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Review

Influenza virus resistance to neuraminidase inhibitors

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ABSTRACT

In addition to immunization programs, antiviral agents can play a major role for the control of seasonal influenza epidemics and may also provide prophylactic and therapeutic benefits during an eventual pandemic. The purpose of this article is to review the mechanism of action, pharmacokinetics and clinical indications of neuraminidase inhibitors (NAIs) with an emphasis on the emergence of antiviral drug resistance. There are two approved NAIs compounds in US: inhaled zanamivir and oral oseltamivir, which have been commercially available since 1999–2000. In addition, two other NAIs, peramivir (an intravenous cyclopentane derivative) and laninamivir (a long-acting NAI administered by a single nasal inhalation) have been approved in certain countries and are under clinical evaluations in others. As for other antivirals, the development and dissemination of drug resistance is a significant threat to the clinical utility of NAIs. The emergence and worldwide spread of oseltamivir-resistant seasonal A(H1N1) viruses during the 2007–2009 seasons emphasize the need for continuous monitoring of antiviral drug susceptibilities. Further research priorities should include a better understanding of the mechanisms of resistance to existing antivirals, the development of novel compounds which target viral or host proteins and the evaluation of combination therapies for improved treatment of severe influenza infections, particularly in immunocompromised individuals. This article forms part of a symposium in *Antiviral Research* on "Treatment of influenza: targeting the virus or the host."

Contents

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1. Introduction

Two classes of antiviral drugs are currently approved for the management of influenza infections: the adamantanes and the neuraminidase inhibitors (NAIs). The adamantane drugs or matrix (M)-2 blockers, amantadine and rimantadine, were developed in the 1960s and approved since then in many countries. 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 (CDC, 2006). Hence, since 2010, the neuraminidase inhibitors are the only class of antivirals recommended by the WHO for the treatment and prophylaxis of influenza A and B infections (Pizzorno et al., 2011a).

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 U.S. 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 (Birnkrant and Cox, 2009). Peramivir is approved in Japan as Rapiacta and also available in South Korea as Peramiflu. Laninamivir octanoate (CS-8958), which is 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 US and other countries. In this article, we review the mechanism of action, pharmacokinetics and clinical indications of NAIs with an emphasis on the emergence of antiviral drug resistance.

2. Mechanism of action of NAIs

Along with the hemagglutinin (HA), the neuraminidase (NA) is the other major influenza surface antigen. The latter is a mushroom-shaped homotetrameric glycoprotein with a stalk domain anchored to the viral membrane and a globular head that contains a catalytic site. 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 2,3 and 2,6 sialic acid moieties. Therefore, the enzymatic activity of the influenza NA 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.

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. The crystallographic data of NAs from N2 (Colman et al., 1983), N9 (Baker et al., 1987) and B (Burmeister et al., 1992) viral backgrounds

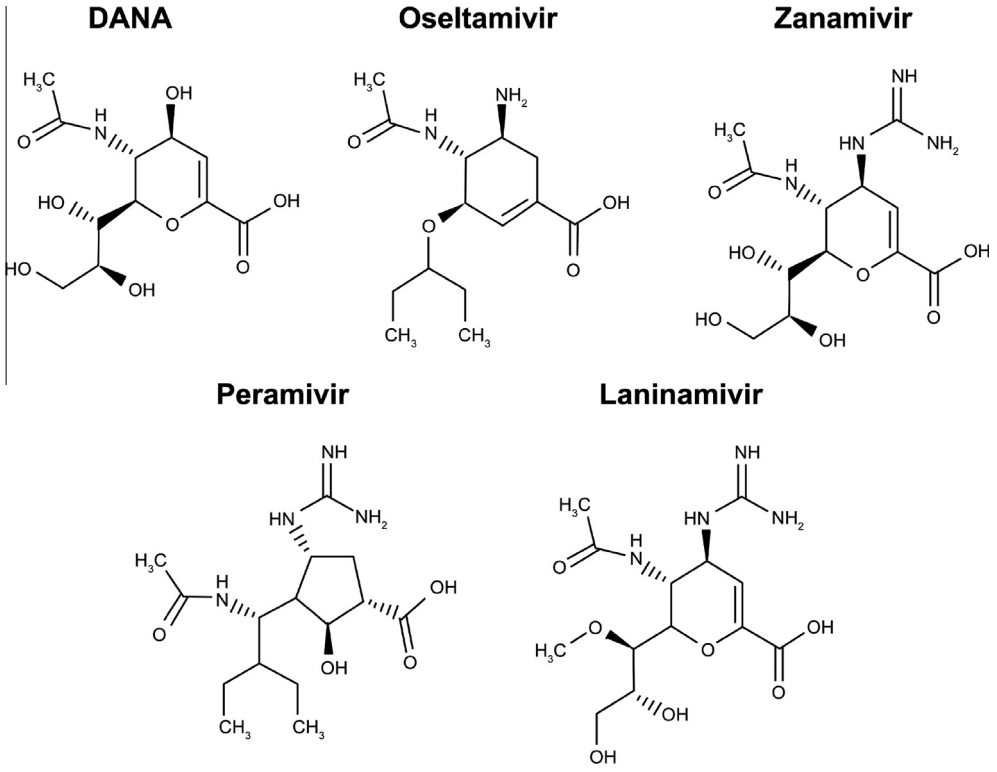

Fig. 1. Chemical structure of neuraminidase inhibitors (NAIs). All these agents are based on the structure of the 2,3-didehydro analog of the *N*-acetyl-neuraminic acid (DANA). The bioavailable prodrug of oseltamivir is an ethyl ester that is converted into the active carboxylate by hepatic esterases. 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.

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 (Fig. 1). Since these NAIs are based on the structure of the 2,3-didehydro analog 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.

3. Structure, clinical indications and pharmacokinetics

Oseltamivir (GS4104) is an ethyl ester prodrug which requires ester hydrolysis to be converted to the active form oseltamivir carboxylate (GS4071). This compound was developed through modifications of the sialic acid analog framework, including the addition of a bulky lipophilic side chain, that allows the drug to be given orally (Kim et al., 1998) (Fig. 1). Like other NAIs, oseltamivir acts as a competitive inhibitor. 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. By contrast, oseltamivir shows little or no activity against NA of other viruses, bacteria or human liver microsomes (Mendel et al., 1998).

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 6–10 h (Davies, 2010; Dutkowski et al., 2003; He et al., 1999). 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 (Brennan et al., 2012). 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 (Brennan et al., 2012).

Zanamivir (GG167) is a 4-deoxy-4-guanidino analog of DANA that was approved as an anti-influenza agent in 1999 (Fig. 1). 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 (Diggory et al., 2001). 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 (Ison, 2010).

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 (CDC, 2009a; Sheu et al., 2008). Conversely, A(H3N2) strains are more sensitive to oseltamivir than to zanamivir (Ferraris et al., 2005). 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 $\geqslant$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 (FDA, 2011). After a 10 mg dose inhalation, zanamivir is rapidly deposited mainly in the oropharynx (78%) and in the lungs (13%). Only 10–12% 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

Table 1
Recommended dosage of neuraminidase inhibitors (NAIs) for chemoprophylaxis and therapeutic use.

NAIs	Age	Route of administration	Prophylaxis dosing	Treatment dosing
Oseltamivir[a]	Children	Oral Suspension		
(Tamiflu)	⩽15 kg		30 mg once daily/10 days	30 mg twice daily/5 days
	>15–23 kg		45 mg"	45 mg"
	>23–40 kg		60 mg"	60 mg"
	>40 kg		75 mg"	75 mg"
	Adolescents and adults	Oral capsule		
	⩾13 years		75 mg once daily/10 days	75 mg twice daily/5 days
Zanamivir[a]	Children and adults	Oral inhalation		
(Relenza)	⩾5 years (prophylaxis)		10 mg once daily/10 days	10 mg twice daily/5 days
	⩾7 years (treatment)			
Peramivir[b,c]	Children	Parenteral		
(Peramiflu)	0–30 days		–	6 mg/kg once daily/5 days
(Rapiacta)	31–90 days		–	8 mg/kg"
	91–180 days		–	10 mg/kg"
	⩾181 days–5 years		–	12 mg/kg"
	⩾6 years		–	10 mg/kg"
	Adolescents and adults	Parenteral		
	⩽17 years		–	10 mg/kg once daily/5 days
	⩾17 years		–	600 mg"
Laninamivir[d]	Children and adults	Oral Inhalation		
(Inavir)	⩽10 years		–	20 mg single inhalation
	⩾10 years		–	40 mg"

[a] Approved dosage in U.S.
[b] Approved dosage in Japan and South Korea.
[c] Maximum daily dose: 600 mg.
[d] Approved dosage in Japan.

inhaled dose supports the twice daily treatment regimen since drug concentrations remain above the IC_{50} values after 24 and 12 h, respectively (Peng et al., 2000).

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 (Cass et al., 1999). 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 (Calfee et al., 1999).

Peramivir (BCX-1812, RWJ-270201) is a cyclopentane derivative with a negatively-charged carboxylate group, a positively-charged guanidino group and a lipophilic side chain (Babu et al., 2000) (Fig. 1). Due to its low bioavailability, this drug needs to be delivered parenterally (Bantia et al., 2006; Yun et al., 2008). 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 (Babu et al., 2000; Boltz et al., 2008; Kitano et al., 2011). Peramivir was first developed for oral administration and showed *in vivo* activity in both mice and ferrets (Bantia et al., 2001; Sidwell et al., 2001; Sweet et al., 2002). 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 (Barroso et al., 2005).

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 (Bantia et al., 2006). Another mouse experiment has demonstrated that a single IV injection of peramivir provided a significant therapeutic effect that was superior to that of oral oseltamivir (Yoshida et al., 2009). Moreover, preclinical studies in mice and ferrets demonstrated that parenteral administrations of peramivir rapidly produced high plasma concentrations (Bantia et al., 2006; Yun et al., 2008).

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 (Kohno et al., 2011). 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. On the other hand, an interim analysis of IV peramivir phase 3 clinical trials in US has recently shown little difference with placebo on influenza outcomes, which halted the development of this drug in this country.

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 5–10 days (Table 1). The IV administration of peramivir (600 mg daily for 5 days) resulted in a peak plasma concentration of 45,200 ng/ml (Kohno et al., 2011). Peramivir is approximately 90% eliminated as an unchanged drug by the kidneys with an apparent half-life of 7.7–20.8 h in adults with normal renal function (FDA, 2009).

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 (Fig. 1). Laninamivir has shown good inhibition against a broad range of influenza A (N1–N9) and influenza B strains (Kubo et al., 2010; Yamashita et al., 2009). 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 $\leqslant$10 years old is a single 20 mg inhalation dose, whereas for patients $\geqslant$10 years old the dose is doubled to 40 mg (Table 1) (Ikematsu and Kawai, 2011).

Once inhaled, this inactive prodrug is converted solely 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 (Chairat et al., 2013). This high retention time confers long-lasting NA inhibition (Koyama et al., 2009; Yamashita et al., 2009), 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 (Kubo et al., 2010). 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 (Ikematsu and Kawai, 2011). 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 (Biota Holdings Ltd., 2009a,b; Ishizuka et al., 2010a,b; Sugaya and Ohashi, 2010; Watanabe et al., 2010). Noteworthy, 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 H274Y (H275Y in N1 numbering) variants (Watanabe et al., 2010).

4. Mechanisms of resistance

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. The catalytic site of the NA is constituted of eight functional residues (R-118, D-151, R-152, R-224, E-276, R-292, R-371, and Y-406), surrounded by eleven framework residues (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 system) implicated in the stabilization of the active site structure. These residues are conserved in all influenza A and B viruses (Colman et al., 1993).

Because oseltamivir has a large hydrophobic side chain, the NA must undergo rearrangements to accommodate drug binding. To form this pocket, the amino acid E276 must rotate and bond with R224 (Collins et al., 2008; Malaisree et al., 2008). Any mutations that affect this rearrangement may reduce the binding affinity of oseltamivir leading to lower efficiency. Up to now, mutation H274Y, that is more commonly associated with oseltamivir resistance, as well as mutations R292K and N294S commonly associated with oseltamivir reduced sensitivity, have been shown to inhibit the rotation of the E276 residue and to prevent pocket formation (Wang et al., 2002). The molecular structure of zanamivir includes a guanidino group, instead of the hydrophobic group found in oseltamivir. The guanidino group interacts with the conserved E119 residue in the active center pocket (Zürcher et al., 2006).

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 (Gubareva, 2004). 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, struc-

tural rearrangements are necessary to accommodate the peramivir hydrophobic side chain (Babu et al., 2000) and mutations that affect the activity of oseltamivir and zanamivir can also affect peramivir activity. No laninamivir-resistant mutations have been reported yet. However, since zanamivir and laninamivir share highly similar binding properties with the NA protein (Vavricka et al., 2011), mutations of resistance to zanamivir are also expected to confer resistance to laninamivir (although this needs to be confirmed).

Besides NA mutations, NAI resistance could also emerges *in vitro* due to mutations in or near of the HA receptor binding site (Ginting et al., 2012). Such HA changes are thought to reduce viral dependency on NA activity. However, only the role of NA mutations will be reviewed in this article since they are the ones considered to be clinically relevant (Abed et al., 2002; McKimm-Breschkin, 2000). Resistance mutations differ according to the type of NAI used and they have been found to be subtype specific (Pizzorno et al., 2011a) (Tables 2–4). It should be noted that among substitutions in the NA that are known to occur clinically, only the H274Y is unequivocally considered to cause clinical resistance (WHO, 2012a).

4.1. Resistance to oseltamivir

Oseltamivir-resistant influenza viruses have been rarely detected in clinical samples before the availability of NAIs. During the first 5 years (1999–2004) following the introduction of oseltamivir and zanamivir, the incidence of oseltamivir resistance seen in clinical trial samples was 0.33% in adults ($\geqslant$13 years) and 4.0% in children ($\leqslant$12 years) (Aoki et al., 2007; Monto et al., 2006; Ward et al., 2005). However, more significant resistance levels were observed in some clinical therapeutic settings, such as in young hospitalized children (up to 18%) (Kiso et al., 2004), immunocompromised patients, (Baz et al., 2006; Ison et al., 2006; Weinstock et al., 2003) and human cases of influenza A(H5N1) infections (de Jong et al., 2005; Le et al., 2005). Unexpectedly, high rates of natural resistance to oseltamivir were reported worldwide during the 2007–2008 influenza season. During the subsequent season (2008–2009), 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 (CDC, 2009c). Epidemiological studies reported no evidence of an association between the development of resistance and

Table 2
Influenza A viruses of the N1 subtype with reduced susceptibility to neuraminidase inhibitors.

Influenza Subtype	NA mutation[a]	Virus source/NAI used for selection	Phenotype in NA inhibition assays:[b]			Reference
			Oseltamivir	Zanamivir	Peramivir	
A(H1N1)	H274Y	Clinic/oseltamivir	HRI	S	HRI	Mishin et al. (2005)
	Q136K	*In vitro* (clinic?)/none	S	HRI	–	Hurt et al. (2009b)
A(H1N1)pdm09	N294S	Reverse genetics	HRI	S	RI	Pizzorno et al. (2011b)
	H274Y	Clinic/oseltamivir	HRI	S	HRI	Baz et al. (2009)
	S246N/H274Y	Clinic/oseltamivir	HRI	S	HRI	Hurt et al. (2011b)
	I222V/H274Y	Clinic/oseltamivir	HRI	S	–	CDC (2009a)
		Reverse genetics	HRI	S	HRI	Pizzorno et al. (2011b)
	I222R/H274Y	Clinic/oseltamivir	HRI	RI	HRI	Nguyen et al. (2010a)
		Reverse genetics	HRI	RI	HRI	Pizzorno et al. (2012)
	I222R	Clinic/oseltamivir	RI	RI	–	Eshaghi et al. (2011)
		Reverse Genetics	RI	RI	RI	Pizzorno et al. (2012)
	E119G	Reverse genetics	S	HRI	RI	Pizzorno et al. (2011b)
	E119V	Reverse genetics	RI	HRI	RI	Pizzorno et al. (2011b)
A(H5N1)	H274Y	Clinic/oseltamivir	HRI	S	HRI	Le et al. (2005)
	N294S	Clinic/oseltamivir	RI	S	S	Le et al. (2005)
	D198G	*In vitro*/zanamivir	RI	RI	S	Hurt et al. (2009a)
	E119G	*In vitro*/zanamivir	S	HRI	RI/HRI	Hurt et al. (2009a)

[a] Numbers indicate the position of the substituted residue in the NA amino acid sequence (N2 numbering).
[b] S, susceptibility or normal inhibition (<10-fold increase in IC_{50} over WT); RI, reduced inhibition (10–100-fold increase in IC_{50} over WT); HRI, highly reduced inhibition (>100-fold increase in IC_{50} over WT). A(H1N1): seasonal H1N1 viruses, A(H1N1)pdm09: Swine origin H1N1 viruses responsible for the 2009 pandemic.

Table 3
Influenza A(H3N2) viruses with reduced susceptibility to neuraminidase inhibitors.

Influenza Subtype	NA mutation[a]	Virus source/NAI used for selection	Phenotype in NA inhibition assays:[b]			Reference
			Oseltamivir	Zanamivir	Peramivir	
A(H3N2)	N294S	Clinic/oseltamivir	HRI	S	–	Kiso et al. (2004)
	R292K	Clinic/oseltamivir	HRI	–	–	Carr et al. (2002)
		Clinic/oseltamivir	HRI	S	–	Sheu et al. (2008)
		Reverse genetics	HRI	RI	–	Yen et al. (2006)
	Deletion 245–248	Clinic/oseltamivir	HRI	S	S	Abed et al. (2009)
	D151A/D	Clinic?/none	S	HRI	–	Sheu et al. (2008)
	Q136K	Clinic/none	S	RI	–	Dapat et al. (2010)
	E119V/I222V	Clinic/oseltamivir	HRI	S	S	Baz et al. (2006)
	E119V	Clinic/oseltamivir	HRI	S	S	Mishin et al. (2005)
	R224K	Reverse genetics	HRI	HRI	–	Yen et al. (2006)
	R371K	Reverse genetics	RI	RI	–	Yen et al. (2006)

[a] Numbers indicate the position of the substituted residue in the NA amino acid sequence (N2 numbering).
[b] S, susceptibility or normal inhibition (<10-fold increase in IC_{50} over WT); RI, reduced inhibition (10–100-fold increase in IC_{50} over WT); HRI, highly reduced inhibition (>100-fold increase in IC_{50} over WT).

Table 4
Influenza B viruses with reduced susceptibility to neuraminidase inhibitors.

Influenza Type	NA mutation[a]	Virus source/NAI used for selection	Phenotype in NA inhibition assays:[b]			Reference
			Oseltamivir	Zanamivir	Peramivir	
B	R371K	Clinic/none	HRI	RI	–	Sheu et al. (2008)
	N294S	Clinic/none	HRI	–	–	Carr et al. (2011)
	D198N	Clinic/oseltamivir	HRI	HRI	S	Mishin et al. (2005)
	R152K	Clinic/zanamivir	HRI	RI	HRI	Mishin et al. (2005)
		Reverse genetics	HRI	RI	HRI	Sleeman et al. (2011)
	E119A	Reverse genetics	HRI	HRI	HRI	Jackson et al. (2005)
	E119D	Reverse genetics	HRI	HRI	HRI	Jackson et al. (2005)
	E119G	Reverse genetics	HRI	RI	HRI	Jackson et al. (2005)
	E119V	Reverse genetics	HRI	S	HRI	Jackson et al. (2005)
	R292K	Reverse genetics	RI	RI	HRI	Jackson et al. (2005)
	E105K	Clinical/none	RI	S	HRI	Fujisaki et al. (2012)
	H274Y	Clinical/?	RI	S	RI	Fujisaki et al. (2012)
	I222T	Clinical/none	RI	S	–	Wang et al. (2012)

[a] Numbers indicate the position of the substituted residue in the NA amino acid sequence (N2 numbering).
[b] S, susceptibility or normal inhibition (<5-fold increase over WT); RI, reduced inhibition (5–50-fold increase over WT); HRI, highly reduced inhibition (>50-fold increase over WT).

oseltamivir use (Dharan et al., 2009; Hauge et al., 2009; Meijer et al., 2009).

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 (Renaud et al., 2011): ~1% in the United States (Storms et al., 2012),<1% in Canada (PHAC, 2011), ~2.5% in Europe and <1.6% worldwide (Hurt et al., 2012a). 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 (Baz et al., 2009; Graitcer et al., 2011), the fraction of drug-resistant cases not associated with oseltamivir exposure has increased significantly in US, from 11% in the 2009–2010 season to 74% in 2010–2011 (Storms et al., 2012). 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 (HPA, 2009; Lackenby et al., 2011), Australia (Hurt et al., 2011a, 2012b) and Vietnam (Mai et al., 2010).

4.1.1. Resistance to oseltamivir in influenza A viruses of the N1 subtype

In influenza viruses of the N1 subtype, including seasonal A(H1N1), A(H1N1)pdm09 (Baz et al., 2009; Memoli et al., 2010a) and highly pathogenic A(H5N1) (de Jong et al., 2005; Le et al., 2005) strains, oseltamivir-resistant clinical isolates typically contain the H274Y mutation (H275Y in the N1 numbering system) (Table 2). Additional NA mutations in oseltamivir-resistant A(H1N1)pdm09 isolates, such as I222R/K/V (I223R/K/V in N1 numbering) (CDC, 2009a; LeGoff et al., 2012; Nguyen et al., 2012a; Pizzorno et al., 2012, 2011b; van der Vries et al., 2010), S246N (S247N in N1 numbering) (Hurt et al., 2011b) and I117V (Hurt et al., 2012c) 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. Noteworthy, the A(H1N1)pdm09 I222R clinical mutant has been shown to retain its virulence and transmissibility in the ferret model (van der Vries et al., 2011). The I222R substitution could even restore the mildly reduced fitness of the H274Y mutant (Pizzorno et al., 2012). It should indicated that only the H274Y NA mutation was shown to confer resistance to oseltamivir in the human A(H5N1) background (Le et al., 2005). Moreover, the reduced sensitivity to oseltamivir conferred by the N294S mutation (N295S in N1 numbering), was shown to occur without NAI pressure in humans infected with highly pathogenic A(H5N1) viruses (Earhart et al., 2009; Le et al., 2005).

Similar observations have also been reported during *in vitro* studies. In NA inhibition assays, the H274Y mutation was associated with significant increases in IC_{50} values (450- to 1000- fold) for seasonal A(H1N1) and A(H1N1)pdm09 variants (Abed et al., 2011a; Pizzorno et al., 2011b). Seasonal A(H1N1) variants with N294S mutations also demonstrated decreased sensitivity to oseltamivir (Abed et al., 2006, 2008).

4.1.2. Resistance to oseltamivir in influenza A viruses of the N2 subtype

In influenza viruses of the N2 subtype, the R292K substitution in the catalytic site, is rather common and markedly reduces sensitivity to oseltamivir (Table 3). Decreased sensitivity to oseltamivir is also frequently conferred by the E119V mutation; in that 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 (Kiso et al., 2004; Whitley et al., 2001). The N294S variant also showed decreased susceptibility to oseltamivir in seasonal A(H3N2) viruses (Abed et al., 2006; Kiso et al., 2004). It should be noted that, in addition to amino acid substitutions, small NA deletion mutations conferring reduced susceptibility to NAIs were also reported in A(H3N2) viruses (Abed et al., 2009; Memoli et al., 2010b).

4.1.3. Resistance to oseltamivir in influenza B viruses

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) (Wang et al., 2012).

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 (Ison et al., 2006; Mishin et al., 2005; Sheu et al., 2008) (Table 4). 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 (Higgins et al., 2012) whereas a N294S variant was detected in a 7-year old patient with cancer prior to oseltamivir therapy (Carr et al., 2011).

Reduced susceptibility to oseltamivir associated with the R152K mutation has also been reported during *in vitro* studies. Indeed, this variant emerged under NAI pressure and led to a 100-fold increase in oseltamivir IC_{50} value (Mishin et al., 2005), whereas a

>250-fold increase in IC_{50} values was observed with a genetically modified virus (Jackson et al., 2005). A genetically modified influenza B virus with the R292K substitution was also associated with increased oseltamivir IC_{50} values (Jackson et al., 2005); however, this mutation have not yet been observed in the clinic.

4.2. Resistance to zanamivir

So far, resistance to zanamivir has remained quite infrequent in the clinical setting for both seasonal and pandemic viruses as compared to oseltamivir (Thorlund et al., 2011). The higher structural homology to the NA natural substrate (sialic acid) as well as the lower use of this drug compared to oseltamivir are the most probable factors that may account for this observation.

4.2.1. Resistance to zanamivir in influenza A viruses of the N1 subtype

Surveillance data from the 2006 to 2008 seasons in Australia and Southeast Asia identified zanamivir-resistant influenza A(H1N1) viral isolates harboring the Q136K substitution (Hurt et al., 2009b) (Table 2). 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 (Okomo-Adhiambo et al., 2010). 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 (Hurt et al., 2010). 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 (Eshaghi et al., 2011; Nguyen et al., 2010a; van der Vries et al., 2010).

Substitutions at residues 119 (E119G) and 198 (D198G; D199G in N1 numbering) were selected *in vitro* under zanamivir pressure among influenza A(H5N1) viruses (Hurt et al., 2009a). In adition, zanamivir resistance was observed for recombinant influenza A/WSN/33 (H1N1) (Abed et al., 2004) and A(H1N1)pdm09 (Pizzorno et al., 2011b) viruses carrying the E119V mutation. Interestingly, the E119V mutation severely compromised the fitness of the latter two viruses (Abed et al., 2004; Pizzorno et al., 2011b).

4.2.2. Resistance to zanamivir in influenza A viruses of the N2 subtype

Influenza A(H3N2) variants containing zanamivir resistant D151A/E/G/V mutations were identified in A(H3N2) variants during NAI resistance surveillance programs (McKimm-Breschkin et al., 2003; Sheu et al., 2008) (Table 3). Nevertheless, there are uncertainties about the clinical relevance of such changes. Contrasting with the situation in A(H1N1) viruses, where the Q136K mutation was only detected in supernatants of infected cells, the Q136K mutation was detected in two clinical samples of influenza A(H3N2) viruses isolated in Myanmar in 2007 and 2008 with reduced susceptibility to zanamivir (Dapat et al., 2010).

Several other A(H3N2) variants such as R224K, R292K and R371K that exhibited 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 (Yen et al., 2005, 2006). Zanamivir-resistant recombinant H3N2 viruses harboring different substitutions at residue 119 (E119G/D/A) were also rescued by reverse genetics (Zürcher et al., 2006). The fitness of these recombinant variants was significantly impaired due to altered NA enzymatic activity/affinity and stability.

4.2.3. Resistance to zanamivir in influenza B viruses

In clinic, an influenza B variant with the R152K substitution (R150K in B numbering) has been recovered from an immunocompromised child treated with zanamivir (Gubareva et al., 1998) (Table 4). Also, the E119G (E116G in B numbering), R152K (R150K in B numbering) and R292K variants with reduced susceptibility to zanamivir were generated *in vitro* (Jackson et al., 2005). 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 (Jackson et al., 2005).

4.3. Resistance to peramivir

In clinic, emergence of peramivir-resistant viruses has been 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 (CDC, 2009b). These strains showed high levels of resistance to oseltamivir and lower level of resistance to peramivir (Hurt et al., 2012a). Other cases of peramivir resistance have been reported after shorter courses of oseltamivir treatment (i.e., 9 and 14 days) (Memoli et al., 2010a).

4.3.1. Resistance to peramivir in influenza A viruses of the N1 subtype

Peramivir resistance is mainly conferred by the H274Y mutation in influenza viruses of the N1 subtype (Table 2). For instance, a H274Y mutant emerged in a patient with severe A(H1N1)pdm09 infection during IV peramivir therapy (Renaud et al., 2010). During the 2007–2010 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% (2007–2008), 0.1% (2008–2009) and 6.1% (2009–2010) 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 (Baz et al., 2007). Of note, both seasonal and A(H1N1)pdm09 viruses containing the H274Y mutation showed peramivir IC_{50} values that were lower than those of oseltamivir (Abed et al., 2004; Pizzorno et al., 2011b).

4.3.2. Resistance to peramivir in influenza A viruses of the N2 subtype

There are no reports on peramivir resistance in influenza viruses of the N2 subtype.

4.3.3. Resistance to peramivir in influenza B viruses

Two NA mutations (V94I and R152K: respectively V89I and R150K in B numbering) were detected in an influenza B isolate from a pediatric patient (Table 4). These mutants were associated with a reduction in susceptibility to peramivir (Yoshida et al., 2011). 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 (Higgins et al., 2012).

The influenza B H274Y mutant was also generated *in vitro* under peramivir pressure (Baum et al., 2003). 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 wild-type strain (Jackson et al., 2005). In another study, drug-selected R152K

variants also exhibited a reduction in susceptibility to peramivir (Mishin et al., 2005).

4.4. Resistance to laninamivir

To date, no laninamivir-resistant mutants have been reported. Moreover, it is legitimate to infer that both zanamivir and laninamivir might be effective against the most frequent oseltamivir-resistant mutants i.e., H274Y and E119V. Nevertheless, it is important to note the higher potency of laninamivir against group 1 NA viruses in which the more open configuration of the 150-loop may facilitate the access to the active site for laninamivir contrasting to group 2 NAs with a relatively closed configuration of the 150-loop that may hinder the entry and binding of zanamivir and laninamivir (Vavricka et al., 2011).

4.5. Transmissibility of NAI-resistant influenza viruses

Two recent reports have suggested that the drug-resistant A(H1N1)pdm09 virus with the H274Y mutation may have become more transmissible. The first report refers to a cluster that occurred in Newcastle, Australia, in 2011 and involved the detection of an oseltamivir-resistant strain in over thirty community patients. Ninety percent of cases lived within 50 km, although 3 cases were detected 120, 380 and 4000 km away from Newcastle. Only one case (not the index one) received oseltamivir.The H274Y drug-resistant strains were genetically very closely related, suggesting the spread of a single variant (Hurt et al., 2011a, 2012b). The second report refers to two Dutch travelers infected with an oseltamivir-resistant H274Y A(H1N1)pdm09 strain in early August 2012. Both cases were probably infected during separate holidays on the Catalonian coast (Spain). No epidemiological connection between the two cases was found, and neither of them was treated with oseltamivir before specimen collection (Meijer et al., 2012).

The replication capacity and transmissibility of oseltamivir-resistant mutants appear to vary depending on the genetic background of the viruses (Baz et al., 2010). It has been shown that secondary NA mutations such as D344N that emerged in A(H1N1) variants isolated after the 2006–2007 season were associated with higher NA activity and affinity and could have facilitated the subsequent emergence of the H274Y mutation (Collins et al., 2009; Rameix-Welti et al., 2008). Two other permissive NA mutations (V234M and R222Q) were shown to restore the viral fitness and counteract the compromising impact of the H274Y mutation on NA activity and cell surface expression (Bloom et al., 2010). Consequently, such permissive NA mutations in A/Brisbane/59/2007(H1N1)-like viruses provide an enzymatic context that allowed the introduction of the H274Y substitution without compromising viral fitness (Abed et al., 2011b).

Subsequently, potential permissive NA substitutions that could facilitate the replicative capacity of the A(H1N1)pdm09 H274Y mutant were assessed by computational analysis (Bloom et al., 2011). Such analysis identified some NA substitutions that could indeed exert a permissive effect on the emergence and spread of the H274Y change in the A(H1N1)pdm09 background. Interestingly, one of these predicted mutations (N369K) was recently identified, along with two additional non-predicted NA changes (V241I, N386S), in clinical A(H1N1)pdm09 variants from the Newcastle outbreak in 2011 (Hurt et al., 2012b). Strains harboring the same NA genotype (H274Y, V241I, N369K and N386S) were also detected in Dutch travellers returning from Spain in 2012 (Meijer et al., 2012).

Although these two clusters of strains carry the same NA substitutions that can potentially facilitate accommodation of the H274Y substitution, the HA genes from these groups belonged to separate genetic clusters. The Australian cluster carried an HA from genetic clade 7 whereas the Spain group carried an HA from genetic clade 6. It is important to mention that clade 6 HA and NA carrying the V241I, N369K and N386S substitutions represented a substantial proportion of A(H1N1)pdm09 viruses detected worldwide in 2012 (Meijer et al., 2012).

5. Management of NAI-resistant infections

In contrast to oseltamivir, resistance to zanamivir has remained infrequent among seasonal and pandemic influenza isolates to date. 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 (Gubareva, 2004; Pizzorno et al., 2011b). Accordingly, zanamivir is the antiviral of choice for the management of influenza cases or outbreaks involving oseltamivir-resistant viruses (Chen et al., 2011; Mandelboim et al., 2009). However, inhaled zanamivir is not approved in children aged less than 5 years (WHO, 2012b). In addition, the safety and efficacy of inhaled or nebulized zanamivir in severely ill patients remains to be proven. Finally, the zanamivir disc inhaler is not adequate for patients who are intubated as the lactose carrier can interfere with ventilator filters (Kiatboonsri et al., 2010).

Parenteral drugs are therefore highly needed for treatment of severe influenza infections. The IV formulation of zanamivir is in phase 3 clinical trials but can be obtained on a compassionate basis for severely ill patients with suspected or confirmed oseltamivir-resistant infections (Härter et al., 2010). 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 pharmacokinetics properties. Indeed, in healthy volunteers, a single IV dose of 600 mg of peramivir has resulted in plasma concentration of 34,100 ng/ml, which is much higher than the IC_{50} value for H274Y variants (4.26 ng/ml) (Shetty and Peek, 2012). Accordingly, mouse studies revealed that a single IM dose of peramivir provided important prophylactic and therapeutic benefits against the lethal A/WSN/33 (H1N1) virus and its H274Y variant (Abed et al., 2011a). However, controlled trials are needed to confirm the efficacy of parenteral peramivir against H274Y mutants in clinic. Laninamivir, which is a multimeric zanamivir compound given by the inhaled route, may also be useful against most oseltamivir-resistant viruses (Kubo et al., 2010; Yamashita et al., 2009).

6. Investigational agents and combination therapy

This review highlighted the increasing challenges of public health authorities and clinicians for the control of influenza infections during epidemics and occasional pandemics. It particularly emphasized the need to develop additional anti-influenza strategies to compensate for the inefficacy of adamantanes, for which influenza A(H1N1)pdm09 and A(H3N2) strains are resistant, and to address the issue of NAI-resistant variants that may emerge either during drug pressure or as a result of natural genetic evolution. Indeed, new anti-influenza targets involving the polymerase (T-705 or favipiravir) (Furuta et al., 2002, 2005, 2009) or host cells receptors (DAS181 or Fludase) (Triana-Baltzer et al., 2009a,b) are at various stages of clinical development.

Combination therapies may also be particularly useful in immunocompromised patients for synergistic or additive antiviral effects and for reducing the emergence of drug resistance (Govorkova et al., 2004; Hayden et al., 1984; Stein et al., 1987). Recently, a triple combination regimen of oseltamivir, amantadine and the nucleoside analog ribavirin, exhibited a strong synergistic activity

against seasonal and A(H1N1)pdm09 viruses *in vitro* and *in vivo* (Nguyen et al., 2010b, 2009, 2012b). In addition, lethal mouse studies demonstrated a significant increase in survival for animals that received peramivir combined to ribavirin (Smee et al., 2002) and rimantadine combined to oseltamivir (Galabov et al., 2006) versus respective monotherapies. Interestingly, the combination of oseltamivir to ribavirin (Ilyushina et al., 2008) or to amantadine (Ilyushina et al., 2008) showed benefits against highly pathogenic influenza A(H5N1) infections in BALB/c mice. On the other hand, combination of two NAIs (oseltamivir and zanamivir) has been shown to be inferior to oseltamivir monotherapy in a French clinical trial (Duval et al., 2010).

7. Conclusion and perspectives

NAIs constitute an important tool for the control of influenza infections. 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. This observation was highlighted by the worldwide dissemination of the drug-resistant seasonal A(H1N1) H274Y variant during the 2007–2009 annual influenza epidemics. The detection of oseltamivir-resistant A(H1N1)pdm09 variants in untreated individuals and from a few community clusters is also of growing concern. Rapid evolution of influenza surface genes may select for permissive substitutions with the potential to improve the fitness of drug-resistant variants. This observation has an impact on the global monitoring of NAI resistance; such monitoring should look not only for drug resistance mutations but also for potential permissive substitutions. It is also imperative to perform additional studies evaluating different combinations of existing anti-influenza agents as this approach may provide the advantage of reducing the rate of resistance in severely-infected patients. Finally, there is a need of developing new antiviral strategies including not only new antiviral agents but also immunomodulatory compounds to improve the outcome of influenza infections.

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