Antiviral resistance in influenza viruses

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ARTICLE INFO	ABSTRACT
Review *Article history:* *Received: December 02, 2022* *Accepted: October 26, 2023* *Published: December 10, 2023* *Keywords:* *Influenza, antivirals, resistance, neuraminidase inhibitors, baloxavir*	Influenza represents a major threat to public health worldwide, vaccination is the most effective strategy to reduce infections. However, achieving adequate vaccination rates is challenging and vaccination does not always guarantee complete protection. For this reason, antiviral drugs represent an important measure to reduce the risk of complications in high-risk patients. However, influenza viruses have a high mutation rate which causes genetic, biochemical, and pathogenic changes that represent a challenge both for the constant replacement of vaccines and reduce their susceptibility to antiviral action. This makes it necessary to determine the mechanisms of these processes, as well as their epidemiological surveillance and, of course, the development of new therapeutic options that may be available in the event of a widespread resistance phenomenon. In this article we review some of the relevant aspects of the replicative cycle of influenza viruses, the antivirals currently used, as well as their resistance mechanisms.

Introduction

Influenza, commonly called the flu, is an acute respiratory infection caused by negative-strand RNA viruses of the *Orthomyxoviridae* family (1). Each year these viruses cause epidemics from fall to early spring. Influenza viruses are highly contagious, and symptoms can be mild or severe. Morbidity and mortality from this infection are more frequent in some populations such as children, individuals with chronic diseases, immunocompromised individuals, pregnant women, and older adults (2). The World Health Organization (WHO) estimates that influenza viruses infect between 3 and 5 million people annually, of which between 290,000 and 650,000 ends in death (3).

On the other hand, there is another type of respiratory infection that is generally mild, and the symptoms disappear within two weeks. It is known as the common cold, which is caused by viruses other than influenza (4). Among others, the causes of the common cold are the parainfluenza virus, respiratory syncytial virus, metapneumovirus and the coronaviruses NL63, OC43, HKU1 and 229E (5). As of 2020, the landscape of respiratory infections has changed with the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in late 2019. This infection named COVID-19 caused a pandemic with important consequences, since a high percentage of patients can present severe conditions and may be lethal (6). Due to social measures to reduce the impact of the COVID-19 pandemic, the cases of influenza and other respiratory infections decreased drastically between 2020 and 2021, however, it is very possible that as soon as the restrictions

and care due to the pandemic, influenza rebound in incidence (7).

Classification of influenza viruses

Influenza viruses are within the family *Orthomyxoviridae*, in which there are four genera of influenza viruses, which are *Alphainfluenzavirus* (Influenza type A), *Betainfluenzavirus* (Influenza type B), *Gammainfluenzavirus* (Influenza type C), and *Deltainfluenzavirus* (influenza type D). In each of them, only one species is located. There are three other genera in the family, which are of little relevance to human health: *Thogotovirus*, *Isavirus* and *Quaranjavirus* (8).

Influenza C virus infects humans but usually causes mild or asymptomatic infections without causing epidemics, however, hospitalization in children has been reported in some cases. Influenza D virus causes infections in farm animals and infection in humans is not well documented but appears not to be a substantial cause of disease (9). The most important for human health are the influenza A and B viruses, which cause epidemics every year. The influenza A virus can cause pandemics, some of which have been associated with millions of deaths. Influenza A viruses are divided into subtypes based on the antigen-antibody reactivity of their two surface proteins: hemagglutinin (HA) and neuraminidase (NA). There are 18 hemagglutinin subtypes (H1-H18) and 11 neuraminidase subtypes (N1-N11). Influenza B viruses are not divided into subtypes, they are classified into two lineages: Yamagata and Victoria (10).

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Structural characteristics of influenza viruses

The size of the viral particle ranges between 80 and 120 nm. It has a pleomorphic lipid membrane (envelope) that protects the nucleocapsid of helical symmetry, which is made up of negative single-stranded ribonucleic acid (RNA) chains intimately interacting with the nucleoprotein (NP). Associated with the nucleocapsid is the RNA polymerase, which consists of three subunits: acidic polymerase (AP), basic polymerase 1 (PB1), and basic polymerase 2 (PB2). In a virion, there are eight viral RNA-protein complexes, each containing a different RNA segment (11) (Figure 1). Table 1 summarizes the characteristics of each of the segments for influenza A and B viruses (12, 13).

Neuraminidase (NA) is a mushroom-shaped tetramer. Its globular part consists of 4 coplanar and spherical subunits, which are supported by a stem that has a hydrophobic portion that is anchored in the virus membrane. The main function of NA is the release of newly synthesized virions since it catalyzes the breaking of the bond between the sialic acid (receptor) to which the virus can bind through its HA and the adjacent carbohydrate residues, generally galactose, for virions to diffuse into the tissue (14).

The replicative cycle of influenza viruses

The replicative cycle begins when the influenza virus interacts with the host cell surface. The attachment of the virion to the cell surface occurs through the action of HA, which is an antigenic glycoprotein found on the surface of the virus and interacts with sialic acid residues on the epithelial cells of the respiratory tract. HA from influenza viruses that infect humans preferentially binds α2,6-linked sialic acid to galactose, whereas HA from avian viruses prefers α2,3-linked sialic acid. This receptor specificity, which is driven in part by the structural features of the

Figure 1. Schematic diagram of influenza viruses. Four proteins associated with the lipid membrane or envelope (HA, NA, M1, and M2) are shown; inside the virion, the eight RNA segments associated with the NP and polymerase proteins (PB1, PB2 and PA) are depicted, numbered from 1 to 8 and with their abbreviated name in italics. The nuclear export protein (NEP) is observed inside the virion. The information was obtained from (9, 13).

HA active site, is one of the most important determinants influencing virus tropism, including cross-species adaptation and transmissibility. Therefore, infection of humans with avian viruses and vice versa is unlikely; if it occurs, human-human transmission is practically nil. However, through genetic modification, some viruses could be adapted to infect humans (15, 16).

Once the HA-receptor complex is formed, the virions are internalized by endocytosis. In the endosome, the viral M2 ion channel plays a key role in the fusion of the viral and cellular membranes, in addition to allowing the dissociation of the internal components of the virion. This occurs by pumping hydrogen ions from the endosome into the viral particle, which acidifies it internally and breaks protein-protein interactions to permit viral ribonucleoproteins to be released from the viral matrix in addition to inducing a conformational change in HA that favors the fusion of the viral and the endosomal membranes, which allows the just released ribonucleoproteins to enter the cytoplasm and be transported to the interior of the nucleus (17-19).

Ribonucleoprotein complexes are composed of viral genomic RNA, NP proteins, and the polymerase complex (PA, PB1, and PB2). Within the nucleus, viral genomic RNA is transcribed and replicated by the action of the viral RNA polymerase complex. The transcription of the different viral genes occurs in a process in which the viral polymerase interacts with the cellular RNA polymerase during the transcription of its own genes and cleaves the newly synthesized cellular transcript to trap a segment of 10 to 13 nucleotides including the 5'cap (a process known as "cap snatching"), which is used as a primer for the synthesis of viral messenger RNAs (20).

Membrane protein messenger RNAs (HA, NA, M1, M2) are translated by ribosomes associated with the endoplasmic reticulum. These proteins migrate to the cell membrane directly, where virion assembly takes place. The mechanism by which the viral genome is incorporated into new virions has not been fully elucidated, however, RNA-RNA and RNA-nucleoprotein interactions are determinants in packaging (21, 22). Packaging signals within RNA sequences have been shown to be important for genome incorporation and in viral replication and genetic reassortment processes (23, 24). The accumulation of the M1 protein on the cytoplasmic side serves as a signal for the anchoring of ribonucleoproteins. The release of the newly formed virions is favored by NA, which removes sialic acid residues from the viral glycoproteins and prevents the aggregation of the virions among themselves and their accumulation in the membrane of the infected cell, which is important to retain its infectious and spreading capacity. A general scheme of the cycle is represented in Figure 2 (25, 26).

Drugs for the treatment of influenza

The genetic and antigenic variation of influenza viruses necessitates continuous replacement of vaccines. Vaccines are produced each year against influenza viruses that are likely to be circulating next season. There are different technologies used to produce vaccines, among which we can mention: inactivated virus vaccines, recombinant protein vaccines or attenuated virus vaccines. Usually, in any of the types of technology, these are trivalent vaccines de-
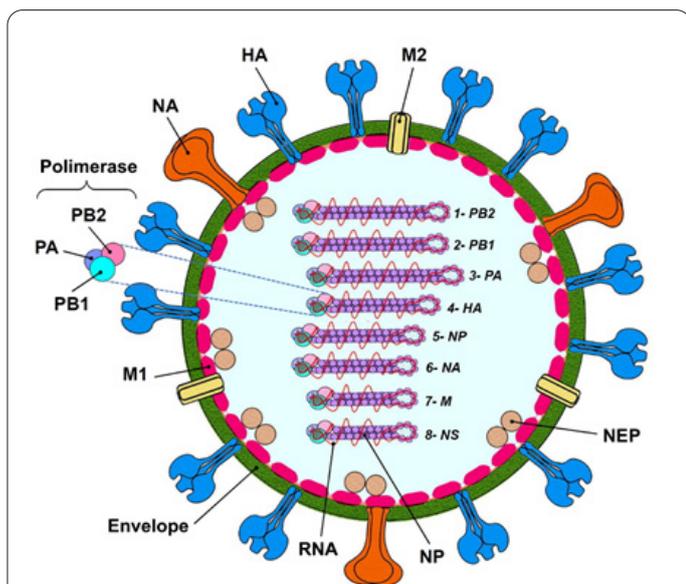
Table 1. Genes and proteins of influenza A and B viruses and their associated functions.

Segment	Influenza A virus				Influenza B virus				Associated functions
	Gene	Size (nt)	Protein	Size (aa)	Gene	Size (nt)	Protein	Size (aa)	
1	PB2*	2341	PB2	759	PB1*	2386	PB1	752	PB2 – Component of RNA polymerase; 5'cap recognition of host mRNA
			PB2-S1	510					Probable inhibitor of type I interferon response
2	PB1	2341	PB1	757	PB2	2396	PB2	770	PB1 – Component of RNA polymerase; RNA synthesis
			PB1-F2	87					Induction of apoptosis
3	PA	2233	PA	716	PA	2304	PA	726	PA – Component of RNA polymerase; endonuclease, cleaves 5'cap from host mRNA; protease activity
			PA-X	252					Probable modulation of immune response
4	HA	1778	HA	565	HA	1882	HA	584	Receptor binding, membrane fusion
5	NP	1565	NP	498	NP	1841	NP		Viral RNA protection contains nuclear localization signal for nuclear import of viral ribonucleoproteins (RNPs)
6	NA	1413	NA	454	NA	1557	NA	466	Removes sialic acids to release new virions
							NB	100	Probably ion channel function
7	M	1027	M1	252	M	1191	M1	248	Interaction with RNPs and viral glycoproteins; nuclear export of RNPs and assembly
			M2	97			M2	109	Proton channel, HA activation for membrane fusion
8	NS	890	NS1	230	NS	1096	NS1	281	Inhibits type I interferon response
			NEP	121			NEP	122	Export of RNPs from the nucleus to the cytoplasm

For the influenza A virus, the first segment encodes for PB2, for the influenza B virus, the first segment encodes for PB1; PA+PB1+PB2 form the viral RNA polymerase; data shown is for influenza A/Puerto Rico/8/1934 H1N1 virus and influenza B/Lee/1940 virus (https://www.uniprot.org/).
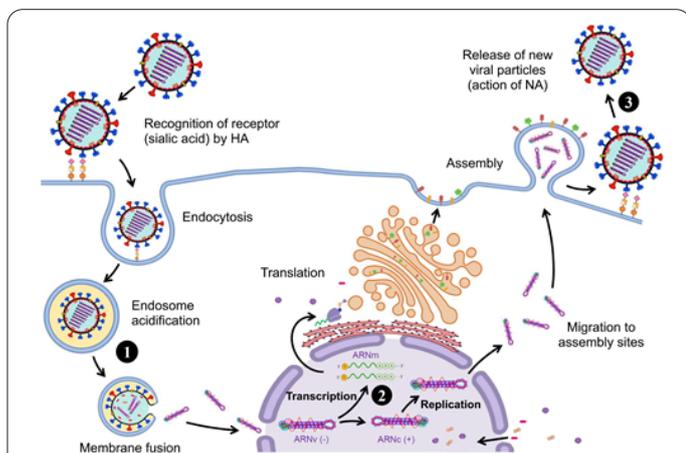
Figure 2. Replicative cycle of influenza viruses. The main events from the interaction of the virion with its receptor to the formation of the new viral particles are shown. The numbers enclosed in a black circle indicate the key points of action of the antivirals: 1) adamantanes block M2 protein inhibiting the acidification of the endosome and preventing membrane fusion; 2) baloxavir inhibits PA exonuclease activity and the generation of viral messenger RNA, and 3) neuraminidase inhibitors hinder the release of viral progeny and thus prevent the dissemination of virions. The information was obtained from (9, 19).

signed to provide protection against two serotypes of influenza A viruses, which are H1N1pdm2009 and H3N2, as well as one lineage of the influenza B virus, usually the Victoria lineage. Another version of the vaccine, the so-called tetravalent vaccine, also includes the Yamagata lineage of influenza B virus (27).

Vaccines are especially important for prevention, however, once the infection is established, antivirals play a key role in treating the disease. In a normal influenza season, antivirals are used to treat patients with severe illness, particularly young children, and the elderly or those with compromised immune systems. In a pandemic setting, before vaccines are available, antivirals are essential for treating patients who have been infected and for preventing infection in those individuals who have been exposed. This has shown benefits in terms of shortening the duration of the disease and avoiding the risk of complications and death if the treatment is applied on time (14).

The adamantanes, which include amantadine and rimantadine, were the first drugs for the treatment of influenza and have been licensed for use since the 1960s. Adamantanes block the viral M2 ion channel, which interrupts the replicative cycle at an early stage (28, 29). However, over the years, influenza viruses acquired mutations that reduced the activity of these drugs to the point that their use was no longer recommended since 2006 (30).

There are currently four antiviral drugs approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) (Figure 3). There are three neuraminidase inhibitors: zanamivir (Relenza, GlaxoSmithKline), oseltamivir (Tamiflu, Hoffmann-La Roche Inc.), and peramivir (Rapivab, BioCryst Pharmaceuticals), besides an acidic polymerase (PA) endonuclease inhibitor, baloxavir marboxil (Xofluza, Genentech Inc.) (31).

The three neuraminidase inhibitors are analogs of sialic acid, the natural substrate of viral neuraminidase. The first to be authorized for human use was zanamivir in 1999, followed by oseltamivir in 1999, while peramivir was au-

thorized in 2014. Baloxivir marboxil (BXM) was the most recently authorized, in 2018. Neuraminidase inhibitors, which are active against all subtypes of influenza A and the two main lineages of influenza B, have been the main class of antivirals for the control of influenza epidemics and pandemics for several years (32).

In commercial preparations, oseltamivir is included as oseltamivir phosphate, a rapidly metabolized prodrug by hepatic carboxylesterases to the active metabolite, oseltamivir carboxylate. In the presence of this neuraminidase inhibitor, the virions remain attached to the membrane of infected cells and become trapped in respiratory secretions. *In vitro* studies have shown that oseltamivir is active against all influenza subtypes with 50% inhibitory concentrations (IC50) of ≤2.0 nM on viral replication. Activity is greater against influenza A viruses than against influenza B viruses (33).

The mechanism of action of zanamivir is like that of oseltamivir, the difference between the two lies in the fact that zanamivir is administered through inhalation due to its low lipid solubility, which is why it has a very low bioavailability (2%). Given the low systemic circulation of zanamivir, the formulation is not recommended for the treatment of complicated forms of influenza. An intravenous formulation of zanamivir has recently been licensed in Europe for use in complicated forms of influenza (14).

Peramivir's mechanism of action differs slightly from other neuraminidase inhibitors because the molecule establishes multiple interactions with the active site of NA. For this reason, the compound retains its activity against some influenza A and B strains resistant to zanamivir and oseltamivir. Peramivir is only available as an intravenous formulation and has been approved for clinical use in the United States, Europe, South Korea, China, and Japan (14).

On the other hand, baloxavir, derived from the prodrug baloxavir marboxil, is a cap-dependent endonuclease (PA) inhibitor, which blocks the viral replicative cycle by inhibiting the initiation of mRNA synthesis. Baloxavir
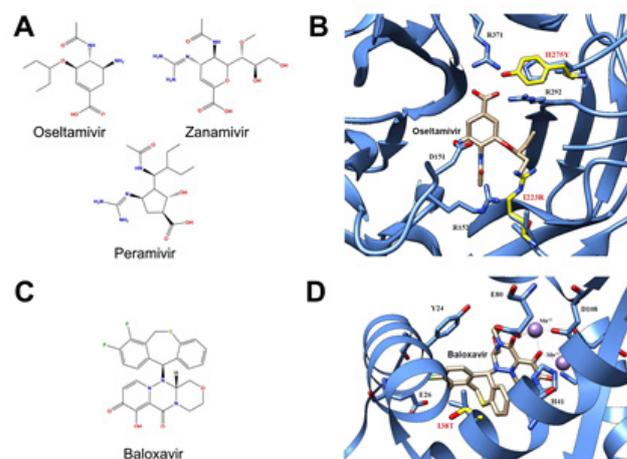

Figure 3. Main antiviral drugs for the treatment of influenza. A) structure of the main authorized neuraminidase inhibitors; B) oseltamivir in the active site of the viral neuraminidase (PDB ID: 3CL0), two of the most relevant mutations associated with resistance (I223R and H275Y) are shown; C) structure of baloxavir marboxil; D) baloxavir in the active site of the PA protein (PDB ID: 6FS6), the main mutation associated with resistance (I38T) is shown. Illustrations of inhibitors in the active sites were obtained in UCSF Chimera 1.16.

is effective against influenza A and B viruses, including oseltamivir-resistant strains and avian strains H7N9 and H5N1 (34).

Drug resistance in influenza viruses

Antiviral resistance is associated with the ability of influenza viruses to acquire mutations, caused by errors of viral RNA polymerase during the RNA synthesis since it has no proofreading activity. This genetic variability of viruses is also associated with the appearance of variants that evade the immune response acquired by natural infection or by available vaccines (35). Table 2 summarizes the main mutations associated with resistance to the most common antivirals.

WHO collects data from National Influenza Centers and WHO Collaborating Centers through the Global Influenza Surveillance and Response System (GISRS). The clinical significance of susceptibility to neuraminidase inhibitors determined *in vitro* is still unclear, but the correlation between the IC_{50} value, the concentration required to inhibit enzyme activity by 50%, is commonly accepted as a parameter to define whether an isolated virus is or is not susceptible to inhibitors (36).

For this purpose, influenza A viruses that have reduced inhibition is considered to have increased IC_{50} values compared to an IC_{50} of a reference virus (37). Influenza A viruses are considered to have reduced inhibition (RI) when there is an increase in the IC_{50} value relative to a reference IC_{50} value (virus of the same type/subtype) of at least 10-fold and a highly reduced inhibition (HIR) if it is 100-fold or more. Values are 5-fold for RI and 50-fold for HRI for influenza B viruses because baseline values for these viruses are higher (37, 38).

Variations in the genetic and biochemical characteristics of influenza virus neuraminidase may reduce the efficacy of oseltamivir. Mutations H275Y in influenza A/H1N1, R292K or E119V in influenza A/H3N2, and R152K or D198N in NA of influenza B virus are detected more frequently, mainly after prolonged treatment (33).

The emergence of resistance has also been associated with the use of lower-than-recommended doses of oseltamivir. This has been suggested by data collected in Japan, where oseltamivir is typically administered at a dose of 2 mg/kg twice daily, and resistance rates in post-treatment isolates were found to be 16-18%, which is significantly higher than the percentages reported in countries where recommended doses ensure an approximately 20% higher plasma level of the active metabolite of oseltamivir (54).

On the other hand, in February 2018, the use of baloxavir marboxil (S-033188), was approved for the treatment of patients infected with influenza A and B viruses (55), however, different studies have reported that the I38T substitution in PA is associated with decreased susceptibility to baloxavir by influenza A(H1N1), A(H3N2), and influenza B viruses (51, 56). It is noteworthy that viruses with resistance to baloxavir were detected during the phases II and III clinical studies; high percentages of sequences with the I38T/M mutation, 20% in A(H1N1) and more than 50% in A(H3N2) have been reported (52).

This problem of resistance to both baloxavir and neuraminidase inhibitors warns of the need to promote research on the prevalence of resistance and the development of new antivirals.

It is not considered that there is currently an emergency related to the increase in resistance to antivirals, however, analyses are periodically reported that show the frequency of viruses with mutations associated with resistance or decreased effectiveness of antivirals in tests that demonstrate biochemically or in cell culture.

For example, in an analysis of several countries in the Western Pacific, Americas, and Europe regions, the proportion of viruses with decreased effectiveness to neuraminidase inhibitors in 8,457 samples tested during 2016 and 2017 by *in vitro* enzymatic assays was estimated to be 0.2%. When analyzing the sequences present in databases, it was shown that 0.5% of the sequences presented markers of resistance to these drugs. This same type of study has been carried out in previous years and the percentage of resistant viruses has been calculated between 0.6% and 1.9% from 2012 to 2016 (37).

On the other hand, in Japan, a recent study conducted on viral samples from 2019 to 2020 showed that 1.6% of influenza A(H1N1)pdm2009 viruses showed resistance to neuraminidase inhibitors. Extending the analysis from 2010 to 2020, the calculated value was 1.1%. Interestingly, this study showed that treatment with neuraminidase inhibitors in a group of patients induces mutations that decrease their sensitivity to these drugs, something important to consider in monitoring this phenomenon (57).

In Latin America and in Spain there are few articles related to the study of antiviral resistance in influenza viruses. In Argentina, an increase in resistance to neuraminidase inhibitors of 0.1% (58) to 3.5% (59) has been reported; while, in Mexico, an analysis of influenza virus sequences from 2000 and 2017 showed markers of resistance to neuraminidase inhibitors of 3.9% in A(H1N1) pdm2009 and 1.5% in A H3N2 (35).

In Spain, a surveillance study in Catalonia with samples from 2006 to 2012 reports that no resistant viruses were found, except for the seasonal A H1N1 virus, which practically disappeared with the emergence of the A H1N1 pandemic in 2009. In the same region, has been reported that between 2015 and 2016 only three viruses with resistance markers were detected among 1223 samples analyzed (60).

In a study conducted in China during the COVID-19 pandemic period (2020-2021), it was found that most influenza viruses were type B, particularly of the Victoria lineage; no viruses with resistance to neuraminidase inhibitors or baloxavir were found (61).

These data show that current drugs, both neuraminidase and PA inhibitors, can continue being effective for the treatment of influenza, however, it is necessary to continue carrying out epidemiological surveillance throughout the world and be prepared with the development of new antivirals, if necessary, by an increase in resistance.

Final remarks

Influenza viruses have historically represented a constant public health problem. Vaccines have been available for many years and are effective in preventing severe disease during epidemic seasons. Once the infection is acquired, there are also several antiviral treatment options. However, influenza A viruses can periodically present genetic, biochemical, and pathogenic changes that represent a challenge both for the constant replacement of vaccines

Table 2. Most used drugs for the treatment of influenza.

Name	Authorization	Major substitutions associated with resistance	Type/subtype of influenza virus	Reference
Neuraminidase inhibitors				
Oseltamivir	1999	E119A/D/V, D151N, R152K, D199E/G, S247R, **H275Y**, R292K, **N294S**	A/H1N1	(39-47)
		E119I/V, D151E, **R224K, R292K, N294S**, R371K, Q391K	A/H3N2	
		R152K, D198N	Influenza B	
Zanamivir	1999	S110F, E119A/D/G, Q136K/R, S247R	A/H1N1	(37, 40, 41, 43, 45, 48, 49)
		E119D/I, **D151A/N/G/D**, R224K, **E276D**, R292K, N239K, R371K, Q391K	A/H3N2	
		A200T, P76S, T43A, P124T, S246P	Influenza B, Lineage Victoria	
		R150K, K125T	Influenza B, Lineage Yamagata	
Peramivir	2014	E119D/G, **Q136K/R, S247R, H275Y**	A/H1N1	(39, 41-43, 48)
		R292K, Q391K	A/H3N2	
		A200T, I221V, H134Y	Influenza B, Lineage Victoria	
		R150K, D197N	Influenza B, Lineage Yamagata	
PA inhibitors				
Baloxavir marboxil	2018	E23G/K/R, A36V, A37T/A, I38T/M/F/L, E119D, E199G	A/H1N1pmd09	(50-53)
			A/H3N2	
			Influenza B	

The key residues for the reduction of susceptibility or resistance to antivirals are shown in bold.

and for the effectiveness of antivirals. It is necessary to maintain molecular epidemiological surveillance to detect changes in time and to develop new antivirals to reduce the impact of an eventual increase in the resistance of these viruses.

Interest conflict

The authors declare that they have no conflicts of interest in relation to this article.

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