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Review Article

Detection and management of antiviral resistance for influenza viruses

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Neuraminidase inhibitors (NAIs) are first-line agents for the treatment and prevention of influenza virus infections. As for other antivirals, the development of resistance to NAIs has become an important concern particularly in the case of A (H1N1) viruses and oseltamivir. The most frequently reported change conferring oseltamivir resistance in that viral context is the H275Y neuraminidase mutation (N1 numbering). Recent studies have shown that, in the presence of the appropriate permissive mutations, the H275Y variant can retain virulence and transmissibility in some viral backgrounds. Most oseltamivir-resistant influenza A virus infections can be managed with the use of inhaled or intravenous zanamivir, another NAI. New NAI compounds and non-neuraminidase agents as well as combination therapies are currently in clinical evaluation for the treatment for severe influenza infections.

Keywords Antiviral, influenza, neuraminidase inhibitor, oseltamivir, resistance, zanamivir.

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Classes of antiviral agents for influenza virus infections

There are two classes of antiviral agents approved for the treatment and prevention for influenza viruses: the adamantanes and the neuraminidase inhibitors. The adamantane compounds amantadine and rimantadine act by blocking the M2 ion channel preventing viral uncoating. Influenza B viruses are intrinsically resistant to these compounds due to their lack of M2 target protein. In 1999–2000, two neuraminidase inhibitors (NAIs) were approved in many countries: the oral agent oseltamivir and the inhaled compound zanamivir. Both antiviral drugs are active against all influenza A virus subtypes as well as influenza B viruses.[1] The NAIs prevent the cleavage of the terminal sialic acid residues on budding virions, a process that prevents infection of new host cells and thereby blocks virus dissemination throughout the respiratory tract. Due to high levels of resistance to the M2 blockers, the NAIs and especially oseltamivir have become the agents of choice for the treatment of individuals with severe influenza infections and for those with underlying diseases predisposing to influenza complications. As it is the case for other antivirals, the development of drug resistance is an important issue that can potentially limit the usefulness of NAIs.

Risk factors and incidence of resistance to anti-influenza drugs

Resistance to the adamantanes has been shown to develop rapidly, that is, within 3–5 days in 30–50% of treated immunocompetent and immunocompromised individuals.[2] Furthermore, resistance to this class of agents has emerged in 2004 among A(H3N2) viruses and some A(H1N1) viruses, in the absence of antiviral pressure. Also, all A(H1N1)pdm09 viruses that circulated during the 2009 influenza pandemic and up to now have been intrinsically resistant to the adamantanes. Thus, at the present time, virtually, all circulating influenza A viruses recovered from humans are resistant to these compounds.

It was previously thought that resistance to NAIs would not be an important clinical problem because the neuraminidase (NA) is a critical enzyme in the virus replicative cycle, and previous oseltamivir-resistant viruses were found to be unfit and poorly transmissible in animal models.[3] Thus, the emergence and predominance of an oseltamivir-resistant A (H1N1) virus (A/Brisbane/59/2007, clade 2B) between 2007 and 2009 came as a surprise.[4] It was subsequently shown that the good fitness of this oseltamivir-resistant viral strain containing the H275Y (N1 numbering; H274Y in N2 numbering) NA resistance mutation was due to the presence of pre-existing permissive NA mutations such as R222Q (N1

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numbering) that increased both the activity and the surface expression of the NA.[5,6] The A/Brisbane/59/2007 strain was no longer detected after the emergence of the A(H1N1) pdm09 virus in 2009. Most circulating A(H1N1)pdm09, A (H3N2) and B viruses remain susceptible to oseltamivir with <1·5% of tested strains exhibiting phenotypic or genotypic evidence of resistance in 2011–2012.[7] However, recent outbreaks of oseltamivir-resistant A(H1N1)pdm09 viruses in Australian citizens and Dutch travelers returning from Spain have been reported in the absence of drug treatment and are reminders of the importance of continuous antiviral susceptibility monitoring.[8–10] In those cases, a new set of permissive NA mutations (such as N369K and V241I in N1 numbering) may have facilitated the emergence of the H275Y resistance mutation and improved virus transmissibility. Factors associated with the selection of drug resistance at the individual level include: the use of post-exposure prophylaxis (with the administration of lower drug dose), infection of an immunocompromised host and prolonged antiviral treatment. During the first wave of the 2009 influenza pandemic, up to 25–30% of oseltamivir-resistant cases were reported in diverse immunocompromised individuals, and thus, immunosuppression represents the most important setting where resistance can develop.[11] Of importance, during the 2011–2012 influenza season in USA, up to 74% of oseltamivir resistance cases were not associated with drug exposure,[12] and this may reflect the emergence of a set of permissive NA mutations as described previously.[10] A similar trend had been previously noted in 2010–2011 in United Kingdom.[13] Fortunately, resistance to zanamivir has remained extremely rare in all influenza subtypes.

Assays for detecting antiviral resistance

Similar to other viruses, phenotypic and genotypic assays can be used for assessing resistance to anti-influenza compounds. Phenotypic assays first require viral propagation and then subsequent determination of the drug 50% inhibitory concentration (IC_{50}) value. For the adamantanes, IC_{50} values are assessed by the conventional plaque reduction assay. For the NAIs, enzymatic assays are preferred to plaque assays and have been shown to more reliably estimate drug susceptibilities.[14] Different types of NA assays can be performed using chemiluminescent, fluorescent, or colorimetric NA substrates. The fluorometric assay allows a better discrimination between susceptible and resistant viruses, whereas the chemiluminescent assay needs less input virus for testing.[15] There exists no standard definition of NAI resistance, but recent WHO guidelines have been proposed: reduced inhibition is defined by 10- to 100-fold and 5- to 50-fold increases in IC_{50} values for influenza A and B virus isolates, respectively, whereas highly reduced inhibition is defined by >100-fold and >50-fold increases in IC_{50} values.[16]

Because of the time needed to grow the isolates and to determine drug IC_{50} values, many laboratories are performing some types of genotypic assays to detect drug resistance mutations directly from the clinical samples after a RT-PCR amplification step. Although more rapid than phenotypic assays, these tests do not determine the level of resistance and are of limited utility in case a novel NA mutation is identified in the absence of susceptibility test results. The primary approach for genotypic testing is to amplify by RT-PCR the targeted gene, that is, M2 in the case of the adamantanes and NA for the NAIs, followed by conventional DNA Sanger sequencing. This strategy is comprehensive, potentially detecting all mutations associated with drug resistance, but it suffers from a lack of sensitivity for detection of minor variants within a viral population. Indeed, a mutant variant must be in excess of 15–20% of the total population to be identified by conventional DNA sequencing. The advent of pyrosequencing and especially next-generation ultra-deep sequencing has allowed the detection of minor variants in excess of 1–2%.[17] Such unprecedented level of detection has improved our understanding of the evolution of drug resistance by showing the presence of drug-resistant variants in samples of individuals before the onset of therapy and the transmission of those drug-resistant variants along with drug-susceptible viruses between hosts.[17] This novel information is of great importance for predicting the speed at which resistance will arise and also to gain insight into the relative fitness of some drug-resistant mutants. However, despite its undoubted potential, deep sequencing also has a number of inherent analytical difficulties including the generation of short sequence reads that could be difficult to link together as well as the problem of PCR and/or sequencing artifacts.

Mutations conferring drug resistance

Genotypic analysis of resistance to M2 blockers is relatively straightforward as only a few substitutions occurring at five codons within the M2 gene (codons 26, 27, 30, 31 and 34) have been linked to amantadine/rimantadine resistance.[18] Importantly, these M2 mutants are fully virulent and transmissible between humans.[19] The NA mutations conferring resistance to NAIs vary according to the viral subtype/type and the NAI[20] (Table 1). In the N1 subtype, the most frequently encountered mutation is the H275Y that confers highly reduced inhibition to oseltamivir, moderate cross-resistance to the investigational agent peramivir and susceptibility to zanamivir.[21] Various amino acid changes at residue 223 (I →R/V) can also confer reduced inhibition to oseltamivir and/or to zanamivir.[22,23] In the N2 subtype, the most frequent mutations conferring highly reduced inhibition to oseltamivir are E119V and R292K, the latter being also associated with reduced inhibition to zanamivir.[24,25]

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Table 1. Selected neuraminidase mutations conferring resistance to neuraminidase inhibitors (mainly reviewed in[52])

Influenza subtype	NA mutation	Virus source / NAI used for selection	Phenotype in NA inhibition assays*		
			Oseltamivir	Zanamivir	Peramivir
A(H1N1)	H275Y	Clinic / Oseltamivir	HRI	S	HRI
	Q136K	In vitro (clinic?) / None	S	HRI	RI
A(H1N1)pdm09	N295S	Reverse Genetics	HRI	S	RI
	H275Y	Clinic / Oseltamivir	HRI	S	RI
	S247N/H275Y	Clinic / None	HRI	S	RI
	I223V/H275Y	Clinic / Oseltamivir	HRI	S	-
		Reverse Genetics	HRI	S	HRI
	I223R/H275Y	Clinic / Oseltamivir	HRI	RI	HRI
		Reverse Genetics	HRI	RI	HRI
	I223R	Clinic / Oseltamivir	RI	RI	-
		Reverse Genetics	RI	RI	RI
	E119G	Reverse Genetics	S	HRI	RI
	E119V	Reverse Genetics	RI	HRI	RI
A(H5N1)	N295S	Clinic / Oseltamivir	RI	S	S
	H275Y	Clinic / Oseltamivir	HRI	S	HRI
	D199G	In vitro / Zanamivir	RI	RI	S
	E119G	In vitro / Zanamivir	S	HRI	RI/HRI
A(H3N2)	N294S	Clinic / Oseltamivir	HRI	S	-
	R292K	Clinic / Oseltamivir	HRI	-	-
		Reverse Genetics	HRI	S/RI	RI
	Deletion 245–248	Clinic / Oseltamivir	HRI	S	S
	D151A/D	Clinic?/ None	S	HRI	-
	Q136K	Clinic?/ None	S	RI	-
	E119V/I222V	Clinic / Oseltamivir	HRI	S	S
	E119V	Clinic / Oseltamivir	HRI	S	S
B	R371K	Clinic/ None	HRI	RI	-
	N294S	Clinic/ None	HRI	-	-
	R292K	Reverse Genetics	S	RI	HRI
	H273Y	Clinic? / ?	RI	S	RI
	D197N	Clinic / Oseltamivir	RI	RI	RI
	R150K	Clinic / Zanamivir	HRI	RI	HRI
		Reverse Genetics	HRI	RI	HRI
	E116A	Reverse Genetics	HRI	HRI	HRI
	E116D	Reverse Genetics	HRI	HRI	HRI
	E116G	Reverse Genetics	RI	HRI	HRI
	E116V	Reverse Genetics	HRI	S	HRI
	E105K	Clinical / None	S	RI	HRI

*S, susceptibility or normal inhibition (<10-fold increase in IC_{50} over WT for A viruses or <5-fold increase for B viruses); RI, reduced inhibition (10- to 100-fold increase in IC_{50} over WT for A viruses or 5- to 50-fold increase for B viruses); HRI, highly reduced inhibition (>100-fold increase in IC_{50} over WT for A viruses or >50-fold increase for B viruses). ? means uncertain or unknown information.

Reported changes associated with NAI resistance in B viruses include mainly R150K and D197N.[26,27] Of note, some NA mutations reported at codons 136 and 151 have a questionable clinical impact because they have been almost exclusively detected after cell passages and rarely in primary clinical samples.[28–30] Finally, very few zanamivir-resistant influenza viruses have been reported in clinical samples so far which may be explained by the modest use of this inhaled antiviral and also possibly by a higher genetic barrier for resistance due to a greater structural homology to the natural substrate, sialic acid.[31]

Impact of the H275Y NA mutation in A/H1N1 viruses

Understanding the impact of the H275Y is important for several reasons: (i) it is the most frequent mutation conferring resistance to oseltamivir and (ii) it was detected and transmitted in some viral backgrounds in the absence of antiviral pressure. Indeed, close to 100% of A/Brisbane/59/2007 (H1N1)-like viruses that circulated in 2008–2009 in Europe and North America were resistant to oseltamivir due to the H275Y NA mutation.[4] Although the detection of this

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mutation in the more recent A(H1N1)pdm09 viral background remains limited (<1·5% of tested strains), there is a recent concern that this problem could increase due to the growing number of resistant strains detected in the absence of therapy.[12] These data indicate that, in the appropriate viral background, that is, with the required permissive NA mutation(s), the H275Y mutant can retain fitness and become more transmissible.[5,32] Based on ferret experiments, many groups have shown that the oseltamivir-resistant A (H1N1)pdm09 virus with the H275Y mutation was as virulent as its wild-type counterpart with the exception of a reduced airborne transmission reported in some but not all studies.[33–37]

Using a mathematical model to analyze a set of *in vitro* experiments that allow for the full characterization of the viral replication cycle, our group showed that the primary effects of the H275Y substitution on A(H1N1)pdm09 strains were to lengthen the mean eclipse phase of infected cells (from 6·6 to 9·1 hour) and decrease (by sevenfold) the viral burst size, that is, the total number of virions produced per cell.[38] However, the infectious-unit-to-particle ratio of the H275Y mutant strain was 12-fold higher than that of oseltamivir-susceptible strain (0·19 versus 0·016 per RNA copy). A parallel analysis of the H275Y mutation in the prior seasonal A/Brisbane/59/2007 background showed similar changes in the infection kinetic parameters but, in this strain, the H275Y mutation also allows the mutant to infect cells five times more rapidly. This model estimated a basic reproductive number (Ro), which is defined by the number of secondary infections caused by a single infectious cell, that was approximately the same for the A(H1N1)pdm09 wild type and its H275Y mutant ($1{\cdot}7 \times 10^3$ versus $3{\cdot}0 \times 10^3$, respectively), whereas it was 25 times higher for the H275Y mutant compared with the wild-type virus in the A/Brisbane/59/2007 background (48×10^3 versus $1{\cdot}7 \times 10^3$, respectively). These replication parameters help to explain why the H275Y mutant replaced the wild-type strain in the seasonal A/Brisbane/59/2007 background, whereas it is still not the case for the 2009 pandemic virus. In addition, another group has suggested that oseltamivir-resistant A(H1N1) strains were less susceptible to antibody inhibition than susceptible strains, which may have favored their selection and dissemination in 2008–2009.[39]

Management of oseltamivir-resistant severe influenza A infections

The selection of the most appropriate antiviral therapy must take into consideration that most cases of oseltamivir resistance are due to the H275Y mutation in the N1 subtype or the E119V and R292K mutations in the N2 subtype and that the first two viral mutants remain susceptible to zanamivir. Thus, inhaled or, for more severe cases, intravenous (where available through compassionate use)[40] zanamivir is the best option in the case of suspected or confirmed resistance to oseltamivir. For patients on mechanical ventilation, if intravenous zanamivir is not available, other therapeutic options include inhaled or systemic ribavirin[41] or parenteral peramivir, a NAI that is currently approved in Japan and South Korea. Although the H275Y mutant exhibits highly reduced inhibition to peramivir *in vitro*, animal studies using the A/WSN/33 (H1N1) virus indicated that a single administration (90 mg/kg intramuscularly) or multiple daily doses (45 mg/kg × 5 days) of this compound successfully prevented mortality and significantly decreased weight loss and lung viral titers after infection with the H275Y mutant.[42] Such clinical benefits are likely attributable to the high concentrations of peramivir (4000- to 8000-fold higher than the IC_{50} value in plasma), its high binding affinity and slow off-rate from the NA.[42,43] However, because of the emergence of the H275Y mutation in a few patients receiving peramivir during the 2009 pandemic (through an emergency access program),[44] other studies are needed before recommending such therapeutic modality in that context. An algorithm for the management of oseltamivir-resistant infections is proposed in the Figure 1.

Selected investigational antivirals in clinical development for the treatment for influenza

An interim analysis of intravenous peramivir phase three clinical trials in USA has recently shown little difference with placebo on influenza outcomes, which halted the development of this drug in this country. Nevertheless, several

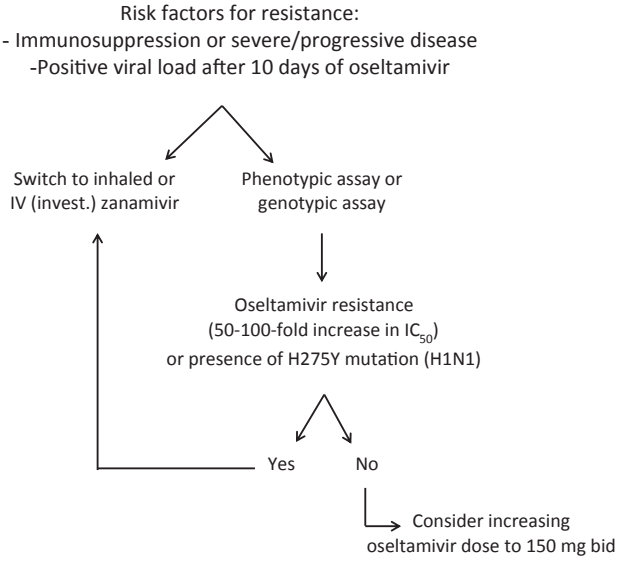

Figure 1. Proposed algorithm for the management of oseltamivir-resistant influenza virus infections.

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antiviral agents are at some stages of clinical development for the treatment for influenza virus infections. In addition to intravenous zanamivir which is in phase three clinical trials in USA (ClinicalTrials.gov #NCT01231620), laninamivir octanoate is another NAI that is already approved in Japan and is in phase three clinical trial in USA (#NCT00803595). This long-acting inhaled prodrug that lasts for 5 days is metabolized to laninamivir, which has a structure and a spectrum of activity similar to those of zanamivir.[45] Favipiravir (formerly known as T-705) is a potent inhibitor of the polymerase of influenza and several other RNA viruses administered by the oral route currently in phase two clinical trials (#NCT01068912).[46] Selection of favipiravir-resistant viruses has not been achieved so far after multiple *in vitro* passages. Another compound with a different mechanism of action is Fludase (formerly known as DAS181), which is an inhaled drug with activity against influenza and parainfluenza viruses.[47] This drug, which is currently in phase two clinical trial (#NCT01037205) for the treatment for influenza infections, acts as a host receptor-destroying enzyme (i.e., it has sialidase activity).

Conclusions

Along with the availability of new compounds with different viral targets, the options for the management of oseltamivir-resistant infections should significantly expand. Consequently, combination therapies for immunocompromised patients will become a feasible strategy. Randomized trials of a triple combination therapy (amantadine, oseltamivir and ribavirin) are in progress based on synergy data for these compounds demonstrated *in vitro* and *in vivo*.[48,49] Another strategy under evaluation consists of combining antiviral agents and immunomodulators such as COX-2 inhibitors.[50] Finally, the administration of broad-spectrum neutralizing antibodies targeting, for example, conserved epitopes of the HA protein is another promising approach.[51] These strategies should increase our therapeutic options and reduce the emergence of NAI-resistant viruses in high-risk patients.

Conflict of interests

Dr Guy Boivin has received research grants from Roche, GlaxoSmithKline and Biota on the topic of influenza treatment.

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