REVIEW

Influenza and antiviral resistance: an overview

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Abstract

Influenza affects approximately 1 billion individuals each year resulting in between 290,000 and 650,000 deaths. Young children and immunocompromised individuals are at a particularly high risk of severe illness attributable to influenza and these are also the groups of individuals in which reduced susceptibility to neuraminidase inhibitors is most frequently seen. High levels of resistance emerged with previous adamantane therapy for influenza A and despite no longer being used to treat influenza and therefore lack of selection pressure, high levels of adamantane resistance continue to persist in currently circulating influenza A strains. Resistance to neuraminidase inhibitors has remained at low levels to date and the majority of resistance is seen in influenza A H1N1 pdm09 infected immunocompromised individuals receiving oseltamivir but is also seen less frequently with influenza A H3N2 and B. Rarely, resistance is also seen in the immunocompetent. There is evidence to suggest that these resistant strains (particularly H1N1 pdm09) are able to maintain their replicative fitness and transmissibility, although there is no clear evidence that being infected with a resistant strain is associated with a worse clinical outcome. Should neuraminidase inhibitor resistance become more problematic in the future, there are a small number of alternative novel agents within the anti-influenza armoury with different mechanisms of action to neuraminidase inhibitors and therefore potentially effective against neuraminidase inhibitor resistant strains. Limited data from use of novel agents such as baloxavir marboxil and favipiravir, does however show that resistance variants can also emerge in the presence of these drugs.

Introduction

The World Health Organization estimates that annually there are approximately 1 billion human influenza cases of which 3 to 5 million are considered severe (especially in children, the elderly and in the immunocompromised) and result in 290,000 to 650,000 deaths [1].

Influenza can be transmitted through the following routes:

1. Respiratory droplets (> 5 μm) generated e.g. by coughing and sneezing. These do not remain suspended in the air and settle to the ground within 1–2 m
2. Contact transmission either through direct transfer of infectious particles from an infected to an uninfected individual or indirectly via contaminated surfaces or objects (i.e. fomites) and influenza can survive for hours on non-porous surfaces
3. Possibly by airborne transmission via small aerosols (< 5 μm) generated from breathing/talking (and can remain suspended in the air for minutes to hours) [2]; however, there is limited data to suggest that infectious particles can be transmitted over long distances (and special air handling and ventilation systems are not considered necessary to prevent spread)

Influenza belongs to the orthomyxovirus family and there are four influenza types A to D of which only influenza A, B and C can infect humans (influenza C is rare and usually causes a mild upper respiratory tract illness) [3]. Influenza A and B contain 8 pieces of segmented single-stranded RNA which encode various proteins including haemagglutinin (which facilitates attachment to the host cell) and neuraminidase (which facilitates release of new virus particles from the host cell). Influenza A has the broadest host range of the influenza viruses and significant interspecies transmission occurs [4]. Eighteen haemagglutinin (H) and 11 neuraminidase (N) subtypes have been described in influenza A (of which 16 H and 9 N subtypes have also been detected within avian species) [5]. Influenza B is far less genetically diverse than influenza A and has no distinct antigenic subtypes (mutates 2 to 3 times slower than influenza A and apart from humans, only seals and ferrets have demonstrated susceptibility) [6–8].

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Influenza achieves antigenic diversity via two main mechanisms:

1. Antigenic drift where mutations readily occur in HA and NA resulting in new antigenic variants (thus avoiding pre-existing host immunity); the error prone nature of the viral polymerase is a significant factor in this
2. Antigenic shift due to reassortment of gene segments between two distinct influenza viruses within the same host giving rise to a novel strain

The 1918 influenza A H1N1 pandemic is thought to have arisen from reassortment between human and avian strains (based on sequencing of fixed, frozen lung tissue from victims) and similarly, the most recent 'swine flu' influenza A H1N1 pandemic was thought to arise from a series of reassortment events between human influenza A H3N2, swine influenza A H1N1 and avian influenza A H1N2 [9, 10]. Lack of influenza B infection in several other species may explain why antigenic shift is not seen with influenza B [11].

This potential for vast genetic variability within influenza viruses and their highly error-prone RNA dependent RNA polymerase does raise concerns regarding the possible emergence of treatment resistant strains and generates further questions regarding their viral fitness and transmissibility as well as which strategies to employ in rapidly identifying and effectively treating these resistance variants. This article discusses these issues including novel agents and experimental strategies that have been used in an attempt to treat as well as prevent the emergence of resistant influenza viruses in humans.

Earlier influenza treatment with the adamantanes

The mechanism of action of the adamantanes is by blocking the M2 ion channel of influenza A thus preventing viral uncoating and the subsequent release of influenza A viral RNA into the host cell. They have activity against influenza A but not influenza B (due to their lack of the M2 protein, influenza B has an alternative ion channel called BM2) [12]. Amantadine was approved for clinical use in 1966 and subsequently rimantadine in 1993. Both drugs were initially very effective in treating and preventing influenza A infection with efficacy rates of up to 90%. The resistance of influenza A to amantadine was first recognised during the 1980 influenza A epidemic [12]. However, resistance to both drugs in seasonal influenza A subtypes was rare (1–2% frequency) until after 2000 when there was a dramatic rise in rates of resistance. By 2013, approximately 45% of all influenza A subtypes in circulation globally were resistant to the adamantanes (> 69% of H1 subtypes and 43% of H3 subtypes) [11]. Resistance (as a

result of the S31N mutation in the M2 protein) to the adamantanes occurs rapidly within 3–5 days of use and occurs in 30–50% of both immunocompetent and immunocompromised patients [13, 14]. Due to such high levels of resistance, the adamantanes are no longer recommended for treatment of influenza A [15].

Neuraminidase inhibitors

Neuraminidase inhibitors are currently first-line treatment for both influenza A and B in the United Kingdom (UK) and most (if not all) parts of the world. They competitively inhibit neuraminidase on the surface of influenza A and B. They act by preventing cleavage of sialic acid residues on budding newly formed virus particles thus preventing release of new virus particles from infected host (ciliated epithelial) cells. Resistance occurs much less readily in comparison with the adamantanes [16]. The neuraminidase inhibitors, if given within 36 h of onset, have been shown to reduce the duration of illness by 30% (with an approximately 40% reduction in illness severity) and, if given within 24 h of symptom onset, even greater reductions in the duration of illness attributable to influenza (approximately 44% reduction) have been observed [17, 18]. A decrease in the incidence of secondary complications, such as otitis media, sinusitis and pneumonia, with the use of neuraminidase inhibitors was also demonstrated [17]. Additionally, when used as prophylaxis (before or shortly after exposure), neuraminidase inhibitors can reduce the incidence of infection by approximately 70–90% [19]. In the UK, oseltamivir (oral agent; typically first line for influenza A and B treatment) and zanamivir (inhaled and also available as an aqueous solution which can be administered intravenously or via nebuliser) are licensed for the treatment of influenza A and B and also for prophylaxis. Oseltamivir is licensed in the UK for use in all ages including neonates, whereas zanamivir is not licensed for children under the age of 5 years. Peramivir (a single-dose intravenous infusion) was licensed in the UK in 2018 but has not been marketed/launched (it is used in the USA, Japan and South Korea). Laninamivir (an inhaled neuraminidase inhibitor) is licensed for use in Japan.

Based on the data from animal models which demonstrated that oseltamivir-resistant viruses were unfit and poorly transmissible, resistance to neuraminidase inhibitors was not envisaged to become an important clinical issue [20]. Prior to 2007, oseltamivir resistance was rarely seen in clinical practice (and low resistance rates of 1–5% were reported in clinical trials) [21]. Human cases of oseltamivir-resistant influenza A H1N1 began emerging during the 2007–2008 influenza season. Many of the cases reported were individuals who had not taken oseltamivir demonstrating that resistant virus could be efficiently transmitted between humans [22]. During the 2008–2009 influenza season, in some parts of the world, such

as the USA, Canada, the UK and Australia, very high rates (> 90%) of oseltamivir-resistant seasonal influenza A H1N1 strains were seen [23, 24]. In 2009, the pandemic (pdm09) influenza A H1N1 strain emerged worldwide but in the majority of cases (< 1.5% resistance) remained oseltamivir susceptible initially. Oseltamivir resistant H1N1 pdm09 cases did emerge but this was mostly immunocompromised patients that had received oseltamivir. Subsequently, however, in the 2010–2011 influenza season in the UK (and other parts of the world), increasing numbers of oseltamivir resistant cases were identified with no previous oseltamivir exposure [25]. Resistance to oseltamivir was still seen with much greater frequency in immunocompromised individuals receiving oseltamivir (with very little cross-resistance seen with zanamivir).

The neuraminidase mutation responsible for the oseltamivir resistance that emerged in the seasonal influenza A H1N1 (A/Brisbane/59/2007-like) strain in 2007 and then in the 2009 H1N1 pdm09 and also subsequent H1N1 strains is the H275Y mutation; a histidine to tyrosine substitution at amino acid 275 of the influenza A N1 neuraminidase [26, 27]. Normally when oseltamivir binds to the neuraminidase on the influenza virion, the neuraminidase active site changes shape to accommodate oseltamivir. A neuraminidase mutation, such as H275Y, prevents this conformational change in the active site and therefore, oseltamivir is unable to bind. Zanamivir, however, does not require this structural change in the neuraminidase active site in order to bind [28]. The H275Y mutation reduces the susceptibility (IC_{50}; the half maximal inhibitory concentration) of influenza A H1N1 to oseltamivir by approximately 400-fold but not zanamivir [13, 29]. Peramivir binds to sialic acid residues in a similar manner to oseltamivir and is also affected by the H275Y mutation [30]. This mutation has been shown to persist even after cessation of treatment, and strains harbouring this mutation are capable of causing outbreaks and significant morbidity and mortality in a similar fashion to their wild-type counterparts [14, 27]. Children and severely immunocompromised patients are at higher risk of developing resistance most likely due to higher viral loads and prolonged viral shedding [31].

Due to certain difference in the neuraminidase enzyme structure, neuraminidase resistance is less likely to occur in influenza A H3N2 and influenza B compared with A H1N1 pdm09 without causing significant loss of neuraminidase enzymatic function and reduced viral fitness [32, 33]. In vitro and in vivo studies have demonstrated that influenza A H3N2 and influenza B neuraminidase inhibitor resistant strains have a lower replicative capacity and less ability to transmit. In many of the case reports of influenza A H3N2 and B resistant strains, resistance only occurs after prolonged treatment (> 10 days but over 1 month in many) and these resistant variants often disappear once treatment is ceased [34, 35]. Public

Health England (PHE) publishes the most frequently observed influenza A and B mutations and their neuraminidase inhibitor resistance profiles in their 'Surveillance and Laboratory Testing of Influenza Neuraminidase Inhibitor Resistance' reports [36]. Table 1 summarises the Centers for Disease Control and Prevention (CDC) and PHE resistance data for the USA and UK, respectively.

The Influenza Resistance Information Study (IRIS) was a multicentre global observational study of neuraminidase inhibitor resistance and clinical outcomes in immunocompetent patients conducted from 2008 to 2013 [37]. This study included patients over 1 year of age presenting within 24 hours of an influenza-like illness and/or had a positive rapid influenza test. Nose throat swabs were collected on days 1, 3, 6 and 10 for influenza typing/subtyping, sequencing and neuraminidase inhibitor phenotypic susceptibility testing. There were 3230 influenza A and B reverse-transcription polymerase chain reaction (RT-PCR) positive patients in the study. Except for 30 patients with pre-treatment (i.e. transmitted) resistant influenza A H1N1 strains, no resistance was detected in day 1 samples. Emergence of oseltamivir resistance after day 1 was detected in 43/1207 (3.56%) of oseltamivir-treated influenza A positive patients; a higher frequency was seen in 1–5 year old (11.8%) compared with those over 5 years (1.4%). All resistant H1N1 viruses had the H275Y mutation and all resistant H3N2 viruses had the R292K mutation (conferring reduced susceptibility to both oseltamivir and zanamivir). Virus clearance was a median of 8.1 days for treated patients with oseltamivir-resistant virus vs 9.9 days for untreated patients vs 10.9 days for treated patients with oseltamivir-resistant virus. Time to alleviation of symptoms was 1 day shorter in treated patients as compared with untreated patients. Interestingly, the oseltamivir-resistant treated group exhibited the shortest duration of symptoms (symptoms resolved by day 6 or earlier).

Dual resistance to oseltamivir and zanamivir is rare. A literature review by Abed et al. identified 14 published cases of human influenza A and B infections with mutations conferring reduced susceptibility to both oseltamivir and zanamivir [38]. Seven had influenza A H1N1 pdm09, 4 had influenza A H3N2, 1 had avian influenza A H7N9 and 2 had influenza B. The age range was 8 months to 88 years and 12 out of the 14 patients were immunocompromised. The other two patients had underlying chronic lung disease. Thirteen out of 14 patients had received neuraminidase inhibitor therapy before emergence resistance mutations (5 oseltamivir alone, 2 oseltamivir then zanamivir, 2 zanamivir alone, 3 zanamivir and oseltamivir simultaneously, 1 oseltamivir then peramivir). There was a mean of 12.76 days (range 0–72 days) treatment before detection of the first mutation. Mortality was high at 71% (10/14 patients). Of the 4 survivors, there was an immunocompetent asthmatic child with H1N1 pdm09 who received no treatment, 1 immunocompromised adult with H1N1

Table 1 Centers for Disease Control and Prevention (CDC; USA data) and Public Health England (PHE; England data) influenza resistance data

Influenza season	CDC	PHE
2013–2014	Influenza A H1N1: 98.8% oseltamivir susceptible to oseltamivir and 100% zanamivir susceptible No specific date for influenza A H3N2 or B identified 'High-level' adamantane resistance	1.9% neuraminidase resistance
2014–2015	Influenza A H1N1: 98.4% oseltamivir susceptible to oseltamivir and 100% zanamivir susceptible Influenza A H3N2 and B: 100% susceptible to oseltamivir and zanamivir	0.5% neuraminidase resistance
2015–2016	Influenza A H1N1: 99.2% oseltamivir and peramivir susceptible and 100% zanamivir susceptible No specific date for influenza A H3N2 or B identified 'High-level' adamantane resistance	0.8% neuraminidase resistance
2016–2017	Influenza A (all subtypes) and B: 100% susceptible to oseltamivir, peramivir and zanamivir 'High-level' adamantane resistance	0.2% neuraminidase resistance
2017–2018	Influenza A H1N1: 99% oseltamivir and peramivir susceptible, 100% zanamivir susceptible Influenza A H3N2 and B: 100% susceptible to oseltamivir, peramivir and zanamivir 'High-level' adamantane resistance	–

pdm09 who received oseltamivir for 14 days then inhaled zanamivir for 40 days, 1 immunocompromised adult with H3N2 who had 5 days of oseltamivir and 1 immunocompromised child with H3N2 who received oseltamivir for 3 months and then inhaled zanamivir for 72 days.

In a recently published UK series of three cases of oseltamivir-resistant influenza A H1N1 pdm09 that occurred in England in immunocompetent patients in the 2018–2019 influenza season, two of the patients (a 7-week old previously well boy and a 39-year old asthmatic woman) made a good recovery with 5 days of oseltamivir despite whole genome sequencing revealing H275Y mutations in 44% and 100% of the virus population, respectively [39]. The third case was a 15-month old girl with a developmental condition (Najer syndrome) admitted with a 1-day history of respiratory illness who showed minimal clinical improvement with 15 days of oseltamivir after which she was switched to intravenous zanamivir (as well as receiving antibiotics) and died soon after. PHE sequencing data later revealed H275Y mutations in 35% of the virus population in a day 5 nasopharyngeal aspirate specimen and this rapidly rose to 80% of the virus population harbouring H275Y mutations 1 day later in a day 6 nasopharyngeal aspirate sample.

The treatment of influenza as recommended by PHE is oseltamivir as first line for immunocompetent children and adults [40]. For immunosuppressed patients, neuraminidase inhibitor choice is based on the dominant circulating strain in that particular season; oseltamivir is recommended when the dominant circulating strain is of lower risk for oseltamivir resistance (i.e. influenza A H3N2 or B) and zanamivir is recommended when the dominant strain has a higher risk of oseltamivir resistance (e.g. H1N1 pdm09). Currently, available PHE date for the 2019–2020 influenza season indicates that influenza A H3N2 is the dominant circulating strain [41]. In many laboratories, typing/subtyping results are becoming available early in the treatment course therefore guiding selection of treatment.

Neuraminidase inhibitor susceptibility testing

Neuraminidase inhibitor susceptibility testing should be considered particularly in young children and immunocompromised patients being treated with a neuraminidase inhibitor for influenza (especially H1N1 pdm09), who are not responding to treatment and/or have persistently high viral loads (low cycle threshold values using RT-PCR) and/or exposed to a suspected or confirmed resistant case. Given that resistance variants can emerge within only 1–2 days of treatment (as well as can be transmitted), resistance testing can be performed at any time prior to, during or after treatment. In England, PHE offers both genotypic and phenotypic influenza A and B susceptibility testing which is summarised in Table 2 [36].

Relatively newer techniques also exist such as pyrosequencing, a high-throughput sequencing method that is able to type/subtype, screen for mutations and delineate the relative proportions of the various influenza variants [42, 43]. Another molecular technique, digital PCR (using a droplet-based system) has been shown to be highly accurate and precise in the identification and quantification of influenza sequence variants with the ability to detect rare single nucleotide polymorphisms present at levels as low as 0.001% of the virus population [44–46]. From a direct clinical perspective, identification of variants at such low proportions may not be relevant but these techniques may provide further insights into the viral dynamics in the emergence of resistant influenza viruses.

Novel influenza therapies

Baloxavir marboxil is a single dose oral agent for the treatment of influenza A and B (no data for it is used of prophylaxis). It was licensed in Japan and USA in 2018 but is not currently licensed in the UK. It suppresses influenza replication by inhibition of cap-dependent endonuclease (an enzyme

Table 2 Summary of Public Health England influenza antiviral resistance testing

Assay type	Mutation(s) detected	When this particular test is used	Considerations with the test
H275Y SNP detection assay	H275Y	Influenza A H1N1pdm09 and treated with oseltamivir	Rapid test
Resistance SNP detection assays	Most common influenza A and B mutations	Influenza A H3N2 or influenza B (regardless of which drug used)	Typically considered for patients with: (i) unsatisfactory clinical response to 10 days of treatment + non-viral causes unlikely + influenza virus remains detectable at a significant Ct value (ii) any patient who has been on neuraminidase therapy for a prolonged period i.e. greater than 1 month
Full-length neuraminidase sequencing	Uses WGS to screen for all previously reported resistance mutations	Influenza A H1N1pdm09 and treated with zanamivir primarily but may also be used for confirmation of susceptibility in influenza A H3N2 and B	May take up to 2 weeks
Phenotypic testing	Not applicable	For all influenza A and B subtypes in specific cases where deemed appropriate	Requires the use of cell grown virus isolates therefore takes a longer period of time No pre-defined 'breakpoint'/IC_{50} cutoff points for drug susceptibility exist

SNP Single nucleotide polymorphism, *WGS* whole genome sequencing, and IC_{50} 50% of the maximal inhibitory concentration

required for initiation of influenza mRNA synthesis) and therefore, its mechanism of action is different to that of the neuraminidase inhibitors [47]. There is limited data on resistance but a recent US/Japan randomised controlled study of healthy adults/adolescents with influenza A and B treated with baloxavir marboxil found that 9.7% (36/370) developed a specific mutation (PA/I38X) 3–9 days after treatment and that the emergence of these PA/I38X variants was associated with higher viral loads, prolonged detection of virus and a longer duration of symptoms compared with baloxavir marboxil treated individuals who did not develop the PA/I38X mutation [48].

Favipiravir is an oral (and intravenous) antiviral which inhibits RNA-dependent RNA polymerases [49, 50]. It has been approved for the treatment of influenza A and B in Japan with very strict regulation for clinical use and is intended to be reserved for pandemics causes by novel/re-emerging influenza strains resistant to other antivirals. A recent study has demonstrated that a specific K229R mutation in the PB1 subunit of the influenza virus polymerase results in reduced susceptibility to favipiravir in vitro and in cell culture. Viral fitness, which was demonstrated to be impaired by this mutation, can be restored by a compensatory second (P653L) mutation [51]. The effects or these mutations in a clinical setting are yet to be determined.

Experimental treatment strategies

Human clinical trials of dual therapy with oseltamivir plus zanamivir for H1N1 have been investigated in various settings (including in ECMO patients) with varying outcomes and no clear benefit in terms of clinical outcome or prevention of drug resistance [52–54]. Studies in mice also failed to show a benefit [55].

Double dose oseltamivir did not reduce the risk of emergence of oseltamivir resistance in patients with influenza A H1N1 pdm09 [56]. This study was a small ($n = 52$), randomised trial of patients treated in community. One patient in the single dose group and one in the double dose group developed oseltamivir resistance. There was no mention of any immunocompromised patients in the study.

A triple-combination of amantadine, oseltamivir and ribavirin (TCAD regimen) was shown in vitro and in the mouse model to have synergistic activity against sensitive and resistant influenza viruses (with greater synergy than any double antiviral regimen) [57]. There was a phase I pilot study of TCAD in 2013 to assess pharmacokinetics and safety in 6 immunocompromised patients with influenza A (H1N1 & H3N2). Five out of 6 tolerated and completed the 10-day course of treatment (1 patient had worsening respiratory failure and TCAD was stopped) [58]. A clinical response and a corresponding viral load reduction in the 5 patients that completed treatment. No drug-drug interactions were seen and no haematological toxicity was seen with ribavirin. No new resistance mutations emerged on treatment. A study of TCAD vs oseltamivir in critically ill mechanically-ventilated patients with pandemic H1N1 showed that TCAD was well tolerated but did not improve outcomes compared with oseltamivir alone [59].

An in vitro study in 2016 of a zanamivir-oseltamivir hybrid inhibitor (MS-257) showed effectiveness against neuraminidase inhibitor-resistant influenza strains but there have been no further published studies to date [60].

Conclusions

Oseltamivir resistance is rare and zanamivir resistance is extremely rare. The presence of resistant virus does not necessarily mean a more severe infection and/or worse outcome, particularly in immunocompetent adults. In some instances, neuraminidase inhibitor resistant virus may actually be less fit (especially with H3N2 and influenza B). There is an increased risk of resistance in the immunocompromised (but resistance does occur in the immunocompetent) and young children (< 5 years); this is primarily with influenza AH1N1 pdm09 but can occur less commonly with influenza A H3N2 and B.

Neuraminidase inhibitor susceptibility testing should be considered primarily in H1N1 infected immunocompromised patients and young children failing to respond to treatment, and clinicians should consider zanamivir in the intravenous form for patients that are critically ill/developing severe complications. There should potentially be a higher threshold for neuraminidase inhibitor susceptibility testing in influenza H3N2 or B infected patients. This should generally be reserved for those that have had extended treatment (at least 10 days) and not responded/deteriorated with persistent low Ct values and no other identifiable cause.

Finally, there is no clearly proven benefit from combination (or double dose) antiviral therapy for influenza in terms of clinical outcome or emergence of resistance.

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