T289M and N369K substitutions. In addition, we investigated the N369K mutation in conjunction with the V241I substitution since variants with the three (H275Y/V241I/N369K) NA mutations have been recently identified among clinical A(H1N1)pdm09 Australian (15) and Spanish (16) isolates. Moreover, NA sequences containing the V241I/N369K changes, without the H275Y mutation, have been deposited on the GISAID public sequence database (15). On the other hand, the T289M change has not yet been reported in A(H1N1)pdm09 viruses.

Our experiments demonstrated the ability of T289M and N369K mutations to partially restore the NA cell-surface expression defect associated with the H275Y mutation. This compensation could be explained by a greater number of NA molecules that reach the cell surface, an increased activity of the enzyme, or a combination of both effects (11, 14). Our results are in agreement with previous NA protein expression analysis (14). Interestingly, the impact of adding the double V241I/N369K permissive mutation to the H275Y in restoring the NA cell-surface expression was higher than that of the single N369K change ($P<0.001$) and may account for the better replication and/or earlier contact transmission of the triple mutant in ferrets as well as its presence in community outbreak isolates.

Our results show that, as predicted, the addition of the potentially permissive NA mutations can enhance the fitness and virulence of A(H1N1)pdm09 viruses containing the H275Y mutation both *in vitro* and *in vivo*. However, it should be noted the efficiency of the PIPS approach to successfully identify permissive NA mutations for both old (A/WSN/33) and contemporary (A/Brisbane/59/07) viruses as well as for the A(H1N1)pdm09 virus. Although, the V241I change which seems to possess a potential permissive role was not predicted by this approach. In addition, the T289M which was predicted with a high score has not yet been described in clinical samples. In particular, the T289M substitution seems to have the most significant compensatory effect by improving NA kinetic parameters, with a 2.2-fold increase in affinity and a 3.8-fold increase in Vmax activity compared to the H275Y mutant (Table 1). This mutation also significantly improved the replicative capacity of the H275Y A(H1N1)pdm09 virus in ST6GalI-MDCK cells (Figure 2) as well as its virulence in C57BL6 mice to levels comparable or even higher than those of the WT virus (Table 2 and Figure 3). We acknowledge that our investigation did not show a perfect concordance between *in vitro* and mice

experiments from one side and transmissibility in ferrets from another one. For instance, whileNo significant differences were observed between the H275Y and H275Y/T289M groups with respect to the nasal wash viral titers of both index and contact ferrets, the triple (H275Y/V241I/N369K) NA mutant resulted in a significant increase in nasal wash viral titers compared to the H275Y group in contact ferrets although this genotype was not associated with an increased virulence in mice. This discrepancy could be attributed to differences in receptor binding cells, which are a major determinant for efficient transmission of influenza viruses (34), between mice (predominantly α2,3 sialic acid receptors) and ferrets (predominantly α2,6 sialic acid receptors). Thus, the triple (H275Y/V241I/N369K) NA mutant enzyme may cleave the α2,6 sialic acid linkage with a better efficiency than the α2,3 sialic acid one. This may result in better airborne transmission, which could not be evaluated in our study. Of interest, A(H1N1)pdm09 viruses containing the V241I/N369K double mutation that demonstrated the potential to counteract the detrimental effect of the H275Y mutation already exist (15). Thus, exposure of individuals infected with A(H1N1)pdm09 V241I/N369K viruses to oseltamivir during prophylaxis or treatment courses, may rapidly select for the H275Y mutation, which could constitute a serious public health threat. Moreover, exposure to NAIs is not necessary to select for the H275Y mutation if permissive mutations are already present in the NA gene, as exemplified by the emergence and global dissemination of oseltamvir-resistant A/Brisbane/59/2007-like variants (9, 35).

In conclusion, our study confirmed that, as was the case for recent seasonal A/H1N1 viruses containing permissive NA mutations that facilitated the replacement of the susceptible virus by the H275Y oseltamivir-resistant variant, permissive mutations that may enhance the fitness of A(H1N1)pdm09 H275Y viruses do exist. Consequently, the potential emergence and dissemination of such variants should be carefully monitored.

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Table 1. Enzyme kinetics parameters of recombinant A(H1N1)pdm09 viruses harboring several neuraminidase mutations.

Recombinant influenza A(H1N1)pdm09 viruses	Km[a] (µM)	Vmax[a] (U/Sec)	Vmax ratio
WT	26.21 ± 1.74***	11.39 ± 0.39	1**
H275Y	80.84 ± 7.93	2.21 ± 0.09	0.19
N369K	46.14 ± 3.42***	4.07 ± 0.6	0.36
T289M	60.76 ± 6.44**	14.87± 0.68	1.3**
H275Y/N369K	94.20 ±3.57*	3.34 ± 0.17	0.36
H275Y/T289M	36.02 ± 3.38***	8.41 ± 0.64	0.74
H275Y/V241I/N369K	54.81 ± 3.58***	5.76 ± 0.21	0.51

[a] Numbers indicate mean Km and relative NA activity (Vmax) values of a representative experiment performed in triplicate, using recombinant viruses standardized to 10^6 plaque forming-units (PFU)/ml.

SD, standard deviation. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to the recombinant H275Y virus.

Table 2. Virulence of recombinant influenza A(H1N1)pdm09 viruses harboring several neuraminidase mutations in C57BL/6 mice.

Recombinant influenza A(H1N1)pdm09 virus	Mean % weight change on day 4 PI ± SD (N = 12)	% Mortality (day 14 PI) (N = 8)	Mean day to death	Mean LVT[a] on day 3 PI ± SD (N = 4)	Mean LVT[a] on day 6 PI ± SD (N = 4)
WT	8.1 ± 1.4	50	7.75	$1.8 \times 10^6 \pm 0.17 \times 10^6$	$1.5 \times 10^6 \pm 0.66 \times 10^6$
H275Y	6.2 ± 1.8	37.5	6.33	$3.9 \times 10^6 \pm 0.13 \times 10^6$	$0.65 \times 10^6 \pm 0.1 \times 10^6$
H275Y/N369K	8.6 ± 1.3	12.5	7	$3.1 \times 10^6 \pm 0.47 \times 10^6$	$1.8 \times 10^6 \pm 0.71 \times 10^6$
H275Y/V241I/N369K	4.2 ± 1.2	0	NA	$4.8 \times 10^5 \pm 0.27 \times 10^5$	$0.15 \times 10^6 \pm 0.05 \times 10^6$
H275Y/T289M	13.2 ± 1.5 **	50	4.75 *	$7.75 \times 10^6 \pm 0.32 \times 10^6$ *	$3.5 \times 10^6 \pm 0.27 \times 10^6$ *
Uninfected	-4.5 ± 0.2	0	NA	--	--

[a]LVT, lung viral titers in plaque forming units (PFU)/ml. *$P < 0.05$, **$P < 0.01$ compared to the recombinant H275Y virus.

PI, post-infection.

Figure 1.

Surface activity of recombinant A(H1N1)pdm09 NA proteins. 293T cells were transfected with plasmids expressing the WT or mutant A(H1N1)pdm09-like NAs. At 24 h post-transfection, cells were treated with a non-lysing buffer and surface NA activity was measured by using a fluorogenic substrate (MUNANA). Percent surface NA activities are shown compared to the WT from triplicate experiments ± standard deviations. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to the recombinant H275Y virus.

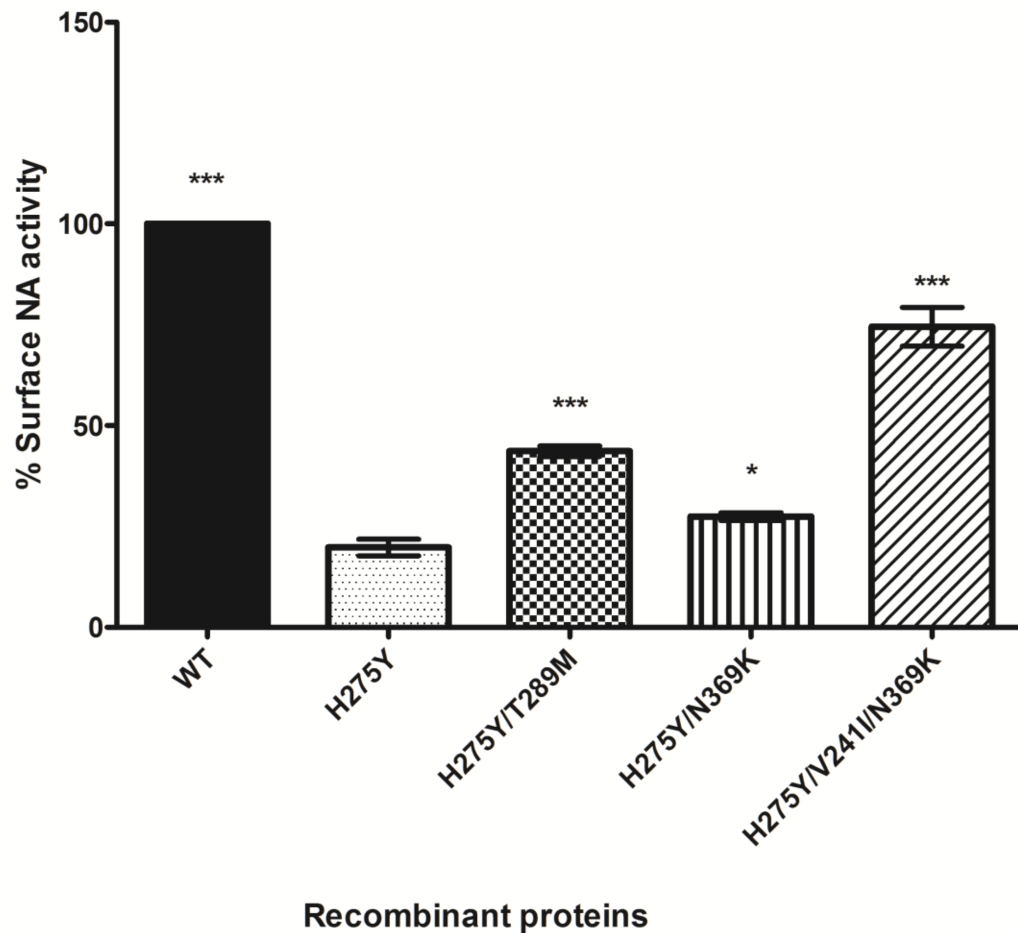


Figure 2.

In vitro replicative capacities of recombinant wild-type (WT) and NA mutant influenza A(H1N1)pdm09 viruses. Viral titers were determined at the indicated time points from supernatants of MDCK-α2,6 cells infected at a multiplicity of infection (MOI) of 0.001. Mean viral titers ± SD from triplicate experiments were determined by using standard plaque assays. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the recombinant H275Y virus.

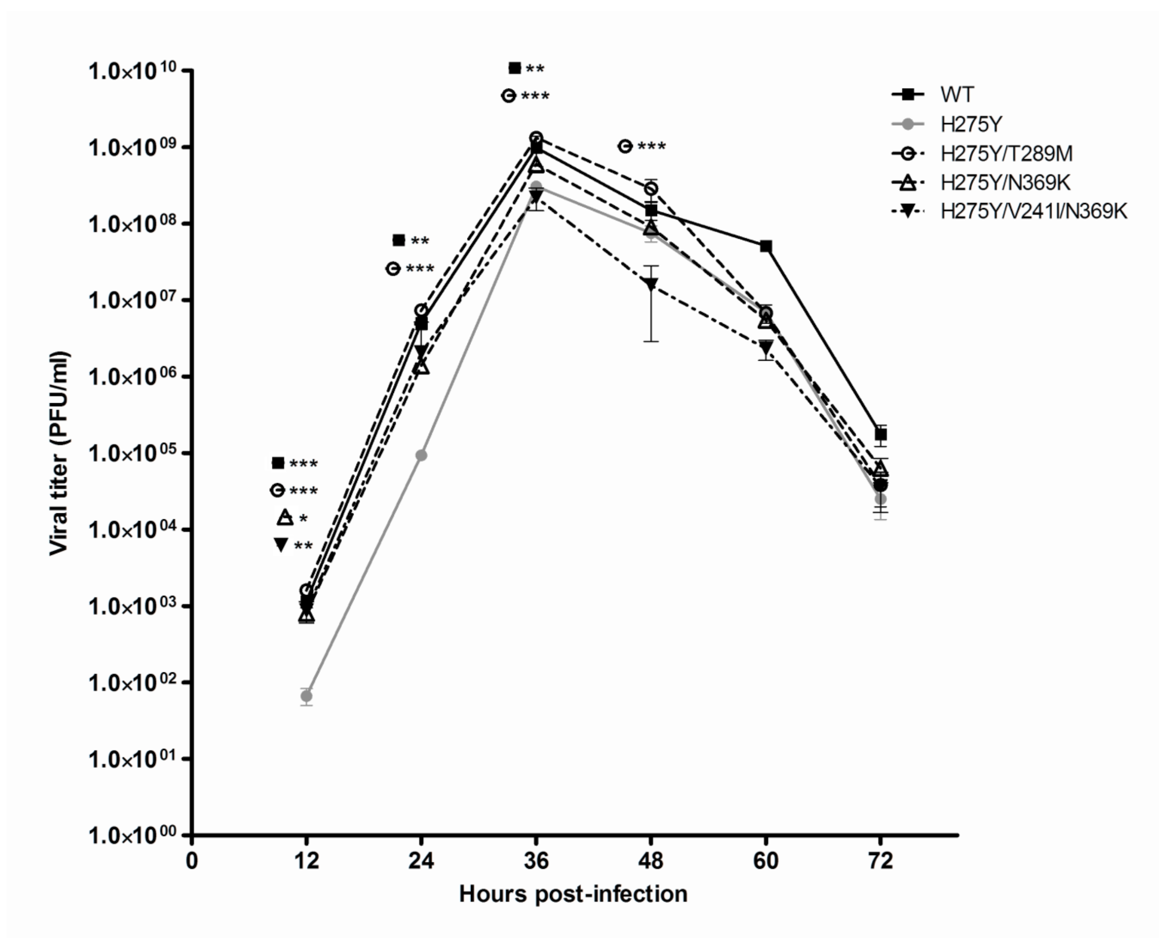


Figure 3.

Mean body weight loss of mice infected intranasally with 10^5 PFUs of the recombinant WT or NA mutant influenza A(H1N1)pdm09 viruses. Percent body weight losses were determined daily up to day 12 post-inoculation. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the recombinant H275Y virus.

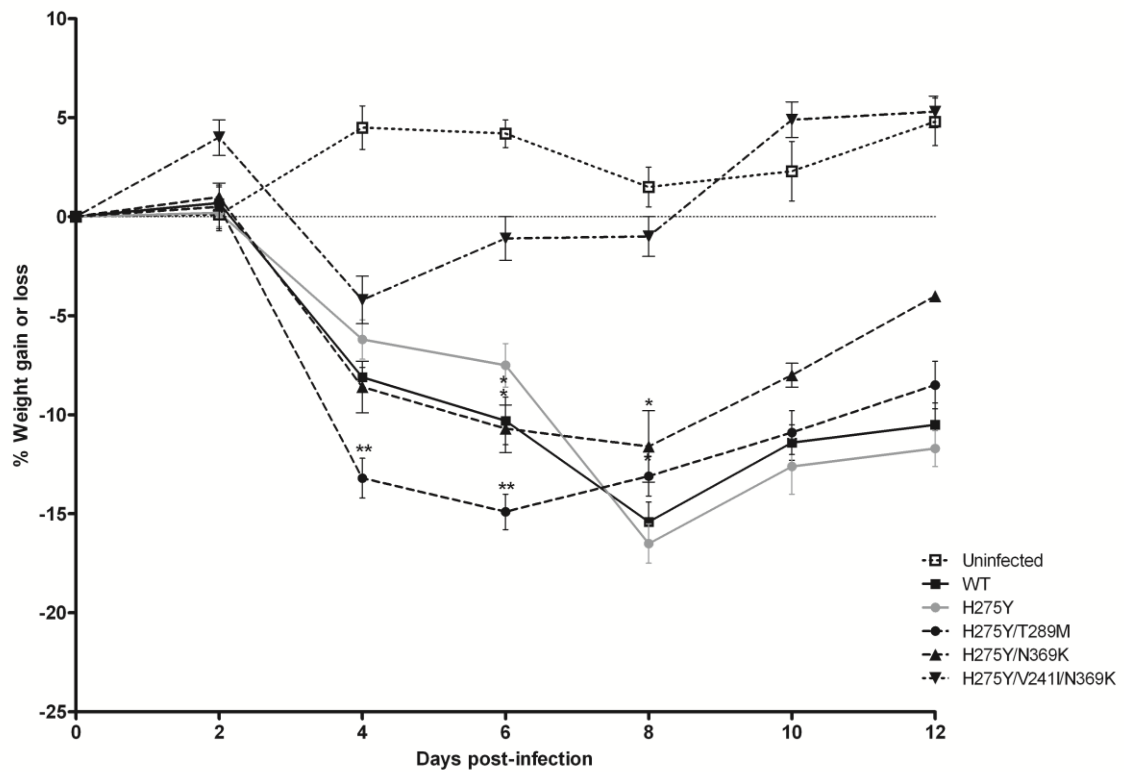
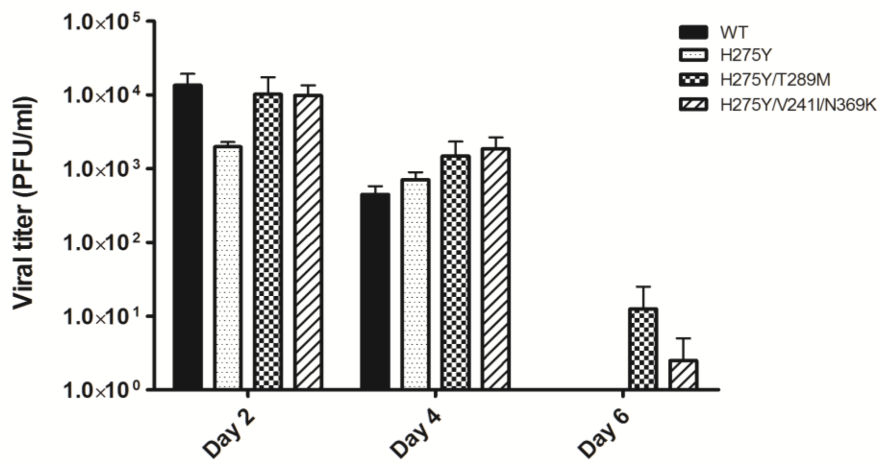


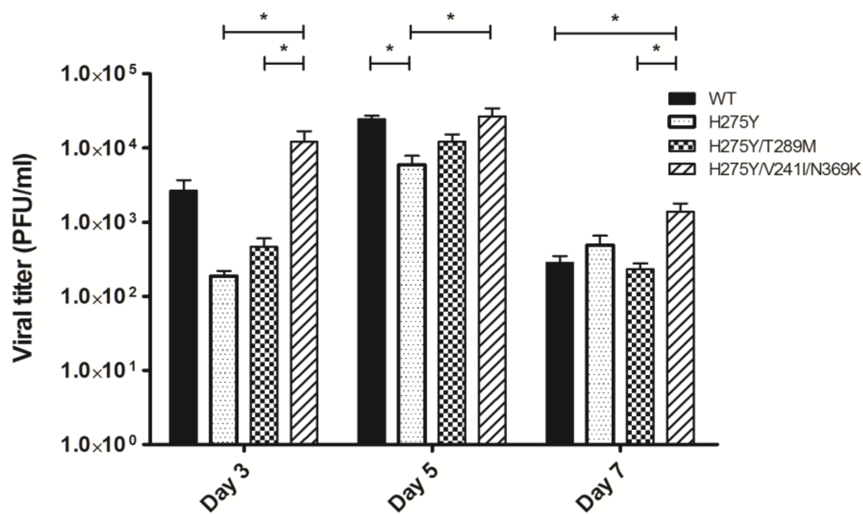
Figure 4.

Mean viral titers ± SD in nasal washes of index ferrets infected with 5 x 10^4 PFUs of the recombinant wild-type (WT) and NA mutant A(H1N1)pdm09 viruses (A) and in samples of their direct contact animals (B). Viral titers were determined at the indicated days post-inoculation by using standard plaque assays. $^{*}P < 0.05$ compared to the recombinant H275Y virus.

A



B



Chapter IX: "Oseltamivir-zanamivir combination therapy is not superior to zanamivir monotherapy in mice infected with influenza A(H3N2) and A(H1N1)pdm09 viruses"

9.1 Foreword

This chapter contains the text of the article **"Oseltamivir-zanamivir combination therapy is not superior to zanamivir monotherapy in mice infected with influenza A(H3N2) and A(H1N1)pdm09 viruses"**, written by Andrés Pizzorno, Yacine Abed, Chantal Rhéaume and Guy Boivin. In this study, we evaluated *in vivo* the potential additional clinical and virological benefit of combining two NAI (oseltamivir and zanamivir) compared to standard monotherapy, on the basis of their differential administration route and interaction with the NA substrate. AP, YA and GB conceived and designed the experiments; AP and CR performed the experiments; AP, YA and GB analyzed the data; AP, YA and GB wrote the article and responded to the reviewer's comments. The article is presented in its final version accepted for publication in **Antiviral Research** in 2014 (May;105:54-8).

9.2 Article

Oseltamivir-zanamivir combination therapy is not superior to zanamivir monotherapy in mice infected with influenza A(H3N2) and A(H1N1)pdm09 viruses

Running title: Oseltamivir-zanamivir combination in mice

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ABSTRACT

The efficacy of oseltamivir-zanamivir combination therapy compared to that of monotherapy was evaluated in mice infected with influenza A(H3N2) or A(H1N1)pdm09 viruses. For A(H3N2) virus, zanamivir monotherapy and oseltamivir-zanamivir combination showed significant reduction of mean weight loss compared to oseltamivir. Zanamivir monotherapy also conferred decreased mortality, weight loss and lung viral titers (LVT) compared to oseltamivir for A(H1N1)pdm09 wild-type virus. Intermediate benefits were observed for the oseltamivir-zanamivir combination. For the oseltamivir-resistant A(H1N1)pdm09 H275Y virus, the efficacy of oseltamivir-zanamivir was comparable to that of zanamivir and significantly higher than that of oseltamivir in terms of survival, weight loss and LVT.

Keywords. Influenza, neuraminidase inhibitors, oseltamivir, zanamivir, combination, resistance.

Neuraminidase (NA) inhibitors (NAIs) such as oseltamivir and zanamivir represent one of the most valuable options for the control of influenza epidemics and pandemics. Combinations of anti-influenza agents have been thought to provide improved therapeutic potency in addition to reducing the emergence of resistance. At this time, available data on the potential effect of combined NAI therapy is very limited. For instance, combinations of oseltamivir and peramivir demonstrated additive activities *in vitro* and in mice infected with A/NWS/33 (H1N1) virus (Smee et al., 2010). On the other hand, a double-blind, randomized clinical trial carried out in France during the 2008-2009 influenza epidemic found that the oseltamivir-zanamivir combination appeared less efficacious than oseltamivir monotherapy in adults with uncomplicated influenza, mostly A(H3N2) infections (Duval et al., 2010). Nevertheless, a more recent study by the same group has suggested greater effectiveness of combined therapy in the reduction of influenza transmission in household contacts (Carrat et al., 2012). In the A(H1N1)pdm09 background, combinations of oseltamivir and zanamivir showed concentration-related additive to antagonistic antiviral effects *in vitro* (Nguyen et al., 2010), whereas a clinical trial conducted during the 2009-2010 influenza pandemic failed to conclude whether combined therapy improved or reduced the effectiveness of oseltamivir monotherapy in the treatment of A(H1N1)pdm09 virus infection in community patients, due to small sample size (Escuret et al., 2012).

The aim of this study was to evaluate the efficacy of the oseltamivir-zanamivir combination compared to that of monotherapy for the treatment of mice infected with NAI-sensitive A(H3N2) and A(H1N1)pdm09 viruses as well as for A(H1N1)pdm09 viruses harboring the oseltamivir resistance H275Y NA substitution.

A mouse-adapted A/Victoria/3/75 (H3N2) wild-type (WT) virus (a gift from Dr. D. Smee, Utah State University, Logan, UT) was passaged twice in Madin-Darby canine kidney (MDCK) cells. The recombinant influenza A(H1N1)pdm09 WT and its oseltamivir-resistant H275Y NA variant were generated from the first A(H1N1)pdm09 virus isolated in Québec City (A/Québec/144147/09, GenBank accession numbers FN434457-FN434464) using bidirectional pLLBA/G plasmids as previously described (Pizzorno et al., 2011). The recombinant viruses were amplified in ST6GalI-MDCK cells overexpressing the α2,6 sialic acid receptors (kindly provided by Dr. Y. Kawaoka, University of Wisconsin, Madison, WI). All three viruses were sequenced using the ABI 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA) and titrated by standard plaque assays.

Susceptibility phenotypes to oseltamivir carboxylate (Hoffmann-La Roche, Basel, Switzerland) and zanamivir (GlaxoSmithKline, Stevenage, UK) were determined by NA inhibition assays using the 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA, Sigma, St-Louis, MO, USA) substrate as described elsewhere (Potier et al., 1979), with minor modifications (Pizzorno et al., 2011). Both A/Victoria/3/75 (H3N2) and recombinant A/Québec/144147/09 (H1N1pdm09) WT viruses were susceptible to oseltamivir with 50% inhibitory concentration (IC_{50}) values of 0.26 ± 0.04 nM and 0.46 ± 0.01 nM, respectively. Both viruses were also susceptible to zanamivir, with IC_{50} values of 0.38 ± 0.01 nM and 0.15 ± 0.01 nM, respectively. As expected, the recombinant A/Québec/144147/09 H275Y NA mutant showed highly reduced susceptibility to oseltamivir (IC_{50}: 451.92 ± 26.01 nM), but remained susceptible to zanamivir (IC_{50}: 0.14 ± 0.01 nM).

Groups of twelve 6- to 8-week old female C57BL/6 mice (Charles River, ON, Canada) were inoculated intranasally (i.n.) with either saline (uninfected control), 10^3 plaque forming units (PFU) of A(H3N2) WT virus, or 10^5 PFU of recombinant A(H1N1)pdm09 WT or H275Y viruses. To mimic clinical conditions, treatment was initiated 48h post-infection (p.i.) with either saline (placebo control), oseltamivir 10 mg/kg by gavage (plus saline i.n.), zanamivir 10 mg/kg i.n. (plus saline in gavage), or the oseltamivir-zanamivir combination, twice daily for 5 days. Mortality and weight loss were monitored for 14 days. The humane endpoint was determined at 25% weight loss. On day 4 p.i., four mice per group were sacrificed to determine lung viral titers (LVT) by plaque assay in ST6GalI-MDCK cells. Viruses recovered from lungs were sequenced for the presence of unexpected NA mutations. All animal procedures were approved by the Institutional Animal Care Committee of Laval University according to guidelines of the Canadian Council of Animal Care.

As shown in Figure 1A, i.n. inoculation of 10^3 PFU of A/Victoria/3/75 (H3N2) virus resulted in a 100% mortality rate on day 7 p.i. in untreated mice. On the other hand, treatment with oseltamivir, zanamivir or oseltamivir-zanamivir combination 48h p.i. resulted in 100% survival. Interestingly, zanamivir monotherapy as well as oseltamivir-zanamivir combination showed a moderate but significant reduced maximum mean weight loss compared to oseltamivir monotherapy (4.4%, 0.1% and 8.2%, respectively, $P<0.01$), on day 7 p.i. (Figure 1B). However, no significant differences in mean LVT on day 4 p.i. were observed among the groups treated with either oseltamivir (4.9 ± 1.1 $x10^5$ PFU/ml), zanamivir (1.5 ± 0.6 $x10^5$ PFU/ml), or oseltamivir-zanamivir combination (4.6 ± 1.8 $x10^4$ PFU/ml) (Figure 1C).

In mice infected with 10^5 PFU of recombinant A/Québec/144147/09 (H1N1pdm09) WT virus, treatment with zanamivir or oseltamivir-zanamivir resulted in 100% survival, compared to 50% (P<0.05) for the oseltamivir group and 12.5% (P<0.05) for the saline group (Figure 2A). Zanamivir monotherapy also led to a greater reduction in mean weight loss compared to oseltamivir as well as lower mean LVT on day 4 p.i. ($5.7 \pm 1.9 \times 10^6$ vs $1.7 \pm 0.6 \times 10^7$ PFU/ml, respectively, P<0.05). Intermediate values were observed for the oseltamivir-zanamivir combination group (Figure 2B-C). For the A/Québec/144147/09 H275Y infection (Figure 3A-C), the efficacy of oseltamivir-zanamivir combination was comparable to that of zanamivir monotherapy and significantly higher than that of oseltamivir in terms of survival (85.7% and 100% vs 12.5%, respectively, P<0.05), weight loss and mean LVT on day 4 p.i. ($5.4 \pm 1.6 \times 10^6$ and $3.5 \pm 0.6 \times 10^6$ vs $2.5 \pm 1.3 \times 10^8$ PFU/ml, respectively, P<0.01). In all cases, comparison of NA viral sequences collected from lung homogenates with those of inoculated viruses confirmed the absence of unexpected mutations or mixed viral populations.

In this study, we used a controlled animal model to evaluate the clinical and virological responses to the treatment of severe influenza infections with NAI-sensitive and NAI-resistant influenza viruses. With the focus of improving oseltamivir therapy, we hypothesized that the combination of anti-influenza agents having different interactions with the NA substrate, namely oseltamivir and zanamivir, could potentially improve antiviral effectiveness while reducing the emergence of drug-resistant variants. Our results showed that, in mice infected with A(H1N1)pdm09, the efficacy of the oseltamivir-zanamivir regimen was comparable to that of zanamivir alone but greater than that of oseltamivir, when treatment was initiated at 48h p.i. This trend was also seen in the A(H3N2) infection model, but with lesser differences among groups.

Interestingly, our findings are not in line with what has been reported in the randomized placebo-controlled trial by Duval and colleagues (Duval et al., 2010), in which oseltamivir showed greater clinical and virological efficacy as compared to either zanamivir or oseltamivir-zanamivir combination in 541 patients with uncomplicated influenza. Nonetheless, this discrepancy could be attributed to many factors. Firstly, in the mentioned clinical trial, almost all influenza viruses detected were of the A(H3N2) subtype, whereas the most important differences in treatment outcomes observed in our study were found for the A(H1N1)pdm09 virus, for which the only clinical trial reported to date failed to be fully informative (Escuret et al., 2012). Secondly, patients enrolled in the study by Duval and colleagues had uncomplicated influenza, whereas we studied the effects of single

or combined NAI therapy in a model of severe infection. Finally, the French group pointed out the possibility of a slight underestimation of performance in both zanamivir and oseltamivir-zanamivir arms of their study. Actually, in a subset analysis of the same cohort, treatment of index patients with oseltamivir-zanamivir combination within 24h of onset of symptoms proved to be more effective than monotherapy for reducing household transmission (Carrat et al., 2012).

The fact that the influenza A(H3N2) strain used in our study is a mouse-adapted strain may represent a limitation of our model. Also, even if clinical trials have not shown any significant pharmacokinetic differences between inhaled and intranasal zanamivir in humans (Cass et al., 1999), no such data are available in mice. Finally, only one dose of each NAI was tested in this study. Although the 10 mg/kg dose of oseltamivir given to mice is considered a good estimate of the normal dose given to humans (75 mg), a similar correlation has not yet been defined in the case of zanamivir. Based on a previous report using the same route of zanamivir administration in mice (Kubo et al., 2010), we arbitrarily used the same concentration of the two drugs; therefore the results presented here should be interpreted with caution.

Although most of the oseltamivir-resistant viruses remain susceptible to zanamivir, the unpredictable concentrations of zanamivir in peripheral lungs after inhalation as well as the potential induction of bronchospasm, especially in young children (Moscona, 2005), constitute an important limitation for such treatment. However, the advent of intravenous zanamivir, currently in phase III clinical trials, may represent a promising alternative. In that regard, a crossover study carried out on healthy Thai adults found no clinically significant pharmacokinetic interactions between oseltamivir and zanamivir (Pukrittayakamee et al., 2011).

The main conclusion of our study is that zanamivir was superior to oseltamivir therapy in A(H3N2) and particularly A(H1N1)pdm09 infections in our mouse model. Moreover, there was no additional benefit of combination therapy over zanamivir alone. Such differential effects of the two NAIs could not be explained by their *in vitro* activity but are probably related to their different pharmacokinetic properties. Additional controlled studies are needed to determine the best therapeutic regimens in case of severe influenza infections, while minimizing the emergence of drug resistance.

ACKNOWLEDGMENTS

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TRANSPARENCY DECLARATIONS

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Figure 1.

Mortality (A), weight loss (B) and lung viral titers (LVT) (C) of C57BL/6 mice infected with A/Victoria/3/75 (H3N2) wild-type (WT) virus and treated with neuraminidase inhibitors. Groups of 12 mice were infected with 10^3 PFU of a mouse-adapted strain and treated 48h post-infection with saline (placebo control), oseltamivir 10 mg/kg by gavage, zanamivir 10 mg/kg intranasally, or oseltamivir-zanamivir combination, twice daily for 5 days. An uninfected group (saline) was added as control. On day 4 p.i., four mice per group were sacrificed and viral titers in lung homogenates were determined by plaque assay in MDCK cells. ##**P<0.01 for differences in mean weight loss between mice treated with oseltamivir compared to zanamivir (*) or oseltamivir-zanamivir combination (#), using one-way ANOVA with Tukey's multiple-comparison post-test.

Figure 2.

Mortality (A), weight loss (B) and lung viral titers (LVT) (C) of C57BL/6 mice infected with recombinant A/Québec/144147/09 (H1N1pdm09) wild-type (WT) virus and treated with neuraminidase inhibitors. Groups of 12 mice were infected with 10^5 PFU of the recombinant WT or H275Y strain and treated 48h post-infection with saline (placebo control), oseltamivir 10 mg/kg by gavage, zanamivir 10 mg/kg intranasally, or oseltamivir-zanamivir combination, twice daily for 5 days. An uninfected group (saline) was added as control. On day 4 p.i., four mice per group were sacrificed and viral titers in lung homogenates were determined by plaque assay in MDCK cells. #*P<0.05, ##**P<0.01 for differences between mice treated with oseltamivir compared to zanamivir (*) or oseltamivir-zanamivir combination (#), using Log-Rank (Mantel-Cox) test for comparing Kaplan-Meier survival plots, and one-way ANOVA with Tukey's multiple-comparison post-test for comparing mean weight loss and LVT.

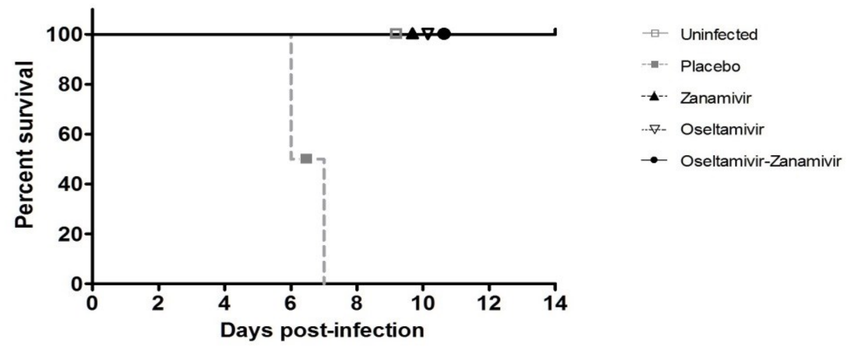
Figure 3.

Mortality (A), weight loss (B) and lung viral titers (LVT) (C) of C57BL/6 mice infected with recombinant A/Québec/144147/09 (H1N1pdm09) H275Y mutant virus and treated with neuraminidase inhibitors. Groups of 12 mice were infected with 10^5 PFU of the recombinant WT or

H275Y strain and treated 48h post-infection with saline (placebo control), oseltamivir 10 mg/kg by gavage, zanamivir 10 mg/kg intranasally, or oseltamivir-zanamivir combination, twice daily for 5 days. An uninfected group (saline) was added as control. On day 4 p.i., four mice per group were sacrificed and viral titers in lung homogenates were determined by plaque assay in MDCK cells. #*P<0.05, ##**P<0.01, ###***P<0.001 for differences between mice treated with oseltamivir compared to zanamivir (*) or oseltamivir-zanamivir combination (#), using Log-Rank (Mantel-Cox) test for comparing Kaplan-Meier survival plots, and one-way ANOVA with Tukey's multiple-comparison post-test for comparing mean weight loss and LVT.

Figure 1.

A



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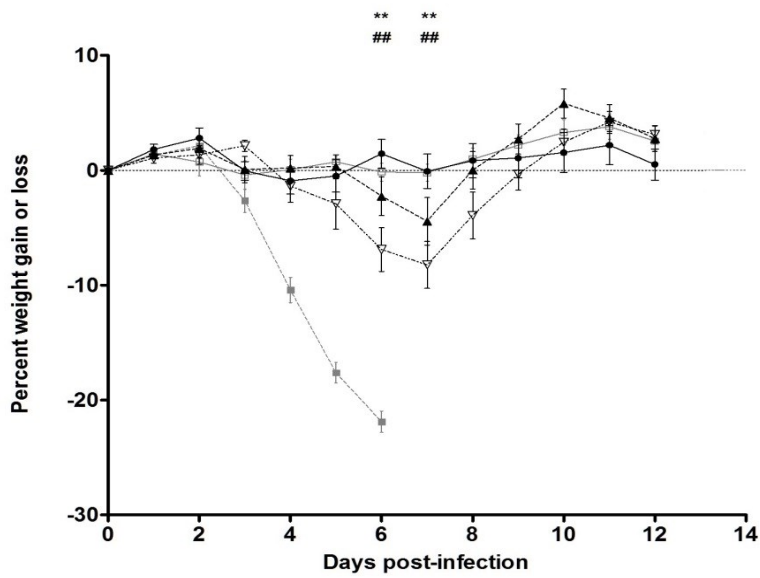


C

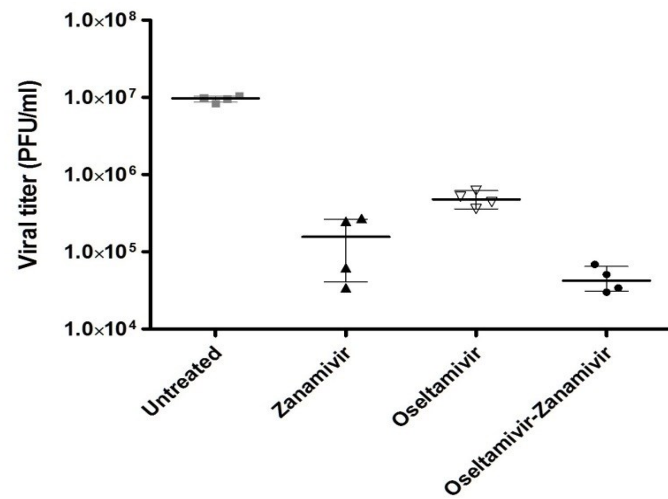
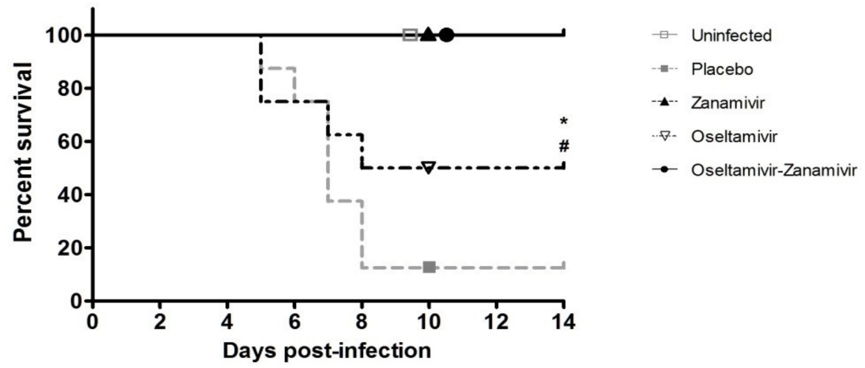


Figure 2.

A



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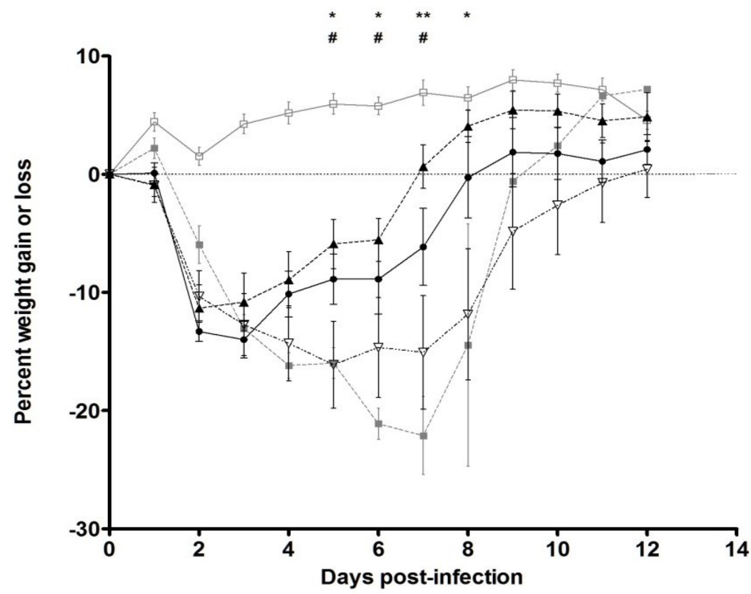

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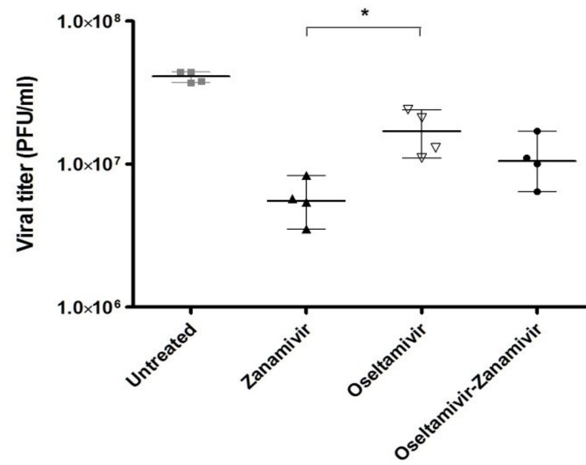
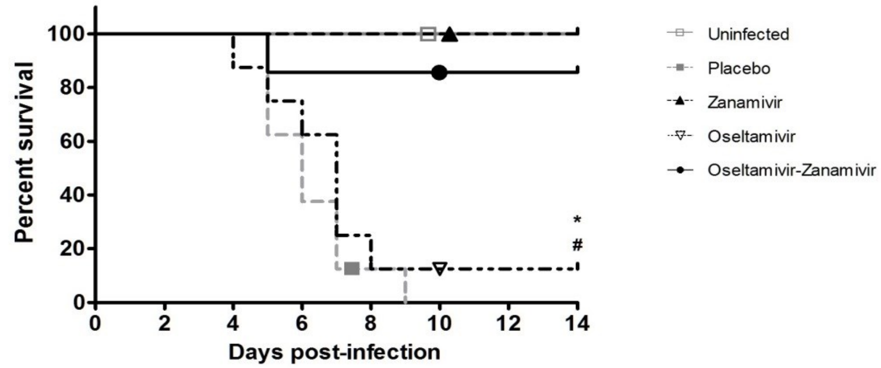


Figure 3.

A



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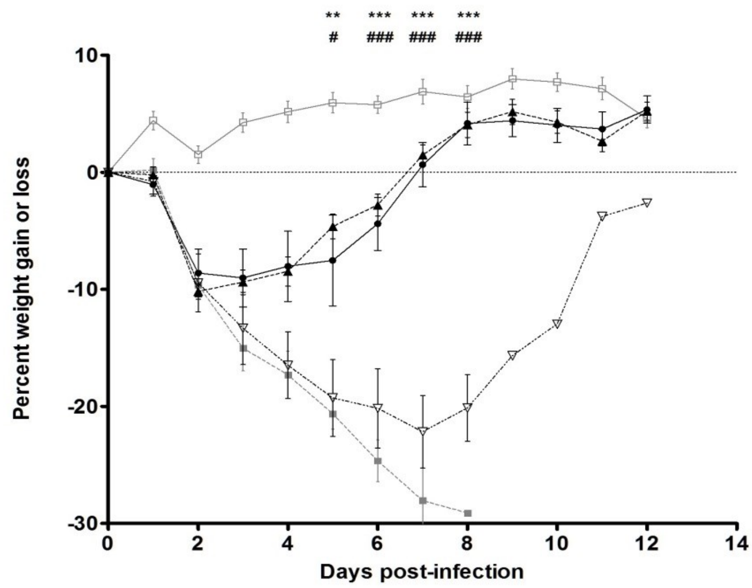
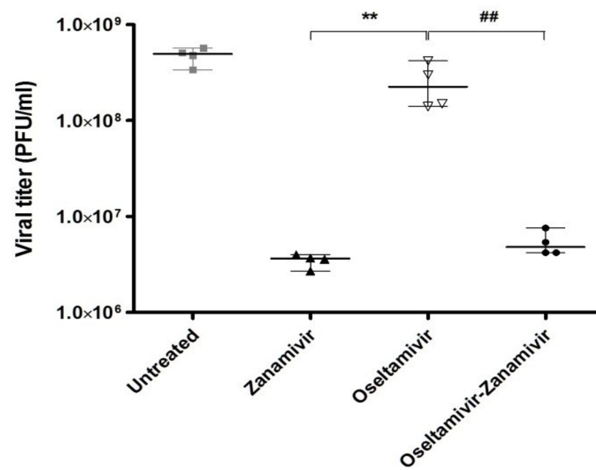


C



Chapter X: Discussion

Apart from annual vaccination, antiviral drugs play a central role in the control of influenza infections, with important implications in the management of outbreaks and pandemics as well as in immunocompromised and other at risk patients, with both prophylactic and therapeutic indications. However, as with many antimicrobial agents, the development of resistance is an important issue that compromises the long-term usefulness of this intervention. In fact, the global circulation of amantadine-resistant virus variants has led to the use of the NAIs oseltamivir and zanamivir as the anti-influenza agents of choice. Nevertheless, resistance to oseltamivir, the most prescribed NAI, has been found to occur not only during treatment and prophylaxis but also in the absence of NAI pressure. The principal NA mutations responsible for conferring clinically relevant resistance to oseltamivir exhibit type and subtype-specific differences. In particular, E119V and, to a lesser extent R292K, are responsible for oseltamivir resistance in N2 viruses whereas for N1 viruses the most usual mutation is H274Y [154]. The problem of resistance to NAIs was highlighted by the worldwide dissemination of the oseltamivir-resistant seasonal A(H1N1) H274Y variant during the 2007-09 annual influenza epidemics [195, 250]. In that case, preliminary observations speculated with the existence of a set of “permissive” mutations that could have facilitated this global transmission [32]. Fortunately, the antigenic shift that enabled the emergence of and global spread of the 2009 pandemic strain meant the replacement of the oseltamivir-resistant seasonal A/Brisbane/59/2007 (H1N1) virus by the naturally NAI-susceptible A(H1N1)pdm09 virus, and, consequently, oseltamivir recovered its clinical utility. In fact, most of the circulating A(H1N1)pdm09, A(H3N2) and B viruses remain susceptible to oseltamivir with only 1-2% of tested strains exhibiting phenotypic or genotypic evidence of resistance [194]. Nevertheless, the growing number of resistant strains recently detected in the absence of therapy raises concern that this problem could increase. In that sense, the different studies presented in this thesis are based on the premise that a better understanding of the mechanisms of antiviral resistance and the impact of these mutations on the therapeutic effectiveness will be useful for treatment management, especially among critically ill patients. We then focused on three aspects: the characterization of NA mutations associated with reduced susceptibility to NAIs; the study of potentially permissive mutations; and the preliminary evaluation of oseltamivir-zanamivir combined therapy.

10.1 Effect of specific NA mutations on antiviral susceptibility

Through this project, we continued the development and optimization of our laboratory platform for the thorough characterization of influenza viruses. This approach relies on the combination of many *in vitro* methodologies, such as culture assays in MDCK and SIAT1-MDCK cell lines, the generation of recombinant WT or mutant viruses and proteins, RT-PCR based characterization and sequencing, NA enzymatic and phenotypic tests, HA-elution and receptor specificity tests, as well as serologic assays. Moreover, *in vivo* replicative capacities, virulence and response to treatment are also assessed in established mouse models (BALB/c or C57BL/6), whereas viral transmissibility is evaluated in ferrets. In that regard, ferrets are considered one of the best animal models for the study of influenza infections since the distribution of α-2,3 and α-2,6 sialic acid receptors along their respiratory tract is highly similar to that of humans. As a result, unlike mice, ferrets are naturally susceptible to unadapted human influenza virus isolates. Ferrets inoculated with human influenza develop signs consistent with human influenza disease, including fever, nasal discharge, lethargy, weakness, and anorexia. Moreover, pathologic changes of bronchitis and pneumonia resemble those seen in humans, and because sneezing is a prominent feature of ferret influenza, they are the model of choice for viral transmission studies [277, 287]. Overall, the combined input from the different NA enzymatic tests with *in vitro* and *in vivo* replicative capacity and transmissibility assays provide valuable insight for the estimation of the viral fitness of NAI-resistant mutants and hence predict, although to a certain extent, the potential risk they might represent.

Our first study (described in chapter III) was conceived during the early days of the 2009 influenza pandemic with the aim to anticipate the viability and impact of NA mutations conferring resistance to NAIs in the A(H1N1)pdm09 virus. We considered that the best strategy to isolate the specific effect of each mutation of interest and minimize the potential interpretation bias due to compensatory/detrimental mutations in other parts of the viral genome was to use recombinant viruses. In that case, differences observed among generated viruses could be attributed to the selected NA mutations, since the rest of the viral genome would be identical. Following the strategy described by Liu and colleagues consisting of an eight bidirectional plasmid system [271], we generated a recombinant virus from the first A(H1N1)pdm09 WT clinical strain isolated in Quebec City (A/Quebec/144147/09), which is closely related to the prototype A/California/07/09 pandemic strain (NA and HA nucleotide identity of 99.7% and 99.6%, respectively). This was an important milestone of our project, since the new A(H1N1)pdm09 reverse genetics system would serve for

many future viral characterizations. At the time of the study, the only mutation reported to confer resistance to NAIs in the A(H1N1)pdm09 background was the prototypic oseltamivir-resistant H274Y mutation, so we decided to assess the effect of several mutations associated with resistance to NAIs in different influenza subtypes on antiviral susceptibilities, enzymatic activities and *in vitro* replicative capacities.

This study was the first to demonstrate that, in addition to the H274Y mutation, other NA mutations were replication competent in the A(H1N1)pdm09 background with some changes conferring specific or multi-drug resistance to currently available NAIs. In particular, the multi-drug resistant E119G/V mutants might represent a problem for immunocompromised patients, although their potential for transmission remains uncertain as suggested by reduced replication kinetics. The I222V NA change, previously described with the H274Y mutation in a case where transmission of oseltamivir-resistant viruses was suspected [57], not only increased the level of oseltamivir- and peramivir-resistance conferred by the H274Y mutation, but also restored NA substrate affinity and viral fitness *in vitro*. Although the absence of animal studies limited the assessment of the potential significance of our findings, they attracted attention to the I222 residue of the A(H1N1)pdm09 NA protein.

Shortly after this study, several reports described the emergence of other I222 NA mutations in either immunocompromised or immunocompetent patients, and related or not to antiviral treatment [57, 264, 317, 436]. Moreover, mutations such as I222R were described to confer reduced susceptibility to multiple NAIs in the absence of the H274Y mutation. We decided to use our reverse genetics system to introduce some of the reported I222 NA mutations combined or not with the H274Y substitution, and assessed the effect of these mutations on the susceptibilities to oseltamivir and zanamivir, and also the investigational NAI peramivir (study described in chapter IV). We then evaluated the replicative capacities of all the mutant viruses *in vitro*, as well as the virulence and transmissibility of some selected variants in ferrets.

We confirmed that I222V/R mutations can alter the susceptibility to one or more NAIs, and their combination with H274Y can significantly enhance the oseltamivir- and peramivir-resistant phenotype of the latter. Also, not only the fitness of I222V/R variants was comparable to that of the WT virus but the presence of I222V/R substitutions could indeed compensate for the slight loss of fitness due to H274Y. In line with that, the ferret experiments showed that the double I222V/H274Y

mutant virus conserved good contact and aerosol transmissibility. We also generated a molecular model to explain the differential and combinatory effect of each mutation on the binding to the NAIs. The hydrophobic faces of I222 and R224 form a hydrophobic pocket to accommodate the glycerol side chain of sialic acid and zanamivir, while E276 forms a hydrogen bond with the O8 and O9 hydroxyls of the glycerol group. Indeed, the proximity of I222 to the R224 and E276 residues determines that mutations in the former aa alter the shape of the hydrophobic pocket and/or its hydrophobicity, therefore interfering with the accommodation of the pentoxyl side chain of oseltamivir. On the other hand, although the binding of zanamivir to the NA active site does not require a conformational change, the presence of a bulky aa as in the I222R and I222K mutations already reported can cause moderate steric hindrance for the correct binding of the N-acetyl and glycerol groups of zanamivir.

While our model shed considerable light on the molecular interactions responsible for the differentially reduced susceptibility to NAIs among I222 NA mutants, with potential implications for the design of new antiviral molecules, it cannot explain the unusual plasticity of this framework residue. In fact, substitutions at residue 222 confer different degrees of reduced susceptibility in N1, N2, and type B viruses, with variable effects on the NA enzymatic properties. Mutations at residue 222 of NA are diverse, including I222V/M/T/R/K in N1, I222V in N2, and I222T/V in type B [290, 315], and have been detected after oseltamivir treatment in patients and *in vitro* exposure, but also spontaneously without drug exposure. Although the incidence of these substitutions is quite low, more functional studies are underscored.

The study described in chapter V, focused on the evaluation of A(H1N1)pdm09 viruses with reduced susceptibility to zanamivir. The increasing reports on the development of oseltamivir resistance among influenza A(H1N1)pdm09 viruses have raised consideration towards a more frequent use of zanamivir. Moreover, clinical studies on an IV formulation (not available for oseltamivir) [388] might constitute an added value for critically ill patients unable to receive NAIs orally. However, in contrast to oseltamivir resistance studies, little information is available on resistance to zanamivir in influenza A(H1N1) viruses in general and specifically in the A(H1N1)pdm09 virus. Of importance, apart from the I222R mutation conferring mild reduced susceptibility to zanamivir previously mentioned, no substitutions associated with highly reduced susceptibility to zanamivir in A(H1N1)pdm09 viruses have been reported. However, the E119G mutation emerged in

different A(H5N1) viruses after *in vitro* passages under zanamivir pressure [198], and also shown >800-fold increase in zanamivir IC_{50} values when introduced in the A(H1N1)pdm09 background in our first study. On the other hand, the Q136K substitution has been reported to confer reduced susceptibility to zanamivir in A(H3N2) clinical samples from immunocompetent [89] and immunocompromised patients [111], but also in seasonal A(H1N1) cultured isolates [199].

We used recombinant WT and mutant viruses to determine whether the E119G and Q136K A(H1N1)pdm09 NA variants retained their fitness and pathogenicity, and demonstrated that both mutations alter enzymatic properties (notably NA surface activity) to levels that significantly compromise viral fitness *in vitro*, in mice and in ferrets. In the case of E119G, the aa change was instable and rapidly reverted to the WT in our ferret experiments. Moreover, although an interesting study explained the mechanism of zanamivir-resistance induced by the Q136K mutation in different N1 proteins [164], multiple reports agree on the notion that the presence of the Q136K mutation is not a natural variant in N1 viruses but an artifact issued from viral propagation in MDCK cells [221, 324, 477]. Of note, an influenza A(H5N1) Q136L variant with highly reduced susceptibility to zanamivir and reduced susceptibility to oseltamivir was recovered from nasal wash samples of a ferret treated with zanamivir [109]. We then concluded that both studied mutations are quite improbable to emerge and disseminate in the clinical setting.

Surprisingly, the detection of the first A(H1N1)pdm09 clinical variant with highly reduced susceptibility to zanamivir was confirmed in October 2014, with the E119D NA substitution being responsible for the zanamivir-resistant phenotype (personal communication from Dr. Laurent Kaiser). In fact, the mutant virus was isolated from a 63-year-old patient that received non-related allogeneic hematopoietic stem cell transplantation, who developed a A(H1N1)pdm09 infection. The patient was initially treated with oseltamivir but developed the H274Y mutation. After 8 days of zanamivir treatment, the E119D NA mutation was identified. The patient died a few days later. Phenotypic studies using recombinant viruses showed that the E119D mutation conferred 827-, 25-, 28-, and 702-fold increases in IC_{50} values against zanamivir, oseltamivir, peramivir and laninanmivir, respectively, when compared to the WT (manuscript in preparation). Furthermore, the double E119D/H274Y mutant increased oseltamivir and peramivir IC_{50}s by 790-fold and >5000-fold, respectively, compared to the WT. These results are in line with those recently reported by Baek and colleagues [14], in which recombinant A(H1N1)pdm09 and A(H5N1) viruses harboring E119A/D/G

substitutions showed reduced susceptibility to many or all the NAIs tested. The E119 carboxylate group is critical for ligand binding through interactions with the 4-guanidino group of zanamivir (Figure 1.17 and [400]). While the E119G mutation causes the complete loss of that interaction as well as alterations in the solvent structure, in the E119D substitution, however, the negatively charged side chain of the aa is still present, although shortened, which might affect the formation of a weak hydrogen bond and hence reduce the binding of zanamivir in a more subtle, yet efficient way. As laninamivir also has a guanidine group with the same orientation, this mutation would also confer reduced susceptibility to this drug but have a milder effect on oseltamivir susceptibility, which contains an acetamide group instead of guanidine at that position. In fact, an *in vitro* study performed by our group confirmed that, as expected due to their structural similarities, the susceptibility profile of laninamivir is very similar to that of zanamivir. In that study, A(H1N1)pdm09 viruses harboring E119A/K NA mutations were selected after serial passages under laninamivir pressure [372]. While the relative total NA activity of expressed recombinant E119A was reduced by 63.5% compared to that of the WT, the total NA activity of the E119K was reduced by 99%. As a result, although a recombinant A(H1N1)pdm09 E119A virus was successfully generated by reverse genetics, it was impossible to generate the E119K mutant. After comparing the susceptibility profiles of the WT and E119A recombinant viruses to laninamivir, zanamivir, oseltamivir, and peramivir, as determined by NA inhibition assays, the E119A NA substitution conferred reduced susceptibility to laninamivir, zanamivir, oseltamivir, and peramivir with 82-, 90-, 17-, and 12-fold increases in IC_{50} values, respectively, compared with those of the WT virus.

It should not be forgotten that, as mentioned before in section 1.5.1.2, phylogenetic analyses have shown that the structure of the A(H1N1)pdm09 NA is quite distinct from those of other N1 viruses [266], which may entail differential effects among group specific NAI-resistance substitutions. Indeed, using recombinant proteins coupled to NAIs, Vavricka and colleagues [440] demonstrated that the open 150-loop of a typical group I NA facilitated the entry of the 4-guanidino group of zanamivir and laninamivir into the NA active site, relative to the closed 150-loop of group II NA, with an important role of electrostatic interactions between E119 and R156 residues. Interestingly, both drugs bound with intermediate affinity to the A(H1N1)pdm09 NA, and it is tempting to hypothesize that the impact of mutations at position E119 could be better tolerated in A(H1N1)pdm09 viruses than in other A(H1N1) or A(H5N1) viruses. Moreover, provided the actual sialic acid receptors on newly formed virions or host cells are more complex than a single sialic acid molecule, but recognizing that

this terminal group plays a crucial role in the recognition process by the NA, it could be suggested that the 150-cavity, which is adjacent to the active site, may also play an important role in the recognition of these receptors by the NA. Further investigation is warranted to determine whether this unusual characteristic of A(H1N1)pdm09 viruses makes them unlike other group I viruses in terms of antiviral resistant patterns as well as the balance between the receptor binding and receptor destroying activities of the HA and NA, respectively.

Our three studies discussed before highlight the tremendous utility of the use of recombinant viruses to discriminate the effect of specific mutations (or combinations of mutations) on antiviral resistance, as well as the specific interactions occurring in the catalytic site of the NA protein. Indeed, our results represented a valuable contribution for the creation of a detailed overview of resistance patterns found *in vitro*, in the clinic and in surveillance programs (see descriptive table in the annex). However, since this experimental approach requires the NA mutations to be chosen in advance, generating and testing recombinant viruses harboring all possible NA substitutions and their combinations would be humanly impossible, therefore limiting our ability to anticipate the potential emergence of unprecedented mutations.

The generation of drug-resistant influenza variants through *in vitro* serial passages under NAI pressure has proved itself as a valid option to overcome this limitation. Indeed, the main NA mutations conferring resistance to oseltamivir in humans in both N1 and N2 influenza subtypes were previously predicted by *in vitro* studies [154, 291, 414]. However, *in vitro* models do not mimic completely the distribution and configuration of sialic acid receptors in the respiratory tract, nor they account for the role of the immune response, for which *in vivo* models are needed. In the study described in chapter VI, we evaluated the effect of treatment with suboptimal doses of oseltamivir on the emergence and selection of drug-resistant viral variants in mice infected with A(H1N1) or A(H3N2) viruses, and also compared the sensitivity of traditional and deep sequencing methods to detect these quasispecies at the NA and HA levels. Our experimental set-up resulted in the rapid selection of an A(H3N2) variant harboring the I222T NA mutation, never reported before in this influenza subtype. Moreover, while traditional Sanger sequencing detected this viral subpopulation for the first time at the end of the second 6-day treatment course with oseltamivir, deep sequencing, on the other hand, already detected the I222T variant at the first sampling time-point (first passage, day 4). Deep sequencing also enabled the detection of differences of ~1% among viral populations

therefore allowing a time course analysis of quasispecies evolution. The observed dose-dependent selection pattern, combined with *in vitro* passages and phenotypic assays on recombinant proteins confirmed that the I222T substitution, never reported before in A(H3N2) viruses, conferred reduced susceptibility to oseltamivir. Surprisingly, the presence of an I222T minor population could be traced back to the initial inoculum, something that could have never been detected by classical methods.

In fact, such unprecedented level of information conferred by deep sequencing is of great utility for predicting the speed at which resistance will arise and also to gain insight on the relative fitness of some drug-resistant mutants. Moreover, and as shown by an increasing number of reports [111, 139, 480], early detection of minor viral populations could be of major clinical importance for the rapid and accurate implementation and adjustment of antiviral therapy in some patients, since resistance variants may be initially masked in the clinical samples containing mixed populations but rapidly favored and transmitted upon treatment. In consequence, although we might not yet have the perfect deep sequencing platform that combines low error rates with long reads and relatively low cost, it is a matter of time before this technology will be applied routinely in clinical virology laboratories.

10.2 Permissive NA mutations and the spread of resistance[2]

In the early days of NAIs, it was generally believed that emergence and selection of antiviral resistance would not be an important clinical problem because mutations in the NA catalytic site would entail a critical reduction of enzymatic activity and viral fitness. In line with that, old and recent oseltamivir-resistant seasonal A(H1N1) viruses harboring the H275Y NA substitution showed compromised fitness and poor transmissibility in animal models [26, 49, 213]. Nevertheless, the unattended emergence and global dissemination of the oseltamivir-resistant A/Brisbane/59/2007 (H1N1) virus during the 2007-09 seasons completely changed the paradigm. In fact, the H275Y (H274Y in N2 numbering) NA mutation conferring resistance to oseltamivir and peramivir was detected with increasing frequency in seasonal A(H1N1) viruses since 2007 to the extent that close to 100% of A/Brisbane/59/2007-like (H1N1) viruses that circulated in 2008-09 in Europe and North

[2] Since all publications on the topic of influenza permissive mutations adopt the N1 numbering system, the same system will be used in this section, instead of the N2 system used throughout this document.

America were resistant to oseltamivir [195, 250], hence demanding an explanation for how this H275Y variant replaced the supposedly "fitter" WT virus in the absence of antiviral pressure.

A gain of fitness might be the result of either specific mutations in other parts of the viral genome (detailed in section 1.8.1) or a secondary compensatory mutation in the NA gene. Compensatory mutations are a form of positive epistasis -a combination of mutations that is substantially more beneficial than the single mutations alone- in which one single mutation is deleterious in relation to a certain phenotype that is measured as a marker of fitness, but a second, despite being neutral or deleterious on its own, brings an organism (i.e. a virus) back to WT fitness. Moreover, these two mutations can occur at distant sites in the native folded protein. In the case of permissive mutations, the "compensatory" mutation is already present before the deleterious mutation (e.g.: the H275Y substitution causing reduced enzymatic properties and viral fitness) occurs, therefore facilitating its selection and fixation. A meta-analysis performed by Poon and colleagues [347] indicated that intragenic epistasis accounted for 83% of all compensatory mutations. It was then not unconceivable when, after phylogenetic and NA surface expression analyses, Bloom and colleagues [32] postulated that the dissemination of the oseltamivir-resistant A/Brisbane/59/2007 (H1N1) strain could have been enabled by the presence of three permissive NA mutations: R222Q, V234M and D344N. These mutations likely buffered the defects in NA folding or transport caused by H275Y substitution. In line with that, a previous study showed that NA proteins from recent (2007-08) A(H1N1) viruses had increased enzymatic activity compared to earlier viruses, which mostly correlated with the presence of a N instead of a D residue at position 344 [355].

In an attempt to further investigate the role of these potential permissive mutations (study described in chapter VII), we developed a reverse genetics system from a sensitive (275H) A/Brisbane/59/2007-like (H1N1) clinical isolate containing the three permissive NA mutations. Using mutagenesis, we first introduced the H275Y substitution and then reverted the codons at positions 222, 234 and 344 to the "non-permissive" aa. After comparing the NA enzyme properties and *in vitro* replicative capacities of the five recombinant viruses (WT, H275Y, H275Y/Q222R, H275Y/M234V and H275Y/N344D), we found that the reversion of the R222Q permissive mutation significantly reduced both the affinity and activity of the NA enzyme, resulting in a virus with a reduced *in vitro* replicative capacity. The further reduced replication shown by this revertant in infected and contact ferrets led us to conclude that the R222Q mutation might have been the major, but not necessarily

the only, permissive NA change that facilitated the emergence and spread of oseltamivir-resistant Brisbane/59/2007-like variants. The significantly reduced nasal wash viral titers observed in ferrets infected with the Q222R revertant point to the hypothesis of a reduced airborne transmissibility of the H275Y virus in the absence of the permissive mutation, which would be a determinant factor for global dissemination. Unfortunately, this premise could not be tested in our animal facility. Finally, although we did not observe a significant effect of the M234V and N344D reversions on viral fitness, we did not revert more than one permissive mutation at the same time so the possibility of a combinatory effect remains to be determined.

Shortly after the publication of our results, Ginting and colleagues [143] proposed that the T82K, K141E and R189K HA mutations could also exert a permissive effect as they improved the viral replication of the A/Tottori/52/2008-H275Y (Brisbane/59/2007-like) variant. We then used a 7:1 reassortant approach for assessing the impact of introducing the A/Brisbane/57/2007-like NA gene containing the H275Y mutation with or without the potential R222Q, V234M and D344N permissive substitutions into two different viral backgrounds, namely A/WSN/33 (H1N1) and A(H1N1)pdm09. We found that the transfer of the NA gene without the permissive substitutions, in particular the R222Q, in old and recent A(H1N1) backgrounds resulted in altered replication kinetics that were comparable to those obtained with non-reassortant recombinant variants in the previous study. Hence, the major impact exerted by the 222 residue of the NA protein on viral fitness was conserved despite the fact that the HA and the other 6 internal genes (NP, PA, PB1, PB2, M and NS) were significantly different from those of the original Brisbane/59/2007-like virus. Thus, the contribution of permissive mutations in other genes was unlikely.

Furthermore, the phylogenetic analysis performed by Bloom and colleagues [32] suggested that the R222Q permissive 'drift' became fixed during the 2006-07 period. By contrast, our Blast analysis revealed that influenza A(H1N1) viruses with the 222Q NA permissive mutation were already present during the 2000-02 period in different regions of the world. We then compared the replicative capacities of WT and oseltamivir-resistant A/Mississippi/03/2001 (H1N1) reference viruses, both of which differed only in the H275Y change but possessed a Q at position 222 of the NA. Of note, none of the other two potentially permissive NA (V234M and D344N) nor the HA (T82K, K141E and R189K) mutations were present in these viruses. The two strains grew at comparable titers at all time-points in multiple cycle kinetics experiments, suggesting once again a major permissive role for

the Q222R substitution. A manuscript reporting these findings has been recently accepted to the Antiviral Research journal.

The identification of permissive mutations in seasonal A(H1N1) viruses immediately raised the question whether a similar scenario could happen in A(H1N1)pdm09 viruses, mostly considering that H275Y mutant viruses were found to be as virulent and only slightly compromised compared to their WT counterparts in the pandemic background [162, 212, 296]. To address this issue, we performed the study described in chapter VIII of this thesis. We used reverse genetics to produce recombinant A(H1N1)pdm09 viruses and proteins harboring NA mutations either predicted as potentially permissive by computational analysis (T289M and N369K) [33] or identified both in an Australian community outbreak (V241I/N369K), as well as among Dutch travellers returning from Spain, in which transmission of H275Y viruses in the absence of antiviral use was reported [197, 294]. After testing the effect of these mutations on the *in vitro* replicative capacities, enzymatic properties, and virulence in mice, we found that the T289M and N369K mutations partially restored the NA activity, replicative capacity and virulence of the H275Y A(H1N1)pdm09 variant *in vitro* and in mice. We then selected the double H275Y/T289M and triple H275Y/V241I/N369K mutant viruses as the best candidates for ferret experiments. Surprisingly, no differences were observed between the H275Y and the H275Y/T289M mutants, although the latter conferred the most significant compensatory effect in all other tests and had been predicted with one of the highest scores in the computational analysis. On the other hand, despite the triple H275Y/V241I/N369K was attenuated in the mouse model, it showed significantly increased replication in ferret nasal wash samples suggesting the potential of improved transmission. Once again, airborne transmissibility -the most effective means of transmission in influenza viruses- could not be assessed in our study. Our results had considerable impact, since they demonstrated that although the detection of the H275Y mutation in A(H1N1)pdm09 viruses remains relatively low, with the appropriate permissive NA mutation(s), the H275Y mutant virus could restore its mildly reduced fitness and become more transmissible in the absence of antiviral pressure. Indeed, the reports on the circulation of V241I/N369K A(H1N1)pdm09 variants reinforce the fact that the threat of permissive NA mutations is not restricted to laboratory studies but does exist in the community [197, 294].

In fact, the permissive role of V241I and N369K NA mutations was recently confirmed in a very interesting study using a competitive mixtures ferret model of influenza infection with

contemporary A(H1N1)pdm09 oseltamivir-susceptible and oseltamivir-resistant strains [46]. In that study, apart from enhancing both the surface expression and total activity of H275Y A(H1N1)pdm09 NA proteins, the permissive mutations enabled A(H1N1)pdm09 viruses to maintain robust viral fitness and transmissibility when the virus acquired the H275Y oseltamivir resistance mutation. More importantly, the authors found that these permissive mutations are now present in almost all circulating A(H1N1)pdm09 viruses, meaning that recent A(H1N1)pdm09 viruses are more permissive to the acquisition of H275Y than earlier A(H1N1)pdm09 viruses, therefore increasing the risk of emergence and spread of oseltamivir resistance.

To conclude, intragenic epistasis itself has a rich history of investigation in the context of protein structure-function relationships in other organisms [217, 229, 380, 381, 446], but the few studies discussed above are among the first reports on the role of permissive mutations as an evolutionary mechanism tightly associated with antiviral resistance in influenza viruses. As a result, the specific mechanisms directing the propagation and fixation of these mutations remain mostly speculative, with stochastic drift, genetic hitchhiking and adjustment of the HA-NA balance as well as antigenic variations due to local and global immune pressure as possible factors. Once the permissive mutations are in place, the emergence of H275Y can be the product of any of these same forces and/or of direct selection for oseltamivir resistance. Using epidemiologic and surveillance data, computational models of the global spread pattern of oseltamivir-resistant seasonal A(H1N1) strains were generated [81, 82]. These models showed that the rapid dissemination of the resistant strains could not be explained solely by antiviral use, and postulated that the H275Y must have had a transmission advantage in untreated hosts of 1-2% compared to the WT counterpart. Based on the premise that for an emerging influenza virus strain to become prevalent, it requires fitness in receptor binding (via HA) and cleavage (via NA) mechanisms, replication efficiency and transmissibility, but also adaptation or evasion of the host immune surveillance, Wu and colleagues compared the antigenic properties of WT and H275Y seasonal A(H1N1) viruses [471]. Using H1-positive sera obtained from seasonal flu vaccine immunized and non-immunized individuals, and H1-specific monoclonal antibodies, they showed that oseltamivir-resistant strains exhibited a reduced reactivity to these antisera and antibodies, as compared to susceptible strains. They also observed that the H275Y mutant was less immunogenic than the WT counterpart in experimental infections in mice. They therefore concluded that oseltamivir-resistant seasonal A(H1N1) strains were less susceptible to antibody inhibition than oseltamivir-susceptible strains, and suggested that coupled NA and HA

mutations could be responsible for this observation [471]. In that regard, a thorough phylogenetic analysis performed by Niman proposed that the spread of the oseltamivir-resistant strain was due to genetic hitchhiking of the H275Y resistance marker with specific aa substitutions in the receptor binding domain of the HA protein, particularly A193T, which might have conferred and antigenic advantage for the fixation of the H275Y [322]. Furthermore, a study performed by our group using a mathematical model to analyze a set of *in vitro* experiments enabled the characterization of the viral replication cycle of A/Brisbane/59/2007-like (H1N1) and early A(H1N1)pdm09 viruses [345]. In both backgrounds, the H275Y mutation was shown to significantly lengthen the eclipse phase of the replicative cycle compared to the WT counterparts. Conversely, a significant difference between the infecting times of the A/Brisbane/59/2007-like (H1N1) WT and H275Y viruses was observed, with the latter possessing shorter infecting time and thus higher infectivity, a pattern that was not observed in the early A(HN1)pdm09 strain lacking the potential permissive mutations. Similar analyses with currently circulating A(H1N1)pdm09 viruses harboring the V241I and N369K NA mutations would be of great interest. However, despite the fact that the combination of computational simulations with recombinant viruses and proteins can offer an invaluable insight on the role of different combinations of mutations, characterizing the different properties of protein variants as well as identifying the connections between these properties and viral fitness can be laborious and complex. Anyhow, recognizing the potential permissive aa changes could not only contribute to direct surveillance of circulating viruses but also to better understand the emergence of antiviral resistance, being even of predictive importance. In the meantime, continuous and systematic surveillance of antiviral drug resistance markers is warranted not only in human viruses but also in animal influenza strains.

10.3 Treatment issues for NAI-resistant infections

Oseltamivir and zanamivir therapies were shown to reduce the duration of illness by ~1 day in patients not at risk when initiated within 48 h of symptom onset, being also effective in post-exposure prophylaxis [306]. In the case of patients at risk, many observational studies have shown the effect of NAIs in the reduction of mortality and improved outcome [182, 184, 248, 272, 364]. However, there is a lack of supporting evidence on how resistant mutations affect the clinical effectiveness of antiviral therapies and the alternatives to manage this type of infections.

The susceptibility pattern of circulating influenza strains usually dictates the selection of the most appropriate antiviral therapy. In contrast to oseltamivir, resistance to zanamivir has remained infrequent among seasonal and pandemic influenza isolates to date. As discussed before, due to structural differences between oseltamivir and zanamivir, influenza A variants containing the most frequent mutations conferring oseltamivir resistance (H274Y in the N1 subtype and E119V in the N2 subtype) were found to retain susceptibility to zanamivir [154, 346]. Accordingly, zanamivir positions as the antiviral of choice for the management of influenza cases or outbreaks involving oseltamivir-resistant viruses. However, inhaled zanamivir is not approved in children aged less than 5 years or individuals with underlying pulmonary disease [115, 209]. In addition, the safety and efficacy of inhaled or nebulized zanamivir in severely ill patients remains to be proven. Lastly, the zanamivir disc inhaler is not adequate for patients who are intubated as the lactose carrier can interfere with ventilator filters [232].

Moreover, approved antiviral treatment options for hospitalized patients with influenza are limited to oral or inhaled products. Thus, parenteral drugs other than the nucleoside analogue ribavirin [176] are highly needed for treatment of severe influenza infections. So far, the IV formulation of zanamivir has shown promising results in phase 2 clinical trials among patients with severe or progressive influenza [283]. As a result, IV zanamivir is now undergoing phase 3 studies, but can be obtained on a compassionate basis for severely ill patients with suspected or confirmed oseltamivir-resistant infections [166]. In fact, in a recent case report [187], IV therapy with 600 mg of zanamivir twice daily led to the rapid recovery of a 36-year old woman with severe A(H7N9) infection and previously treated with oral oseltamivir. On the other hand, if IV zanamivir is not available, IV peramivir could be alternatively considered. Despite exhibiting *in vitro* resistance, peramivir may retain significant activity against A(H1N1) H274Y variants *in vivo* due to excellent pharmacokinetic properties. Indeed, in healthy volunteers, a single IV dose of 600 mg of peramivir has resulted in plasma concentration of 34100 ng/ml, which is much higher than the IC_{50} value for H274Y variants (4.26 ng/ml) [389]. Accordingly, our mouse studies revealed that a single IM dose of peramivir provided important prophylactic and therapeutic benefit against the lethal A/WSN/33 (H1N1) virus and its H274Y variant [7]. However, controlled trials are needed to confirm the efficacy of parenteral peramivir against H274Y mutants in the clinic. Finally, the multimeric zanamivir compound laninamivir, which has proved to be useful against most oseltamivir-resistant viruses [247, 474], has also been shown to have “stable NA binder” pharmacokinetic profile comparable to that of peramivir

after one IV administration in mice [220], therefore opening the possibility for the development of an IV formulation.

Despite recent advances and the many promising investigational compounds in different development stages, our arsenal of antiviral strategies is still limited. A medical need exists for clinically proven safe and efficacious treatments with diverse resistance profiles for severe influenza. The situation is even more challenging in the case of very young children and immunocompromised patients as these population groups show a higher prevalence of NAI resistance, owing to higher and more sustained viral load despite antiviral therapy [24, 74, 453]. Thus, it is in this case when the concept of combined antiviral therapies acquires an important dimension. In one clinical study conducted by Kim and colleagues [236], amantadine-ribavirin-oseltamivir triple combination antiviral drug therapy was compared to oseltamivir monotherapy in patients with severe A(H1N1)pdm09 influenza. The study revealed that the 14-day mortality was significantly lower in patients treated with the combination therapy than in the oseltamivir monotherapy group, but no significant differences in survival at 90 days were found. In another small clinical study, Seo and colleagues [383] assessed the tolerability pharmacokinetics and efficacy pharmacokinetics of the amantadine-ribavirin-oseltamivir triple combination in healthy volunteers and six immunocompromised patients with influenza infection, with promising results. Indeed, they found that all but one patient receiving the triple combination therapy showed clinical improvement and earlier viral load reduction even in the presence of amantadine-resistant and/or oseltamivir-resistant strains. Nevertheless, there is still a need for supporting evidence for combined therapies with specific compounds in well-controlled trials, which does not yet constitute a standard clinical practice.

Considering once again the disparity between mutations conferring resistance to oseltamivir or zanamivir, we hypothesized that the outcome of influenza infections could be improved whereas the likelihood of emergence of resistance could be reduced by combining these two antivirals with differential interactions with the viral target NA protein. In that regard, combinations of oseltamivir and zanamivir or peramivir have shown concentration-dependent additive to antagonistic antiviral effects for A(H1N1) viruses *in vitro* [318]. Moreover, a study performed in healthy volunteers concluded that the combined administration of oral oseltamivir and IV zanamivir caused no important pharmacokinetic interactions between the two drugs [350]. We therefore performed a pilot study (described in chapter IX) to assess the potential clinical and virological benefit of the oseltamivir-

zanamivir combination compared to monotherapy in mice infected with NAI-susceptible A(H3N2) and A(H1N1)pdm09 viruses as well as with an oseltamivir-resistant A(H1N1)pdm09 virus. We found that the efficacy of the oseltamivir-zanamivir combination was comparable to that of zanamivir monotherapy and superior to oseltamivir monotherapy in terms of survival, mean life expectancy, weight loss, and lung viral titers for the three studied viruses. Overall, although the oseltamivir-zanamivir combined therapy was well tolerated, neither our pilot study, nor the few clinical trials already reported [51, 107, 109], produced conclusive results on the potential utility of this combination. Actually, Duval and colleagues observed slower virological and clinical responses in patients given the oseltamivir-zanamivir combined therapy compared with oseltamivir alone in adults with uncomplicated influenza [107], although the combination might have been more effective in reducing secondary transmission [51]. However, despite the lack of data on the pharmacokinetics/pharmacodynamics of zanamivir in mice restricts the selection of optimal doses and hence the interpretation of our results, the genetic homogeneity and controlled conditions of our model minimize the possibility of confounders when assessing the specific treatment-related effects. In fact, future preclinical studies with similar design but performed in immunocompromised mice or ferrets [437] could be of great utility to facilitate the evaluation of emergence and selection of resistant variants, something that did not occur in our immunocompetent mice. If the oseltamivir-zanamivir combination maintains its clinical efficacy comparable to that of the most potent drug in monotherapy but with reduced induction of NAI resistance, we will then be one step forward.

Chapter XI: Conclusions and perspectives

This doctoral project addressed a central public health problem with influenza infections, namely antiviral drug resistance. As treatment options for influenza remain limited, the development of resistance is a major factor in the long-term usefulness of antiviral drugs. In that sense, with the objective of "*better describing the mechanisms underlying the development of resistance to NAIs in influenza viruses*" we performed the series of studies described in this thesis.

We were the first to establish that, in addition to the well-known oseltamivir-resistant H274Y NA mutation, other NA mutations are replication competent in the A(H1N1)pdm09 background, with some changes conferring specific or cross-resistance to currently available NAIs. We then demonstrated that mutations at residue I222 can alter the susceptibility to one or more NAIs, and their combination with the H274Y mutation can significantly enhance the oseltamivir- and peramivir-resistant phenotype as well as compensate for the slightly reduced fitness of the latter. In addition, we produced a molecular model that provided important insight on the mechanisms of resistance to NAIs associated with mutations at this particularly polymorphic framework residue. We have also suggested that NA mutations conferring resistance to zanamivir in A(H1N1)pdm09 viruses are quite improbable to emerge and disseminate in the clinical setting given their compromised viral fitness, but should anyway be monitored in immunocompromised patients, thus having important implications for the utility of zanamivir in the management of influenza infections.

Our results also confirmed the role of permissive NA mutations as a newly identified mechanism associated with antiviral resistance, characterized the effect of certain specific mutations and demonstrated that this mechanism was also possible in A(H1N1)pdm09 viruses. As for treatment, we found that the oseltamivir-zanamivir combination does not seem to confer significant clinical advantage over monotherapy, and that treatment with suboptimal doses of NAIs can lead to rapid selection of drug-resistant variants such as the I222T NA mutation, described for the first time in A(H3N2) viruses. Last but not least, during this project we developed and/or optimized a panoply of laboratory techniques, notably the reverse genetics system for A(H1N1)pdm09 viruses, therefore providing a new platform for future characterizations of influenza viruses with mutations in any region of the genome, with relation to antiviral resistance but also to other biological aspects.

Considering all of the above, I can honestly and proudly conclude that the main objective of this thesis has been largely achieved, and that we have contributed to improve the knowledge related to this important viral infection. Nevertheless, numerous functional and adaptive mechanisms developed by influenza viruses remain unknown to us, and for every question we finally answer many others come on the scene. In that regard, we have demonstrated the relevance of combining *in vitro* and *in vivo* methods for the characterization of either permissive or NAI-resistance substitutions, as well as their versatility for the study of new emerging mutations. However, provided the close functional link between HA and NA proteins and the possibility of genetic hitchhiking, the introduction of the specific HA genetic baggage in future experiments assessing the impact and fitness of resistance mutations would be of great interest. It would also be important to incorporate deep sequencing technology to routine *in vitro* and *in vivo* strain competition experiments to assess the fitness and evolution of mutant viral subpopulations in the presence or absence of treatment, as well as to extend our characterization studies to influenza B of the two circulating lineages. In parallel, we will continue to refine our mathematical model to validate the resolution of each of the key parameters defining a specific strain's fitness (e.g.: determining that a strain produces more virus but has a reduction in its NA activity), which could help to predict the outcome of strain competition experiments and also identify a target for therapy, so as to minimize the emergence of that mutant strain. In the meantime, and as already mentioned, antiviral surveillance networks have a major role in the close monitoring and characterization of circulating strains harboring permissive and/or drug-resistance mutations not only in the human population but also among swine and avian reservoirs. Although it may be tempting to focus surveillance efforts in regions where antiviral agents are used the most and where one might expect resistance to arise, the regional prevalence of antiviral resistance might not correlate with usage of antiviral agents. It is therefore necessary to monitor the antigenic and immunogenic properties of circulating viruses, which may confer an evolutionary advantage to drug resistant strains.

As for treatment strategies, it has been clearly stated that current antiviral therapies leave room for improvement and the limited choice of antiviral drugs underscores the development of new anti-influenza compounds. Purposely, the expression of recombinant WT and particularly of mutant NA proteins coupled with sialic acid as well as with different antiviral agents to describe at the atomic level the differential interactions in the catalytic site constitutes a major design tool. Future treatment directions should focus on increasing the genetic resistance barrier, both by using higher doses of the

existing drugs, and/or combining existing and/or novel antiviral therapies, preferably with distinct modes of action and different viral targets. Moreover, targeting host responses instead of viral determinants to reduce viral replication and/or pathogenesis could provide broad-spectrum antiviral effects and limit the risk of emergence of drug resistance, a strategy successfully tested in antiretroviral therapy. Since performing randomized controlled trials is quite difficult and resource intensive, particularly in hospitalized patients with severe infections, initial proof-of-concept studies evaluating virological endpoints and tolerability in controlled animal models can provide important insight on the potential of certain drugs or drug combinations. Also, more flexible clinical trial designs, with planned modifications on sample size and treatment arms based on statistical analysis of data generated in the early stages of the trial in relation to objective endpoints, would enable the study of different treatment combinations simultaneously.

To conclude, it is the commitment of our team not to give up and keep walking this somehow endless path in the search of new viral targets, antiviral compounds and therapeutic strategies. At the end, we expect that our work will have an impact on the management of influenza infections by proposing innovative ways to improve the clinical outcome and minimizing the development of drug-resistant strains.

Chapter XII: Bibliography

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Annex: Influenza viruses with reduced susceptibility to NAIs found *in vitro*, in the clinic and in surveillance programs

Table. Selected neuraminidase mutations conferring resistance to NAIs (mainly reviewed in [373])

Influenza Subtype	NA mutation[a]	Virus source / NAI used for selection	Phenotype in NA inhibition assays:[b] Oseltamivir	Zanamivir	Peramivir
A(H1N1)	H274Y	Clinic / Oseltamivir	HRI	S	HRI
	Q136K	In vitro (clinic?) / None	S	HRI	RI
A(H1N1)pdm09	N294S	Reverse Genetics	HRI	S	RI
	H274Y	Clinic / Oseltamivir	HRI	S	HRI
	S246N/H274Y	Clinic / None	HRI	S	HRI
	I222V/H274Y	Clinic / Oseltamivir Reverse Genetics	HRI HRI	S S	-- HRI
	I222R/H274Y	Clinic / Oseltamivir Reverse Genetics	HRI HRI	RI RI	HRI HRI
	I222R	Clinic / Oseltamivir Reverse Genetics	RI RI	RI RI	-- RI
	E119G	Reverse Genetics	S	HRI	RI
	E119V	Reverse Genetics	RI	HRI	RI

A(H5N1)	N294S	Clinic / Oseltamivir	RI	S	S
	H274Y	Clinic / Oseltamivir	HRI	S	HRI
	D198G	In vitro / Zanamivir	RI	RI	S
	E119G	In vitro / Zanamivir	S	HRI	RI/HRI
A(H3N2)	R371K	Reverse Genetics	RI	RI	--
	R224K	Reverse Genetics	HRI	HRI	--
	N294S	Clinic / Oseltamivir	HRI	S	--
	R292K	Clinic / Oseltamivir Reverse Genetics	HRI HRI	-- S/RI	-- RI
	Del 245-248	Clinic / Oseltamivir	HRI	S	S
	D151A/D	Clinic?/ None	S	HRI	--
	Q136K	Clinic/ None	S	RI	--
	E119V/I222V	Clinic / Oseltamivir	HRI	S	S
	E119V	Clinic / Oseltamivir	HRI	S	S
B	R371K	Clinic / None	HRI	RI	--

	N294S	Clinic / None	HRI	--	--
	R292K	Reverse Genetics	S	RI	HRI
	H274Y	Clinic / ?	RI	S	RI
	I222T	Clinic / None	RI	S	--
	D198N	Clinic / Oseltamivir	RI	RI	RI
	R152K	Clinic / Zanamivir Reverse Genetics	HRI HRI	RI RI	HRI HRI
	E119A	Reverse Genetics	HRI	HRI	HRI
	E119D	Reverse Genetics	HRI	HRI	HRI
	E119G	Reverse Genetics	RI	HRI	HRI
	E119V	Reverse Genetics	HRI	S	HRI
	E105K	Clinic / None	S	RI	HRI

[a] Numbers indicate the position of the substituted residue in the NA aa sequence (N2 numbering)

[b] **S**, susceptibility or normal inhibition (<10-fold increase in IC_{50} over WT for A viruses or <5-fold increase for B viruses); **RI**, reduced inhibition (10-100-fold increase in IC_{50} over WT for A viruses or 5-50-fold increase for B viruses); **HRI**, highly reduced inhibition (>100-fold increase in IC_{50} over WT for A viruses or >50-fold increase for B viruses).