The antiviral resistance of influenza virus

The 2009 pandemic confirmed the increasing role antiviral treatment plays in influenza disease management in severe cases. In the 1960s adamantane derivatives were developed and used to treat influenza A virus infections. However, their limitations emphasized the need for the development of new classes of antivirals, such as neuraminidase inhibitors. Nowadays, different licensed neuraminidase inhibitors are available, but we still need new drugs in the anti-influenza pharmacopea. This article will provide the explanation of the mode of action of two classes of antivirals against influenza viruses, describe the mechanisms of resistance that viruses have developed against these products, and explain the evolution of the susceptibility of the influenza virus subtypes and types against these antivirals. We shall also address the expected evolution of the susceptibility of the viruses, the perspectives regarding new therapeutic options that have been used and/or new drugs that may be available in the near future.

KEYWORDS: A(H1N1) • A(H3N2) • A(H5N1) • adamantane • influenza B virus • influenza viruses • neuraminidase inhibitors • oseltamivir • resistance • zanamivir

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The last pandemic has prompted the use of neuraminidase inhibitors (NAIs) against influenza virus infection and highlighted the problem of the resistance of influenza viruses to antiviral agents. Before NAI, amantadine and rimantadine were used to treat influenza A infections but adamantane derivatives are no longer recommended. From 2005 to 2006 there was an important increase in the resistance to adamantanes and in 2010–2011 most of the A(H1N1) and A(H3N2) circulating viruses were adamantane-resistant. The NAIs oseltamivir and zanamivir were introduced into clinical practice between 1999 and 2002 and are the current antiviral agents used against influenza. Peramivir and laninamivir are more recent NAIs. Peramivir is approved in Japan and South Korea and laninamivir is approved in Japan.

The susceptibility to NAI of isolates recovered from 1996 to 1999 did not reveal any resistance to NAI in any of the isolates tested [1,2]. After 3 years of NAI use (from 1999 to 2002), 0.33% of viruses with a decrease in susceptibility to oseltamivir were isolated at a population level from various parts of the world [3]. Between 2004 and 2007, the percentage of NAI-resistant viruses remained low [4,5]. However, higher rates of oseltamivir resistance were described in isolates collected from NAI-treated patients, in particular from children. In this population, mutations responsible for resistance to NAI were detected in 5% [6], 18% [7] and 8.3% [8] of oseltamivir-treated children, respectively. These high rates of resistance could be explained by a prolonged and higher viral excretion in children as compared with adults. In addition, the NAI dose used in these children in Japan was suboptimal and could explain the rapid selection of resistant variants. Indeed, for children, oseltamivir is given at the dose of 2 mg/kg twice daily in Japan whereas in other countries, it is given according to weight groups (e.g., ≤15 kg, 30 mg twice daily; 15–23 kg, 45 mg twice daily; 23–40 kg, 60 mg twice daily; >40 kg, 75 mg twice daily). For example, a child weighing 10 kg will receive 40 mg/day in Japan and 60 mg/day in other countries [7]. In immunocompromised patients, influenza can become a chronic infection. The prolonged influenza virus shedding in the presence of drug treatment may lead to the selection of drug-resistant viruses in immunocompromised patients. These data suggested that the problem of NAI resistance mainly concerned treated children and/or immunocompromised patients.

In 2007–2008, seasonal oseltamivir-resistant A(H1N1) viruses emerged and became predominant the following winter season. These oseltamivir-resistant A(H1N1) viruses predominantly occurred in individuals who were not under treatment. The emergence and dissemination of this virus was unexpected as it was thought that oseltamivir-resistant viruses had an impaired fitness as compared with oseltamivir-sensitive ones.

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In April 2009, the emergence of the pandemic A(H1N1) 2009 virus displaced the seasonal oseltamivir-resistant A(H1N1) viruses. As a consequence, the circulating A(H1N1) viruses were again sensitive to NAI, and, as before 2007, most of the resistant viruses that have been isolated since 2010 have been isolated in patients receiving antivirals for treatment or prophylaxis.

The aim of this article is to describe the evolution of the resistance to adamantane derivatives and NAI for human [A(H1N1), A(H3N2) and B], and avian [A(H5N1)] influenza viruses. We report the evolution of the NAI-resistance for influenza A(H1N1) viruses and the main mutations responsible for NAI-resistance in influenza A(H3N2) and B viruses isolated in clinical human cases. The potential risks of emergence of new resistant viruses and the future perspectives for treatment or prevention are also discussed.

The evolution of the resistance to adamantanes

Mechanism of the resistance to adamantanes

Before the discovery of NAIs, adamantanes were used for more than 30 years for the prophylaxis and treatment of influenza A virus infection. Adamantane derivatives, amantadine and rimantadine, are inactive on influenza B but inhibit influenza A virus replication by blocking the proton channel activity preventing the acidification of the virus particles and the occurence of uncoating [9]. These drugs can reduce influenza illness by about 1 day if given within 48 h of infection but have gastrointestinal adverse effects and amantadine has adverse effects on the CNS [10]. Adamantane resistance emerges in 30% of the patients after 3 days of treatment. This frequent level of resistance could be explained by the fact that the mutations conferring resistance to adamantanes do not alter the function of the proton channel. A single mutation at one of the six residues of the transmembrane domain of the M2 protein (positions 26, 27, 30, 31, 34, 38 amino acid) can confer cross resistance to both amantadine and rimantadine, but the most frequent one is the S31N mutation (Figure 1) [11,12]. Mutations in M2 responsible for the resistance to adamantanes had no consequences on NAI resistance, which is linked to mutations in neuraminidase (NA).

Increasing resistance to adamantanes

A substantial worldwide increase in amantadine-resistant viruses, unrelated to adamantane use, has been reported for A(H1N1), A(H3N2) and also A(H5N1) viruses.

Between 1991 and 1995, the global surveillance reported that only 0.8% of the A(H3N2) circulating viruses were resistant to adamantanes. But the global incidence of adamantane resistance among A(H3N2) reached 15% in 2004–2005, with the highest frequency of resistance detected in isolates from China (93.4%), and by 2005–2006 it had reached 90.6%. The global incidence of adamantane resistance A(H1N1) viruses reached 4.1 and 15.5% of resistance in 2004–2005 and 2005–2006, respectively, with the highest level found in China (71.7% in 2005–2006) [13].

The phylogenetic analysis of the M gene sequences of A(H3N2) and A(H1N1) viruses, and a large-scale sequence analysis of the M gene from different species, revealed that the spread of resistance was neither due to reassortment between the two antigenic subtypes [13] nor due to reassortment between different hosts or subtypes [14]. Adamantane-resistant viruses spread even in the absence of drug pressure [14]. This is probably due to the lack of fitness impairment of these mutant viruses, however, an association with polymerase genes that favor viral replication should still be explored [13].

More recent seasonal influenza A(H1N1) viruses resistant to adamantanes remained at a low level in the USA (10.7% in 2007–2008 and 0.7% in 2008–2009) but were elevated in southeast Asia (33–100%) since 2007. Phylogenetically, the seasonal influenza A(H1N1) viruses formed two distinct clades, 2B and 2C. As the clade 2B A(H1N1) viruses (oseltamivir-resistant and adamantane-susceptible) increased, the clade 2C A(H1N1) viruses (oseltamivir-susceptible and adamantane-resistant) decreased [15].

In 2010–2011, seasonal A(H1N1) viruses were very rarely detected and the pandemic A(H1N1) 2009 viruses linked to the A/California/07/2009 strain, as well as the A(H3N2) viruses, were all adamantane-resistant. Indeed, the M gene of the pandemic A(H1N1) 2009 virus was homologous to the Eurasian swine lineage M gene bearing the S31N mutation responsible for resistance to adamantanes [16,201].

The A(H5N1) clade 1 viruses circulating between 1996–2005 in Vietnam, Thailand and Cambodia (southeast Asia) were all adamantane-resistant and bore the double mutation S31N and L26I [17]. However, the more recent influenza A(H5N1) subclade 2.2 and 2.3.4 strains were susceptible to adamantanes [18], although, some clade 2.3.4 isolates with the S31N mutation were reported [19].

In 2006, the US Centers for Disease Control and Prevention (CDC) recommended discontinuing the use of adamantanes to treat influenza virus infection owing to high levels of resistance for A(H3N2) viruses. However, in 2008–2009, when seasonal oseltamivir-resistant A(H1N1) viruses were the predominant viruses in circulation, the US CDC recommended the use of adamantanes as these A(H1N1) viruses were sensitive to adamantanes [20]. With the emergence of the pandemic A(H1N1) 2009 viruses resistant to adamantanes, these drugs are no longer recommended [21]. However, adamantanes could be useful in the future in the case of the emergence of a pandemic virus that would be sensitive to this drug [22].

Resistance to NAIs

Mechanism of the resistance to NAI

The role of neuraminidase for influenza virus multiplication

Influenza viruses present on their surface two major glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA). The HA is linked to the sialic acids at the cell surface and mediates the virus entry. The NA facilitates the virus entry by cleaving the sialic acid present in mucus secretion and allows the release of new virions from infected cells by cleaving the sialic acids from cellular glycoproteins and glycolipids and from both of the viral glycoproteins [23,24].

The NA of influenza B viruses are structurally distinct from the NA of influenza A viruses which fall into two distinct groups: group-1 NA containing N1, N4, N5, N8 enzymes and group-2 NA containing N2, N3, N6, N7, N9 enzymes [25]. Amino acid residues of active site interacting directly with the sialic acid are referred to as catalytic residues (in N2 numbering, R118, D151, R152, R224, E276, R292, R371, Y406) and amino acid residues that permit the stabilization of the active site are framework residues (in N2 numbering, E119, R156, W178, S179, D/N198, I222, E227, H274, E277, N294, E425) (Figure 2) [26]. The interactions of the NA active site and the sialic acid were described previously [27].

NAIs, their mechanism of action & NAI resistance

The NAIs are synthetic analogs of sialic acid. The discovery and development of oseltamivir and zanamivir have been reviewed [28]. Their interactions with the NA active site are different as zanamivir has a strong resemblance to the natural substrate sialic acid, whereas oseltamivir needs a conformational change in the NA active site in order to be linked. These structural differences between NAIs can explain the lower rate of resistance observed with zanamivir than with oseltamivir [29]. The most frequent NA mutations found in treated patients *in vivo* differ with the drug used and the influenza virus subtypes due to the various NA subtypes and their structural differences [24,25].

Some mutations conferring resistance to NAIs can occur during virus isolation and viruses bearing NAI resistance mutations can be selected in cell culture with drug selective pressure. *In vivo*, only some of these mutations responsible for NAI resistance were reported. In particular, the mutations on the catalytic active site usually induce a fitness cost and a drop in virus replication and transmissibility. However some permissive mutations can restore the fitness and the influenza virus variability can permit some mutations to emerge. The structural differences in the NA explain that the mutations responsible for NAI resistance are different according to the influenza virus subtype: H275Y for A(H1N1) viruses, R292K and E119V for A(H3N2) viruses and R150K and D197N for influenza B viruses [23]. In this article NA mutations are given mostly in the specific NA numbering; the correspondence in N2 numbering is indicated in Table 1.

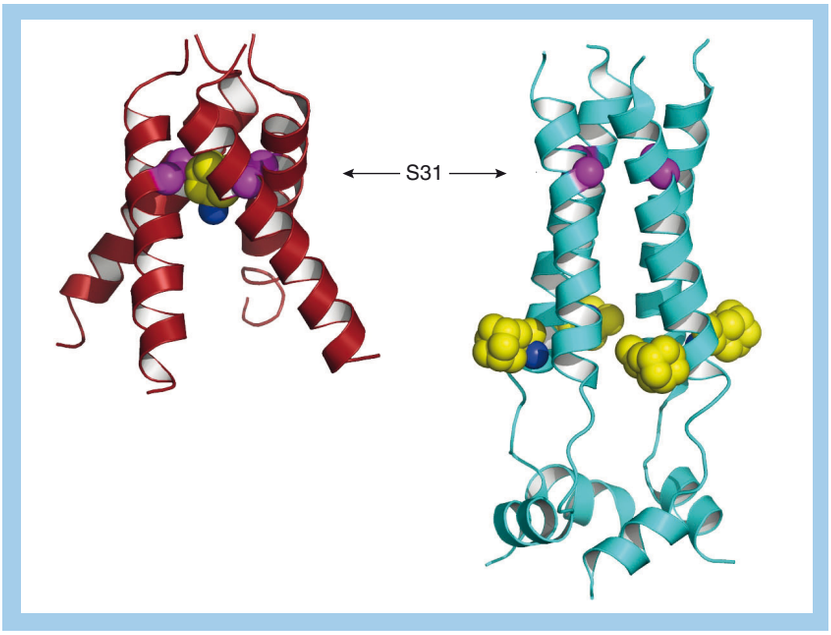

Figure 1. Cartoon representations of the M2 protein showing the structure determined by x-ray crystallography on the left and the structure determined by NMR on the right. The structure of the M2 protein determined by x-ray crystallography [145] (Protein Data Bank (PDB) code 3C9J) and by NMR [146] (PDB code 2RLF) are presented in complex with amantadine and rimantadine, respectively. Amantadine (x-ray) and rimantadine (NMR) are shown as spheres. The location of residue S31 is highlighted as a magenta sphere.

These mutations can confer resistance to one NAI or crossed resistance to different NAIs (Table 1). Usually, mutations in the active site, such as R292K and R152K in N2 numbering, confer resistance to both oseltamivir and zanamivir. In A(H1N1) viruses, the H275Y mutation confers resistance to oseltamivir and peramivir but retains susceptibility to zanamivir. In A(H3N2) the E119V mutation confers resistance to oseltamivir only. For influenza B viruses, the D197N mutation confers resistance to both oseltamivir and zanamivir but retains sensitivity to peramivir (Table 1).

The HA/NA balance

The fitness of a virus can be defined as its ability to survive and reproduce, which can be approximated by its ability to replicate and to be transmitted [30]. The viral fitness is linked to many factors and in particular to an optimal HA/NA balance.

The NA mutations responsible for NAI resistance have consequences on the neuraminidase affinity and activity that can be measured by enzymatic testing. Mutations in HA can modify the HA affinity for sialic acid. HA and NA have the same substrate, the sialic acid, but antagonistic functions; HA allows the entry of the virus into cells, whereas NA allows the release of new virions. If the NA had too high an affinity and activity, it could cleave the sialic acid on the cell surface impeding viral entry. If the NA had too low an activity, it could prevent the release of new virions. An optimal functional balance between HA and NA activity is therefore required for efficient viral replication [31]. Some *in vitro* experiments with drug-selective pressure led to the emergence of variants with mutations in the HA receptor-binding site conferring a lower affinity for sialic acid and a viral multiplication in the presence of the NAI [32]. Some variants with mutations in HA could compensate some of the NA mutations. *In vitro* drug selection experiments identified mutations in HA that decreased the affinity of HA for sialic acid and made the mutant viruses less dependent on NA activity to be released from the infected cells. The decrease in HA affinity often results from extra glycosylation of HA near the sialic acid binding site [24].

Surveillance of the NAI resistance of influenza viruses

The drug resistance of circulating influenza viruses is monitored by national and world influenza centers. This global surveillance permits the identification of mutations in NA or HA associated with the detection of *in vitro* resistance to current antiviral agents against influenza viruses. The isolates tested are from various geographical regions, from patients of different ages and from mild to severe cases of influenza virus infections.

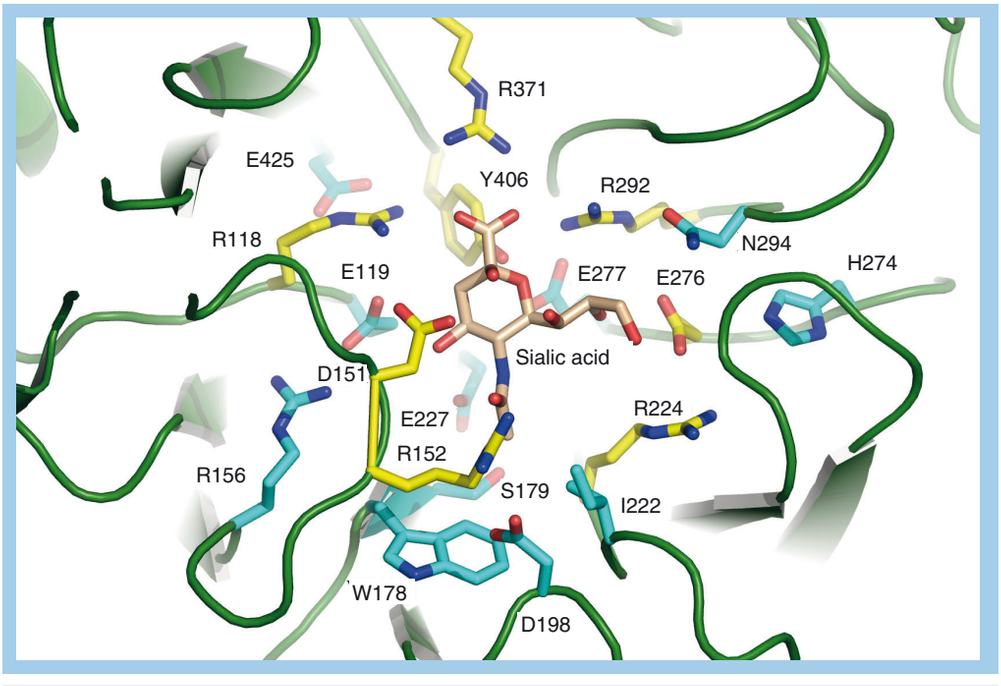

Figure 2. The active site of N2 NA bound to sialic acid. The active site of N2 NA in complex with sialic acid [27] (PDB code 2BAT) is presented. Residues involved in catalysis are shown as yellow sticks and framework residues in cyan sticks. The positions of some key binding residues are shown with nitrogens in blue and oxygens in red.

Studies performed in immunocompetent patients and clinical reports of treated immunocompromised patients allow the identification of mutations responsible for NAI-resistance in influenza viruses from human cases (TABLE 1). However, NAI resistance is related to laboratory assays and at this time, very limited data exist on the clinical relevance of any of the described mutations. A few studies conducted in Japan tried to correlate mutations and NAI-resistance measured *in vitro* with clinical outcomes *in vivo*. Virus shedding after oseltamivir therapy was longer in influenza B than in influenza A virus infected patients, which is correlated with higher IC_{50} for influenza B than influenza A viruses [33]. Oseltamivir had a lower clinical effectiveness, estimated by body temperature, for the treatment of seasonal A(H1N1) viruses bearing a H275Y mutated NA than for the treatment of non-mutated seasonal A(H1N1) viruses [34].

Directed mutagenesis and reverse genetics allow the construction of mutant viruses and can help to predict the impact of mutations on the enzymatic properties of the neuraminidases and the capacities of such viruses to replicate in cell culture. *In vivo* studies in animal models can be performed with such mutant viruses in order to evaluate their capacity of infection, their virulence and their transmissibility. These studies can help in understanding how mutant viruses have emerged and can help predict the mutations that could preferentially emerge *in vivo*.

In this article we will focus on the NA mutations reported from influenza viruses isolated in human cases, according to the influenza virus subtype.

Each mutation is not reported with the same frequency. The H275Y mutation responsible for oseltamivir resistance in A(H1N1) viruses is frequently reported. In N1, this H275 residue can switch to 275Y with no fitness cost. As a result, we observed the emergence of H275Y seasonal A(H1N1) viruses in 2007–2008, and several cases of H275Y A(H1N1) 2009 viruses during the pandemic. On the contrary, mutations associated with resistance in influenza A(H3N2) or B viruses are very rarely reported, mainly concern severely immunocompromised patients, and seem to have a fitness cost that impairs transmission of the resistant strain.

Assays to evaluate the susceptibility to NAI

The susceptibility to NAI is evaluated by assays using either a fluorescent [35] or chemiluminescent substrate [36]. The two methods are reliable for the detection of NAI resistance. Due to its reproducibility and ease of automation the NA inhibition assay using a luminescent substrate method was selected for global surveillance by the Neuraminidase Inhibitor Susceptibility Network [37]. However, R292K mutation did not confer a significant increase in the zanamivir IC_{50} with the chemilumiscent substrate compared with the fluorescent one [4]. Moreover, the chemiluminescent assay gives generally lower IC_{50} than the fluorometric assay that offers a better discrimination between the IC_{50} of the mutant and the wild-type (WT) viruses [38]. Currently, there is no global consensus definition of the resistance. However, efforts are underway to form a global consensus. According to a Neuraminidase Inhibitor Susceptibility Network study, a virus is resistant if it's IC_{50} is superior to tenfold the mean IC_{50} of sensitive relative viruses; a virus is of intermediate susceptibility if it's IC_{50} is superior to the cutoff, defined by the mean IC_{50} value of related viruses +3 standard deviations, but remains inferior to tenfold the mean IC_{50} [4].

Before the introduction of NAIs, the results of global surveillance showed the basal susceptibility of NA. The IC_{50} for oseltamivir and zanamivir are similar between N1 and N2 NA but are lower for the NA of influenza A compared with influenza B viruses [1,2,23]. As an example for the IC_{50} obtained for the different NA subtypes, we present the data obtained globally and in the National Reference Center for Influenza (south of France) (TABLE 2).

These assays require the virus to be isolated in cell culture. This step can amplify some variants that are present in a low proportion in the initial isolate *in vivo* and can constitute a bias by increasing the impact of some *in vivo* mutant viruses [38–40]. Therefore, it is important to develop molecular methods that could be performed directly on the patient sample to avoid the risk of considering mutations that are not of clinical importance.

The NA inhibition assays should be used in conjunction with a NA sequence analysis. A recent study highlights the need to check for the presence of mixed populations of viruses when isolates with reduced susceptibility to NAI are detected. Indeed, cases of A(H3N2) viruses initially reported with a reduced susceptibility to NAI were identified as mixed infection with influenza A and B viruses after plaque purification. Plaque purification is needed to ensure pure populations and to check and confirm the role of potential NA mutations in the susceptibility of influenza viruses to NAI [41].

Table 1. Neuraminidase mutations reported *in vivo* according to the influenza virus subtype.

Subtype	Mutation		Sort of residue	Inhibitor susceptibility *in vitro*				Context of the mutation detection	Ref.
NA	*(N2 numbering)*	*(NA numbering)*		*Zanamivir*	*Oseltamivir*	*Peramivir*	*A-315675*		
N1[†]	H274Y	H275Y	Framework	S	R			Global surveillance	[3–5,50]
	H274Y	H275Y	Framework	S	R	R	S	Oseltamivir/competent	[8,125]
	H274Y	H275Y	Framework	S	R	R	S	Oseltamivir and zanamivir/ immunocompromised	[110,125]
	G248R + I226V			R	R			Global surveillance	[3]
	Y155H			R	R			Global surveillance	
	I222V		Framework	S	I			Global surveillance	
N1[‡]	H274Y	H275Y	Framework					Global surveillance	[52,53]
N1[§]	H274Y	H275Y	Framework	S	R			Oseltamivir treatment	[66,71–74,87]
	H274Y	H275Y	Framework	S	R			No treatment	[75–77,79,80]
	I222R	I223R	Framework	I	I/R	S		Zanamivir/immunocompromised	[88]
	H274Y + I222R	H275Y + I223R	Framework	I/R	R	R		Oseltamivir/immunocompromised	[90]
	H274Y + I222V	H275Y + I223V	Framework	I	R			Oseltamivir/immunocompetent	[74]
	S246N	S247N	Framework	I	I	S		No treatment	[91]
	H274Y + S246N	H275Y + S247N	Framework	I	R	R		Oseltamivir/immunocompromised	[91]
N1[¶]	H274Y	H275Y	Framework	S	R	R		Oseltamivir/severe case	[97,98]
	N294S	N295S	Framework					Before treatment	[99]
	N294S	N295S	Framework		I			Oseltamivir/severe case	[98]
N2[#]	E119V	E119V	Framework	S	R			Global surveillance	[4,5]
	E119V	E119V	Framework		R			Oseltamivir/competent	[7,8]
	E119V	E119V	Framework	S	R	S	S	Oseltamivir/immunocompromised	[38,110,114, 125,126]
	E119V	E119V	Framework		R			Before and during oseltamivir/ immunocompromised	[111]
	E119I	E119I	Framework	R	R	S/I	I	Oseltamivir/immunocompromised	[38]
	I222V	E119V + I222V	Framework	I	R	I		Oseltamivir/immunocompromised	[114,115]
	R292K	R292K	Catalytic		R			Oseltamivir/competent	[7,8]
	N294S	N294S	Framework		I/R			Oseltamivir/competent	[7]

We used the criteria given in a NISN study [4]: a virus was indicated R if its IC_{50} was superior to tenfold the IC_{50} or mean IC_{50} of sensitive relative viruses; a virus was of I if its IC_{50} was superior to the cutoff, defined by the mean IC_{50} value of related viruses plus three standard deviations or remained inferior to tenfold the mean IC_{50} of sensitive relative viruses.

[†]A(H1N1) before 2007.

[‡]A(H1N1) related to A/Brisbane/59/2007.

[§]pandemic A(H1N1) 2009.

[¶]A(H5N1).

[#]A(H3N2).

I: Intermediate susceptibility; R: Resistant; S: Sensitive to the inhibitor.

Table 1. Neuraminidase mutations reported *in vivo* according to the influenza virus subtype (cont.).

Subtype	Mutation		Sort of residue	Inhibitor susceptibility *in vitro*				Context of the mutation detection	Ref.
NA	*(N2 numbering)*	*(NA numbering)*		*Zanamivir*	*Oseltamivir*	*Peramivir*	*A-315675*		
B	R152K	R150K	Catalytic	R	R	R	R	Zanamivir/immunocompromised	[110,124]
	D198N	D197N	Framework	I	I			No treatment	[123]
	D198N	D197N	Framework	R	R	S	S	Zanamivir or oseltamivir/ Immunocompromised	[110,124–126]
	D198E	D197E	Framework	I	R	R		No treatment	[3,127]
	D198Y	D197Y	Framework	R	R			Global surveillance	[50]
	I222T	I221T	Framework	I/R	R			Global surveillance or no treatment	[3,4,123]
	S250G	S249G		R	S			No treatment	[123]
	N294S	N294S	Framework	S	R			Before and during oseltamivir/ immunocompromised	[111]
	R371K	R374K	Catalytic	R	R			No treatment, global surveillance	[4]

We used the criteria given in a NISN study [4]: a virus was indicated R if its IC_{50} was superior to tenfold the IC_{50} or mean IC_{50} of sensitive relative viruses; a virus was of I if its IC_{50} was superior to the cutoff, defined by the mean IC_{50} value of related viruses plus three standard deviations or remained inferior to tenfold the mean IC_{50} of sensitive relative viruses.

[†]A(H1N1) before 2007.

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I: Intermediate susceptibility; R: Resistant; S: Sensitive to the inhibitor.

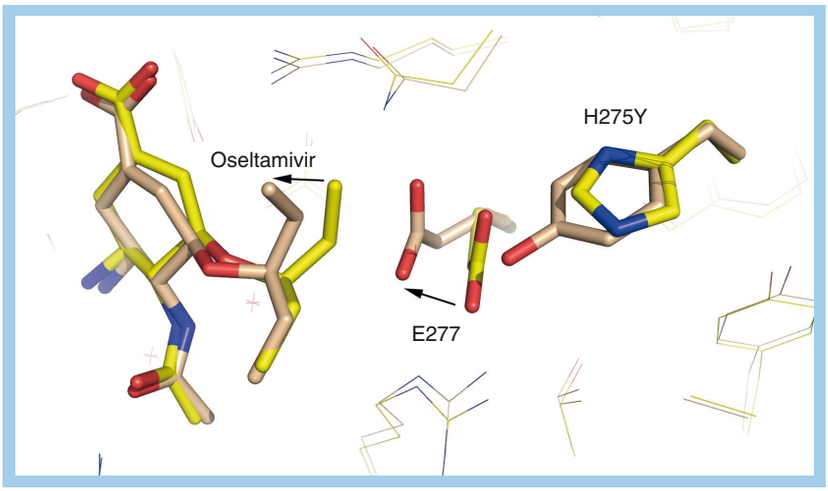

Figure 3. Superposition of the crystal structures of wild-type and H275Y mutant of N1 NAs bound to oseltamivir. The crystal structures of wild-type (ribbons colored yellow) [25] (PDB code 2HU4) and mutant (ribbons colored tan) [48] (PDB code 3CL0) N1 NAs are shown with oseltamivir colored similarly. The positions of some key binding residues are shown with nitrogens in blue and oxygens in red. The shift of residue E277 is highlighted.

Real-time polymerase chain reactions [42,43] or pyrosequencing using specific primers for known mutations can easily detect well identified mutations responsible for resistance and can be performed directly on the initial clinical specimen [44–46]. Pyrosequencing can detect as little as 5% of H275Y mutant virus in a mixed viral population [47]. These techniques allow a rapid diagnosis and are very useful for severe patients. However, the primers should be designed each time a new variant has emerged.

Influenza A(H1N1) viruses & resistance to NAI

Mutation H275Y

This mutation conferred a resistance to oseltamivir and peramivir but retained susceptibility to zanamivir.

Structural studies based on crystallography showed that the mutation H275Y induced an impairment in the conformational change of the E277 to accommodate the hydrophobic chain pentyl ester of oseltamivir (Figure 3). The zanamivir has a glycerol hydrophilic chain and its link to the active site is not impaired by the H275Y mutation [48,49].

Before 2007/2008, the H275Y mutation was uncommonly detected in clinical studies and emerged after oseltamivir treatment [23]. It was also detected in surveillance studies [4,5,50]. It was thought that the low level of isolation of A(H1N1) viruses with the H275Y mutation was due to a lower fitness of resistant viruses [51]. But the emergence of seasonal oseltamivir resistant A(H1N1) viruses in 2007 pointed out that every consideration on drug resistance was dependant on viral subtype and influenza virus variability.

The emergence of oseltamivir-resistant seasonal A(H1N1) viruses in 2007–2008

In 2007–2008, a significant proportion of oseltamivir-resistant A(H1N1) viruses with the H275Y mutation were detected in several countries. Norway gave the alert in December 2007, and the mean proportion of oseltamivir-resistant viruses was then found to be 25% in Europe [52,53]. The following winter, nearly 100% of the seasonal A(H1N1) isolates in the world were oseltamivir-resistant [54–56].

The emergence of these oseltamivir-resistant viruses was apparently not related to any oseltamivir selective pressure as resistant viruses were predominantly isolated in nontreated patients [53,54,57] and only 3% were reported in Japan where NAIs are largely used [58].

The emergence of this oseltamivir resistance was concomitant to the emergence of a new influenza variant related to the A/Brisbane/59/2007 vaccine strain. There was no significant difference in the influenza-like illness caused by oseltamivir-resistant or oseltamivir-sensitive influenza A(H1N1) viruses [53,54,57]. Both the oseltamivir-sensitive and resistant viruses were transmitted efficiently, but the resistant ones became predominant, as if the viruses bearing the H275Y mutation had an advantage leading to the propagation of the oseltamivir-resistant viruses over the sensitive ones. Indeed, *in vivo* assays in ferrets showed higher nasal wash virus titers for oseltamivir-resistant than oseltamivir-sensitive viruses related to A/Brisbane/59/2007 [59]. One hypothesis is that the H275Y mutation has restored a correct balance between the HA binding to sialic acid and the NA affinity for sialic acid that was significantly altered by previous mutations that increased this NA affinity [49,60,61]. Recent studies showed that some mutations in NA (V234M and R222Q) increase surface expression of properly folded NA and can counteract the alterations due to the H275Y mutation and restore viral fitness. The emergence of oseltamivir resistance was then enabled by these 'permissive' mutations and the H275Y mutation could have been selected by restoring a functional HA/NA balance [62,63]. Another hypothesis suggests that some mutations enhancing viral fitness could have been associated with the H275Y mutation.

Some studies report mutations in NS1 [64] and in PB2 [65] that were preferentially associated with the H275Y mutation; however the impact of these mutations on viral fitness is not known.

Pandemic A(H1N1) 2009 viruses & resistance to NAI

With the emergence of the pandemic influenza A(H1N1) virus of 2009 (A(H1N1) 2009), the seasonal oseltamivir-resistant A(H1N1) viruses were very rarely detected [201]. The A(H1N1) 2009 viruses remain oseltamivir-sensitive: more than 98% of the 9300 influenza A(H1N1) 2009 viruses tested by the WHO Global Influenza Surveillance Network were oseltamivir-sensitive [202].

Most of the cases of oseltamivir resistance were described in treated immunocompromised and/or severely ill patients [63,66]. Immunosuppression is associated with a prolonged viral excretion [67] and the persistence of viral replication is an important source of genetic diversity and facilitates the selection of drug-resistant viruses by the treatment [68]. In severely ill patients, the immunity is compromised by the state of sepsis [69] and prolonged influenza virus excretion and detection of drug-resistant viruses has been linked to lymphocytopenia [70]. The transmission of oseltamivir-resistant viruses was mainly observed in immunocompromised patients. Clustered cases were reported for patients admitted to hematological units and the person-to-person transmission of oseltamivir-resistant viruses was confirmed epidemiologically and by sequence analysis [71]. A case of transmission between two hospitalized children with cerebral palsy was described [72].

Some oseltamivir-resistant pandemic A(H1N1) 2009 viruses were reported in people who received prophylaxis treatment. If the patient was already infected, then he received a subtherapeutic level of oseltamivir, which permitted the selection of oseltamivir-resistant variants [73,74].

In a few cases, some oseltamivir-resistant A(H1N1) 2009 viruses were described with no known exposure to oseltamivir. The first case was described in Hong Kong [75,76]. A community cluster of seven cases of resistant viruses was described in Vietnam with the detection of the H275Y mutation before any oseltamivir treatment [77]. Other cases were described in community patients with no known exposure to oseltamivir [78–81]. These observations suggest a possible transmission of resistant strains in the community.

Viral fitness was studied in animal models. The pathogenicity was similar in murine or ferret models for oseltamivir-sensitive or -resistant influenza A(H1N1) 2009 viruses [82–84]. Concerning the capacity of transmission of these viruses, most studies found that the transmission for oseltamivir-sensitive or -resistant viruses was similar by direct contact or aerosol in murine, guinea pig or ferret models [82–86]. However, in one study the oseltamivir-resistant viruses seem to lose their capacity to be transmitted via the respiratory route [83]. The co-infection of a ferret with oseltamivir-sensitive and oseltamivir-resistant A(H1N1) 2009 viruses revealed an increase in the proportion of the WT virus to 100% 6 days post-infection. These results seem to indicate a better fitness of the oseltamivir-sensitive viruses than the oseltamivir-resistant ones [83]. In human infection, the predominance of the oseltamivir-sensitive WT strain after treatment cessation suggests a better *in vivo* fitness of oseltamivir-sensitive versus oseltamivir-resistant A(H1N1) 2009 viruses in the absence of drug selective pressure [75,87].

Mutation I223V/R alone or associated with the H275Y mutation

An I223V mutation in seasonal A(H1N1) virus was observed in surveillance studies [3]. A few oseltamivir-resistant pandemic A(H1N1) 2009 viruses bearing a mutation at residue 223 in NA were described in the world [63,74,88–90]. In an immunocompromised patient, the I223R mutation emerged during oseltamivir treatment for A(H1N1) 2009 virus infection and became predominant after administration of inhaled zanamivir [63]. The I223R mutation was also described in variants isolated from an immunocompromised patient treated with intravenous (IV) zanamivir who first developed H275Y mutation after treatment by oseltamivir. This I223R mutation conferred reduced susceptibility to oseltamivir and zanamivir [88].

The detection of an A(H1N1) 2009 virus with the dual mutations H275Y and I223V was reported for two patients receiving oseltamivir prophylaxis [74]. This I223V mutation could have been implicated in the possible transmission of the oseltamivir-resistant strain. Indeed, recombinant A(H1N1) 2009 virus generated by reverse genetics showed that the I223V mutation could, in part, restore NA affinity and activity altered by the H275Y mutation [89].

An A(H1N1) 2009 virus carrying the H275Y and the I223R mutations in NA was isolated from an immunocompromised child after prolonged treatment with oseltamivir. The dual mutation appeared before the child was treated

Table 2. Neuraminidase inhibitors mean IC_{50} from the global surveillance and from the National Influenza Center (south of France) according to the seasons and influenza virus subtype.

Season years	Viral subtype	n		Fluorescent assay, mean IC_{50} (nM)†		Luminescent assay, mean IC_{50} (nM)‡		Ref.
		Oseltamivir	*Zanamivir*	*Oseltamivir*	*Zanamivir*	*Oseltamivir*	*Zanamivir*	
Values obtained by the WHO for global surveillance								
Before the introduction of NAI	A(H1N1)	Globally	Globally	1.54	0.92	0.92	0.61	Adapted from [2]
Global Monitoring	A(H3N2)	(>1000 isolates)	(>1000 isolates)	0.43	1.48	0.62	2.17	
1996–1999	B			12.46	2.02	5.21	2.57	
After 3 years of NAI use	A(H1N1)	235§/622	622	0.66§	0.37§	0.56	0.42	Adapted from [3,148]
1999–2002	A(H3N2) + A(H1N2)	169§/922	922	0.31§	1.04§	0.37	1.38	
(Mean values)	B	128§/743	743	14.84§	1.40§	5.73	1.87	
2004–2007	A(H1N1)	1128	1134	ND	ND	0.91	1.06	Adapted from [4]
(Mean values)	A(H3N2)	1236	1233	ND	ND	0.44	2.54	
	B	889	889	ND	ND	3.42	3.87	
Values obtained by NIC from south of France								
2002–2003	A(H1N1)	9	9	1.34	0.92	ND	ND	[1]
	A(H1N2)	50	50	0.90	3.09	ND	ND	
	A(H3N2)	128	128	0.67	2.28	ND	ND	
	B	80	80	12.99	4.19	ND	ND	
2003–2004	A(H3N2)	ND	ND	0.43	2.03	ND	ND	[120]
2004–2005	A(H3N2)	ND	ND	0.53	1.87	ND	ND	[120]
2005–2006	A(H1N1)	150	149	1.66	1.17	ND	ND	[50]
	A(H3N2)	8	8	0.37	0.85	ND	ND	
	B	224	224	21.29	9.82	ND	ND	
2006–2007	A(H1N1)	10	10	1.66	1.12	ND	ND	[Lina B, Unpublished Results]
	A(H3N2)	440	441	0.48	1.01	ND	ND	
2007–2008	A(H1N1)¶ H275	232	232	1.36	1.54	ND	ND	[Lina B, Unpublished Results]
	A(H1N1)¶ Y275	142	142	565.00	1.37	ND	ND	[61]
	A(H3N2)	11	11	0.40	1.10	ND	ND	
	B	50	50	13.84	10.79	ND	ND	
2008–2009	A(H1N1)¶ Y275	16	16	649.00	1.47	ND	ND	[Lina B, Unpublished Results]
	A(H3N2)	65	65	0.39	1.13	ND	ND	
	B	10	10	12.45	8.60	ND	ND	
2009–2010	A(H1N1) 2009	192	192	0.46	0.44	ND	ND	[Lina B, Unpublished Results]
	A(H1N1)¶ Y275	4	4	502.00	1.12	ND	ND	
	A(H3N2)	10	10	0.29	0.70	ND	ND	
2010–2011	A(H1N1) 2009	38	38	0.45	0.52	ND	ND	[Lina B, Unpublished Results]
	A(H3N2)	22	22	0.24	0.61	ND	ND	
	B	75	75	14.13	3.06	ND	ND	

†Obtained with fluorescent assay. ‡Obtained with chemiluminescent assay. §Related to the data adapted from [148] concerning influenza viruses tested with a fluorescence-based NA inhibition assay between 1998 and 2002. ¶Related to A/Brisbane/59/2007.
n : Number of isolates tested; NAI: Neuraminidase inhibitor; ND: No data; NIC: National Influenza Center.

with zanamivir IV. The susceptibility of dual I223R/H275Y or I223V/H275Y mutant virus conferred highly elevated IC_{50} to oseltamivir and peramivir and a reduced susceptibility to zanamivir compared with the single H275Y mutant virus [89,90].

Mutations on the residue 223 in NA should play a role in the fitness of H275Y mutant viruses, and assays in animal models to monitor the impact of the I223R mutation are needed. The complete sequencing of the NA and HA genes should be performed when possible to detect other possible variants under treatment. Indeed, a S247N mutation with effects similar to the I223V mutation was recently reported.

Mutation S247N alone or associated with the H275Y mutation

A S247N mutation, was reported in the NA of A(H1N1) 2009 viruses for more than 30% of community specimens from northern Australia in the first months of 2011. This mutation was also described in specimens from Brunei and Singapore. Sequence analysis revealed that this S247N mutation appeared in at least two different clades of pandemic A(H1N1) 2009 viruses suggesting a good fitness and transmissibility of these S247N variants. The S247N mutation conferred a sixfold reduction in oseltamivir susceptibility and a threefold reduction in zanamivir susceptibility. A variant bearing the H275Y mutation associated to the S247N mutation was detected in an immunocompromised patient treated by oseltamivir. The double mutation H275Y and S247N induced a very important increase in the oseltamivir IC_{50}. Structural studies showed that the E277 residue was pushed further in the NA active site and prevented the oseltamivir binding [91].

Risk of reassortment with NAI-resistant viruses

In 2007/2008, the seasonal A(H1N1) viruses resistant to oseltamivir seems to have emerged without any oseltamivir selective pressure and was a consequence of mutations resulting from the genomic variability of influenza viruses. Emergence of resistance could also result from reassortment between drug-sensitive and drug-resistant influenza viruses. With the emergence of the pandemic A(H1N1) 2009 viruses, one threat was the possible reassortment with oseltamivir-resistant seasonal A(H1N1) viruses. Such reassortments were quite easily obtained after *in vitro* co-infections [92]. Such a reassortment is a possibility as human infection with a triple reassortant A(H1N1) virus of swine origin and HA and NA of a seasonal A(H1N1) virus were detected *in vivo* [93] and a natural co-infection of pandemic and seasonal A(H1N1) strains was reported during the 2009 influenza season in New Zealand [94]. However, pandemic A(H1N1) 2009 viruses were largely predominant and oseltamivir-resistant seasonal A(H1N1) viruses were uncommon, which reduced the risk of reassortment.

Other resistance mutations in A(H1N1) viruses described *in vivo*

The N295S mutation was evaluated in recombinant A(H1N1) viruses obtained by reverse genetics, it conferred resistance to oseltamivir but the susceptibility to zanamivir and peramivir was conserved [89,95].

The analysis of A(H1N1) viruses isolated between 2006–2008 from Australasia and Southeast Asia revealed a new Q136K mutation (N2 numbering) in NA responsible for a 300-fold and 70-fold decrease in zanamivir and peramivir susceptibility, respectively. This mutation has no effect on oseltamivir susceptibility. However, this mutation seems to have been amplified during MDCK cell culture passage [39].

Influenza A(H5N1) viruses & resistance to NAI

Avian influenza A(H5N1) viruses are generally restricted to birds, but they can occasionally infect humans. The A(H5N1) viruses diverge into ten different genetic clades. Human infections have been caused by subclades 1, 2.1, 2.2, 2.3 and 7 [96]. The susceptibilities to current antiviral agents are variable across the different A(H5N1) virus clades [96]. However, oseltamivir-resistant A(H5N1) mutated H275Y were isolated from infected Vietnamese patients treated by oseltamivir [97,98].

The N295S mutation conferring a reduced susceptibility to oseltamivir was also detected in patients infected by A(H5N1) virus and treated by oseltamivir [98,99]. This N295S mutation was also detected before the treatment with oseltamivir [99].

The enzymatic properties and crystal structures of mutant neuraminidases from A(H5N1) virus-infected patients explain the molecular basis of resistance due to H275Y (see paragraph on the mutation H275Y) or N295S mutation [48]. The N295S mutation makes a hydrogen bond with the E277 that alters the hydrophobic pocket and the link with oseltamivir [48].

The H275Y and N295S mutations were stably maintained *in vitro* in recombinant influenza A/Vietnam/1203/04(H5N1) and A/Puerto Rico/8/34(H1N1) viruses. In a mice model, the lethality of the WT and mutated recombinant viruses carrying either H275Y or N295S mutation was similar [100]. In the ferret model, recombinant influenza clade 2.2 A(H5N1) viruses carrying the H275Y or the N295S mutation were not attenuated and the N295S mutated viruses even showed significantly higher virus titers than the WT viruses, suggesting an increased virulence [101]. In animal models, the N295S viruses were attenuated compared with the WT viruses but maintained their pathogenicity in the ferret model [102].

In 2007, A(H5N1) clade 2.3.4 viruses replaced clade 1 viruses in northern Vietnam. Some of these viruses showed a reduced *in vitro* susceptibility to oseltamivir as compared with clade 1 viruses, but the IC_{50} for oseltamivir and zanamivir remained low and no known resistance mutation was found [18]. These results reflect the natural variations of susceptibility to NAI due to genetic differences according to the clade and subclade [103]. The analyses of more than 5490 avian NCBI annotated sequences revealed 132 (2.4%) sequences carrying mutations of resistance to oseltamir carboxylate or zanamivir. These mutations can be the result of the natural variations in the virus or could be induced by the selective pressure of xenobiotics (oseltamivir or zanamivir). One question is to know whether the identified mutations actually reduce NA susceptibility to NAI [104]. The study of NAI susceptibility of 55 A(H5N1) avian influenza viruses isolated between 2004 and 2006 revealed that the I117V (in N2 numbering) mutation was responsible for a 16-fold increase in oseltamivir IC_{50} and that the V116A (in N2 numbering) mutation was responsible for a 63-fold and 11-fold increase in zanamivir and oseltamivir IC_{50}, respectively, compared with the mean IC_{50} of the other A(H5N1) viruses tested [105].

Influenza A(H3N2) viruses & resistance to NAIs

Most A(H3N2) viruses are susceptible to NAI, although NAI-resistant A(H3N2) variants were selected under treatment particularly in children and in immunocompromised patients.

To investigate the oseltamivir resistance under treatment, 50 children were treated with oseltamivir. The A(H3N2) viruses (seasons 2002 and 2003) collected before and during treatment were studied by cloning and sequencing. The following NA mutations were detected in viruses isolated from the 50 patients: R292K (six patients), E119V (two patients) and N294S (one patient) [7]. Another study conducted from 2005–2006 to 2008–2009 influenza seasons in Japanese children treated by either oseltamivir (4 mg/kg daily in divided doses twice daily for 5 days) or zanamivir (20 mg daily in divided doses twice daily for 5 days) detected NA mutations in viruses isolated from six among 72 patients treated by oseltamivir. Two seasonal A(H1N1) viruses possessed the H275Y mutation, three A(H3N2) viruses had a R292K mutation and one A(H3N2) virus was mutated at E119V. No NA mutations were detected in viruses isolated from the 72 patients treated by zanamivir [8].

Mutation R292K

The R292K mutation is the most commonly observed mutation among the NAI-resistant A(H3N2) viruses reported in clinical studies (see previously) albeit it concerns a catalytic amino acid residue and confers resistance to both oseltamivir and zanamivir [23,106].

Structural approaches can explain the differences in the frequency of the observed mutations according to the subtype. In N2 NA, the R292K mutation induces a loss of hydrogen bond from R292 to the carboxylate group of oseltamivir, and the K292 interacts with E276, impeding its movement to accommodate the hydrophobic chain of oseltamivir. In N1 NA, the Y347 forms a hydrogen bond with the carboxylate group of oseltamivir and compensates for the loss of the link with the R292, this could explain why the R292K mutation does not confer oseltamivir resistance in N1 NA [25].

The R292K mutant viruses had a reduced infectivity and a lack of transmissibility by contact in ferrets [51,107,108]. This mutation R292K was not maintained and was reversed through multiple cycles of multiplication in cell culture [108]. The co-infection of ferrets with a mixture of R292 and K292 viruses demonstrated that the mutant virus was rapidly outgrown by the WT R292 virus [109].

Mutation E119V/I

In N2 NA, the E119V mutation (**Figure 2**) confers resistance to oseltamivir with a 100-fold increase of the IC_{50} , but these viruses remain sensitive to zanamivir and peramivir [38,110].

The E119V mutation has been detected in A(H3N2) isolates from patients treated

by oseltamivir [23]. This resistance has been detected in immunocompetent and immunocompromised patients [4,7,8]. In a study conducted in children and young adults with hematologic malignancies E119V mutations have been detected before the initiation of antiviral therapy in two patients and during therapy in one patient [111]. A double mutant virus, resistant to both oseltamivir (E119V in N2 NA) and amantadine (V27A mutation in M2) has been observed in an immunocompromised infant [112].

Oseltamivir-resistance was also associated to the presence of a mix of E119V and E119I variants in A(H3N2) virus isolated from an immunocompromised child treated with oseltamivir. These two mutant viruses were present in low proportion (<10%) in the clinical specimens but could be amplified by culture on MDCK cells [38]. The E119I mutation was tested and conferred a high resistance to oseltamivir (IC_{50} 1000-fold greater than WT IC_{50}), to peramivir (IC_{50} 400 fold greater than WT IC_{50}) and to a lesser extend to zanamivir (IC_{50} tenfold greater than WT IC_{50}) [38].

The impact of NA mutations on viral fitness was tested in animal models. A(H3N2) virus with the E119V framework mutation had the same transmission profile as the WT virus [51].

While the A(H3N2) R292K catalytic mutant viruses have compromised virus growth and transmissibility the E119V framework mutant virus did not show a severely altered fitness. Even if the aerosol transmission of E119V mutant virus was impaired, these viruses were efficiently transmitted by contact in a ferret model [113]. The R292K catalytic mutation resulted in a greater loss of NA activity and thermostability than the E119V framework mutation [108].

Mutations I222V

The I222V mutation was found in association with the E119V mutation in an immunocompromised child. Similar to that observed for the I223V and H275Y mutations in A(H1N1) viruses, the single I222V mutation conferred marginal levels of oseltamivir resistance but increased drug resistance when associated with E119V in A(H3N2) viruses [114]. The assays conducted with the isolates from this patient, showed that the impaired fitness of the E119V mutant virus was partially restored by the I222V mutation [115]. *In vivo* studies in ferrets showed that recombinant human influenza A(H3N2) virus (A/Panama/2007/1999(H3N2)) with the E119V mutation or the double mutation E119V and I222V were not altered in their infectivity and transmissibility by contact, but their transmission by aerosol was altered compared with the WT virus in a guinea pig model [113]. Such double mutations that induce a high level of resistance to NAI associated with a good viral fitness have to be monitored [116].

Mutation N294S

The N294S mutation was detected in one child among 50 children infected by A(H3N2) virus and treated with oseltamivir. This N294S mutation conferred resistance to oseltamivir [7], as it was also confirmed by reverse genetic studies in N2 NA [117].

Deletion in NA & resistance to NAI

A four amino-acid deletion (Del 245–248) was observed in the NA of an A(H3N2) virus isolated from an immunocompromised child after 107 days of treatment with oseltamivir. This deletion was responsible for resistance to oseltamivir [118]. The same deletion (Del 245–248) was observed in an immunocompromised patient infected with A(H3N2) virus only after 5 days of oseltamivir treatment. This deletion conferred a decreased susceptibility to both oseltamivir and zanamivir [119].

During the 2003–2004 season, four A(H3N2) viruses were isolated in cell culture albeit they had no NA activity [120]. These viruses had no detectable NA gene but could grow in cell culture. Mutations in HA responsible for a decreased sialic acid affinity allowed virus multiplication even without NA activity [120,121].

Influenza B viruses & resistance to NAI

A study conducted in Japanese children infected by influenza B or A(H3N2) viruses and treated by oseltamivir showed that the mean duration of fever after the start of oseltamivir was greater in the influenza B virus infected group than in the influenza A(H3N2) virus infected group in children aged 1–5 years old. The IC_{50} of oseltamivir was significantly higher for influenza B than for A(H3N2) viruses [122].

To assess the prevalence and transmissibility of resistance to NAI of influenza B viruses, a study was conducted in Japan: 74 children aged from 0 to 15 years, were treated with oseltamivir for 5 days with weight-based doses. In one child, a virus with reduced sensitivity to oseltamivir (IC_{50} × 7.1) and to zanamivir (IC_{50} × 3.9)

was isolated 3 days after initiation of oseltamivir treatment, and sequence analysis on cDNA clones of the NA gene revealed a G402S mutation (N2 numbering). Influenza B viruses with reduced sensitivity to NAI were also detected in untreated patients suggesting a transmission of these NAI-resistant influenza B viruses within the community. These viruses had mutations in N2 numbering: D198N (three patients), I222T (three patients), S250G (one patient) [123]. The most frequent mutations involved in the resistance of influenza B viruses to NAI are presented in Figure 4.

Mutation R150K

The R150K mutation (R152K, N2 numbering) was isolated in an immunocompromised child treated with zanamivir. This mutation of a catalytic residue conferred a high level resistance to zanamivir (IC_{50} × 1000) [124] and also to oseltamivir and peramivir [125].

Mutation D197N/E/Y

Several mutations have been reported at position 197.

The D197N mutation (D198N, N2 numbering) was reported in an immunocompromised patient treated with oseltamivir [125,126]. This D197N mutation conferred a tenfold increase in IC_{50} for both oseltamivir and zanamivir [126]. Influenza B viruses with this mutation were also detected in patients at the onset of disease, prior to treatment. Two of them were from the same family, which suggested a possible transmission of the mutated virus [123]. In this study, the D197N virus has a reduced sensitivity to oseltamivir (IC_{50} × 5) and to zanamivir (IC_{50} × 3.5). According to a second study, this D197N mutation did not alter the viral fitness *in vivo* [110].

A D197E mutation has been detected in an untreated child. This mutation conferred resistance to oseltamivir, peramivir and a low level of resistance to zanamivir [127]. The mechanism of the resistance conferred by this D197E mutation was described [128].

In a global surveillance study in France, a D197Y mutation was also detected in an influenza B virus conferring a 15-fold increase in IC_{50} for both oseltamivir and zanamivir [50].

Mutation I221T

The I221T mutation (I222T in N2 numbering) was observed in global surveillance studies; and conferred resistance to oseltamivir and a low level of resistance to zanamivir [3,4,123].

Mutation N294S

A recent study reported for the first time a N294S mutation responsible for oseltamivir resistance in influenza B viruses. It was detected in an immunocompromised child treated by oseltamivir. This mutation conferred resistance to oseltamivir but retained susceptibility to zanamivir [111].

Other mutation

An extreme outlier resistant to both oseltamivir and zanamivir has been described with a R374K mutation (R371K N2 numbering). This virus was not selected by NAI treatment [4].

Future perspective

Future risks of emergence of drug-resistant influenza viruses

In this article we summarized the mutations responsible for the NAI resistance of influenza viruses. Most of the resistant viruses reported so far are A(H1N1) viruses bearing the H275Y mutation. However, one question for the future, with the increased use of NAIs, is the possible emergence of NAI-resistant viruses in A(H3N2) or B viruses. The rapid rise in the resistance to adamantanes in different subtypes could be explained by the fact that this resistance does not alter the viral fitness. Therefore, mutations like E119V, which are responsible for NAI-resistance but without alteration of fitness in A(H3N2) viruses, could emerge in the future. To prevent this emergence there should be a rationale (good medical practice) for the use of NAIs. Close monitoring for early symptoms and early treatment might be a preferable alternative to the use of NAIs for prophylaxis [21,129]. As patients continue to shed virus while on therapy and as oseltamivir-resistant viruses may develop early while on therapy, hygiene should be encouraged and practiced to avoid the transmission of such viruses.

The emergence of NAI-resistant viruses is not only the result of single mutations. Sustained transmission of resistant viruses is associated to acquisition of compensatory mutations that maintain enzyme activity and viral fitness. The emergence of seasonal A(H1N1) viruses in 2007 pointed out the role of these compensatory mutations [62]. For pandemic A(H1N1) 2009 viruses, the I223V/R and S247N mutations associated with H275Y can lead to a high level of resistance to oseltamivir while viral fitness remains high [78,89]. These mutations may facilitate the emergence and dissemination of oseltamivir-resistant viruses. These compensatory mutations should be monitored closely.

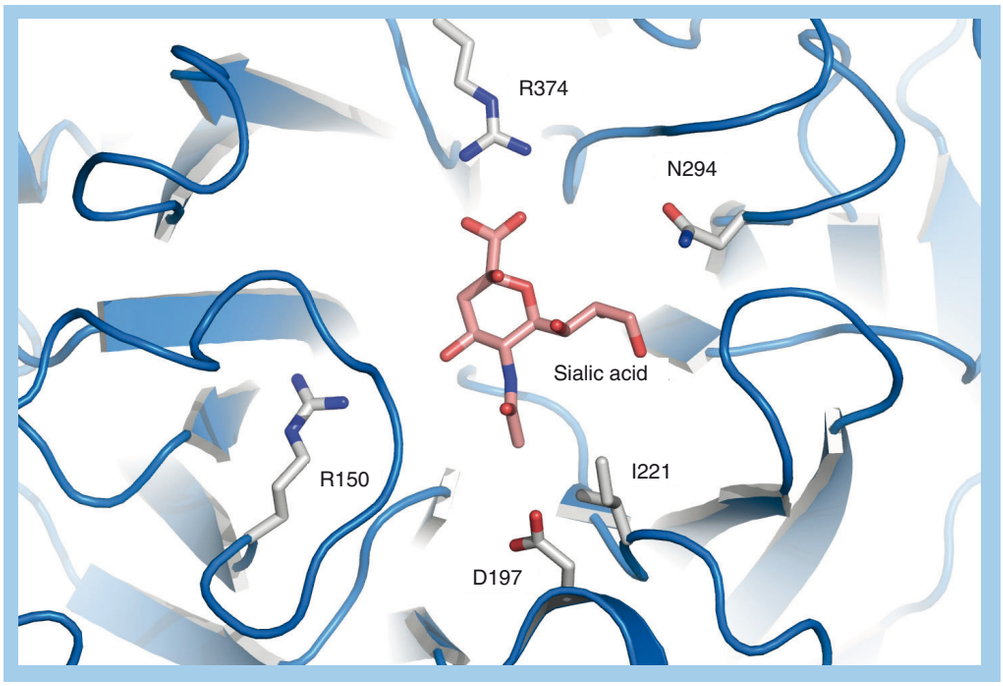

Figure 4. Schematic of the active site of a wild-type influenza B virus neuraminidase in complex with sialic acid. The active site of a wild-type influenza B virus neuraminidase in complex with sialic acid [147] (PDB code 1NSB) is presented. Residues involved in neuraminidase inhibitor resistance are indicated.

As discussed previously, the risk of emergence of resistance could also result from a reassortment between sensitive and resistant viruses. Indeed, the pandemic A(H1N1) 2009 virus can easily reassort with seasonal influenza viruses as it was shown *in vitro* and *in vivo* [92–94].

With the exception of the resistance of seasonal A(H1N1) viruses related to A/Brisbane/59/2007, the problem of antiviral resistance concerns mainly the patients for whom an antiviral treatment is necessary. Annual vaccination is the best method for preventing seasonal influenza virus infection but antiviral agents are at the forefront of defenses against a pandemic influenza virus. Beside the detection of such compensatory mutations, the future challenge is also to propose new treatment options to prevent the emergence of resistant viruses.

Prevention of the emergence of drug-resistant influenza viruses

Vaccination

Influenza virus vaccination is the best prevention against influenza virus infection. Each year, vaccine should be a priority for at risk patients, their families and hospital staff [71].

Monitoring of viral clearance & of drug susceptibility

In the context of the A(H1N1) 2009 pandemic, recent reports have recommended the monitoring of viral excretion and of drug susceptibility in at-risk patients infected by pandemic A(H1N1)2009 virus treated with oseltamivir and whose influenza virus infection was not resolved by oseltamivir treatment [67,130]. Indeed, in immunocompromised patients, the prolonged viral excretion facilitates the development of viral quasispecies and minor populations of oseltamivir-resistant viruses are then selected under treatment.

Immunity restoration

Efficient immune response is required to cure influenza virus infection. In immunocompromised patients, persistent lymphopenia and hypo or agammaglobulinemia have been associated with poor clinical outcome. Treatments that restore immunity or the administration of IV immunoglobulins that can contribute to decrease viral load should be considered [131].

New therapeutic options or new antiviral treatments of influenza virus infections

Inhaled zanamivir & IV zanamivir

During four influenza seasons (from the years 2005–2006 to 2008–2009), a study has been conducted in Japan with children infected with influenza viruses and treated with either

oseltamivir or zanamivir. This study concluded that the virus-shedding period was significantly shorter in zanamivir versus oseltamivir-treated patients. Furthermore, eight oseltamivir-resistant viruses were described whereas no resistant viruses were detected in the group treated with zanamivir. The authors suggest that zanamivir should be preferred for pediatric patients that can use inhaled drugs [8]. Similarly, zanamivir alone should be considered in patients with a high risk to develop drug-resistant viruses [71]. According to the licensed formulation, zanamivir can only be administered by inhalation and has poor systemic absorption, which can limit its efficacy in severely ill patients. For such patients, IV zanamivir should be considered. During the 2009 pandemic, several patients presenting low respiratory tract infection and admitted into the intensive care unit had a prolonged viral excretion and eventually developed an oseltamivir-resistant virus. These patients were treated with IV zanamivir, available on a compassionate use basis, allowing viral clearance. However, all NAIs are virostatic drugs, and the reduction in their viral load has been reported to be concomitant with the immune restoration [131,132].

Intravenous oseltamivir

Intravenous oseltamivir was available for severe influenza A(H1N1) 2009 virus infections during the pandemic phase [203]. Clinical trials are ongoing to evaluate efficacy and tolerance of IV oseltamivir in adults.

Intravenous peramivir

Due to its low oral biodisponibility, IV peramivir was available during the 2009 pandemic [133,204]. Clinical trials showed similar efficacy between IV peramivir and oral oseltamivir [133]. Peramivir should not be used in the case of H275Y oseltamivir-resistant influenza A(H1N1) 2009 virus infection as this mutation confers cross resistance to both antivirals [22,204]. As for oseltamivir, a case of emergence of H275Y mutation has been observed during IV peramivir treatment in an immunocompromised patient infected with A(H1N1) 2009 virus [134].

Association with existing antiviral agents

Theoretically, the use of antivirals in association can prevent the emergence of resistance. *In vitro* studies performed with the A(H1N1) 2009 virus showed that the association of oseltamivir or peramivir with zanamivir was either additive or antagonistic, whereas the triple association oseltamivir, amantadine and ribavirin was synergistic [135]. A clinical study carried out in France in 2008–2009 also found an antagonistic effect of the association oseltamivir and inhaled zanamivir [136]. Combination therapy to decrease the probability of resistance is currently being evaluated. The association of oseltamivir and IV zanamivir has been used for hematology patients infected with A(H1N1) 2009 virus. However, as there is no evident synergy between oseltamivir and zanamivir [135,136], zanamivir alone remains recommended as a first-line treatment in high-risk patients [71].

Laninamivir

The laninamivir (CS-5958) is the prodrug of a compound structurally related to zanamivir allowing a long-acting NA inhibition [137]. *In vitro*, the IC_{50} of the bioactive form of laninamivir (R-125489) were comparable to those of zanamivir and viruses bearing the H275Y mutation remain susceptible to zanamivir and to R-125489 [138]. *In vivo*, a single administration of laninamivir was at least as efficient as a 5-day-long oseltamivir treatment in children and adults infected with influenza virus. Laninamivir was also efficient in case of infection with an oseltamivir-resistant seasonal A(H1N1) virus, allowing rapid symptoms resolution [139,140]. Laninamivir is now approved in Japan and is currently in ongoing Phase III clinical trials in Australia.

Favipiravir

The favipiravir is an influenza virus polymerase inhibitor. Promising results have been obtained in animal models, including mice infected with oseltamivir-resistant A(H5N1) viruses [141,142]. Phase III clinical trials are ongoing in Japan and the USA [22].

Sialidase (DAS 181)

The DAS 181 is a broad recombinant fusion protein that inactivates sialic acid receptors on the cells of the human respiratory tract thereby preventing viral entry into cells. DAS 181 was detected to be efficient in mice infected by high pathogen influenza A(H5N1) viruses [143] or oseltamivir-resistant seasonal A(H1N1) or pandemic A(H1N1) 2009 viruses [144].

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Executive summary

Evolution of the resistance to adamantanes

- Adamantane resistance emerges frequently in treated patients. A single mutation, the most frequent one is the S31N mutation, in the transmembrane domain of the M2 protein can confer resistance to both amantadine and rimantadine.
- There has been a substantial worldwide increase in adamantane resistance since 2005. Nearly all the A(H3N2) and pandemic A(H1N1) 2009 viruses are adamantane-resistant. For A(H5N1) viruses, clade 1 are resistant but most of the more recent strains (subclade 2.2 and 2.3.4) are susceptible to adamantanes.
- Currently, adamantanes (amantadine and rimantadine) should not be used for the treatment of influenza, but may be beneficial in the future if a susceptible virus circulates widely.

Resistance to neuraminidase inhibitors

- Neuraminidase inhibitors (NAIs) are synthetic analogs of sialic acid and effective for the treatment of influenza A and B viruses. NAIs prevent the release and propagation of new virions.
- NAI resistance is due to mutations of NA residues located in the active site or in framework regions of the NA. Most of the mutations responsible for *in vivo* resistance are mutations of framework residues. Structural differences explain that the most frequent mutations responsible for NAI-resistance found *in vivo* differ according to the NA subtype. The H275Y and secondly the N295S mutations are mainly found in N1 NA, the R292K and E119V/I mutations are responsible for N2 resistance and D197N mutation is the most frequent for the NA of influenza B viruses.
- The monitoring of NAI-resistance is frequently based on both luminescent and fluorometric NA inhibition assays. These assays require the use of virus isolates and may be biased by possible *in vitro* selection of quasispecies that may not reflect the initial viral population in the clinical specimen. The sequencing of the NA should be performed to identify mutations responsible for NAI resistance and compensating mutations. Pyrosequencing performed directly on the initial sample provides a rapid and interesting tool that should be generalized in the future for monitoring known mutations and detecting mixed populations.
- The most frequently described mutation is H275Y. Indeed, seasonal A(H1N1) viruses that were bearing this mutation in the absence of any oseltamivir-selective pressure emerged in 2007–2008. With the emergence of the pandemic A(H1N1) 2009 virus, the H275Y mutation appeared mainly next to oseltamivir treatment of infected patients. NAI-resistant influenza A(H3N2) or B viruses are rarely reported and concern mainly children or immunocompromised treated patients.
- The fitness of mutated viruses is difficult to estimate. The frequency of *in vivo* isolation could help to appreciate the fitness of mutated resistant viruses in humans. In the animal models, the pathogenicity of sensitive or resistant A(H1N1) 2009 viruses is similar, but the results concerning their transmission are different between the studies. However, oseltamivir-sensitive A(H1N1) 2009 viruses predominate in the absence of drug-selective pressure, suggesting the better fitness of the sensitive viruses.
- The I223V/R mutation was found to be associated with the H275Y mutation in pandemic A(H1N1) 2009 viruses and the I222V mutation was associated to the E119V mutation in A(H3N2) viruses. In cell culture, the I223V or the I222V mutations restore in part the viral fitness altered by the H275Y and the E119V mutation, respectively. Assays in animal models to monitor the impact of double mutations I223V/R are needed.
- The emergence of the S247N mutation in pandemic A(H1N1) 2009 viruses conferring a decrease in the susceptibility to NAI should be monitored.

Future perspective

- In light of the emergence of oseltamivir resistance in seasonal A(H1N1) viruses in 2007, the risk of emergence of NAI-resistant pandemic A(H1N1) 2009, A(H3N2) or B influenza viruses should be considered. This risk is linked to the viral fitness of these NAI-resistant viruses and to the therapeutic use of NAIs.
- The development of influenza virus vaccination, better monitoring of antiviral susceptibility in patients with prolonged excretions and the use of new therapeutic options/drugs for influenza virus treatment will reduce the risk of emergence/dissemination of resistant viruses.

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