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Oseltamivir Resistance and the H274Y Neuraminidase Mutation in Seasonal, Pandemic and Highly Pathogenic Influenza Viruses

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Abstract

Along with influenza vaccines, the world is currently almost completely dependent on two licensed drugs for the treatment or prevention of seasonal (influenza A and B viruses) and pandemic influenza (influenza A viruses). These drugs – oseltamivir (Tamiflu®) and zanamivir (Relenza®) – are classified as neuraminidase inhibitors (NAIs) because they act by inhibiting one of the key surface proteins of the influenza virus, the neuraminidase, which in turn reduces the ability of the virus to infect other respiratory cells. Our dependence on these drugs has arisen because of high levels of resistance with seasonal influenza viruses to the older class of anti-influenza drugs, the adamantanes (amantadine and rimantadine), combined with the lack of activity of these drugs against influenza B viruses. Recently, however, significant levels of oseltamivir-resistant influenza A(H1) seasonal influenza viruses have also been encountered, which has been associated with a single amino acid change in the viral neuraminidase (H274Y). Oseltamivir is the most widely used and stockpiled NAI and, while these A(H1) viruses are still sensitive to zanamivir, it highlights the ease with which the influenza virus can mutate and reassort to circumvent available drugs. Fortunately, the current pandemic A(H1N1) 2009 virus, which is circulating globally, remains largely sensitive to both NAIs, although a small number of oseltamivir-resistant viruses have been isolated from patients to date, again with the H274Y mutation. Clearly there is a need to use the NAI drugs prudently to ensure they remain an effective defence against future seasonal and pandemic influenza viruses, along with careful monitoring of levels of resistance in the circulating viruses combined with the further development of new anti-influenza drugs.

The neuraminidase inhibitors (NAIs) are a class of anti-influenza drugs that were rationally designed using the 3-dimensional structure of the influenza neuraminidase.[1] The compounds bind to the enzyme active site of the neuraminidase glycoprotein of influenza A and B viruses and inhibit the normal function of the neuraminidase, thereby preventing release of viral progeny from host cells following viral replication.[1] In 1999, two NAIs were brought to the market; the first was zanamivir (Relenza®, GlaxoSmithKline), a structural design that incorporated a guanidine group at the 4-position of the sialic acid analogue Neu5Ac2en, whilst the second, oseltamivir (Tamiflu®, Roche) was a prodrug that contains a bulky lipophilic side-chain at the 6-position (figure 1). Because of these structural differences, zanamivir demonstrated poor bioavailability when given orally and was therefore produced as an inhaled product, whereas oseltamivir has good oral bioavailability and can therefore be administered orally.

Sales of oseltamivir have far exceeded those of zanamivir (figure 2a), due in part to the difference in mode of administration (there is a patient preference for an orally administered drug), and also because oseltamivir is licensed for use in children aged ≥1 year, whereas zanamivir is licensed only for children aged ≥7 years (table I). An oral oseltamivir suspension assists with the treatment of children because of the ease of administration and the ability to easily adjust the dose. Since coming to market, the majority of global oseltamivir sales have occurred in Japan (41 million doses, 71% of global sales), and to a lesser extent in the US (14.9 million doses, 26% of global sales), with very little used by the rest of the world (1.6 million doses, 3% of global sales) [figure 2b].[2] Global use of oseltamivir prescribed for seasonal influenza (and excluding drug purchased for pandemic stockpiling) peaked in 2005, although sales declined over the next 2 years, while a steady decline in sales from Japan has also been observed between 2005 and 2008 (figure 2b).[2] This decline may be a result of reports in Japan of an association of neuropsychiatric episodes in children and teenagers taking oseltamivir that resulted in a number of fatal outcomes,[3] and led to the Japanese Department of Health and Welfare instructing the Japanese distributor of the drug, on 21 March 2007, to include a warning not to give the drug to Japanese children aged between 10 and 19 years. Warnings on potential adverse psychiatric events were also included on the Tamiflu® product information sheets in many other countries. Since then, a number of studies have challenged these initial findings,[4-6] and one study has even suggested that there may be a reduction in neuropsychiatric events for patients with influenza given oseltamivir.[7] Although overall oseltamivir use has declined in Japan since this time, interestingly, the proportion of use in children (aged 0–16 years) compared with adults has remained steady since 2006, ranging from 48% to 50%[2] (figure 2c).

In contrast to the relatively poor global uptake of NAIs for seasonal influenza, since the re-

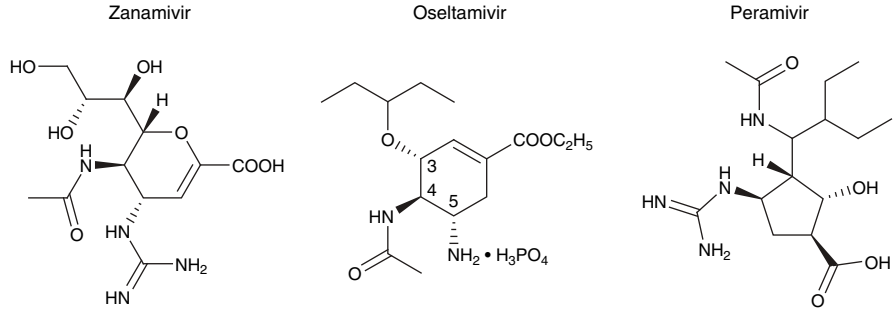

Fig. 1. Structural formulae for zanamivir, oseltamivir (oseltamivir phosphate) and peramivir.

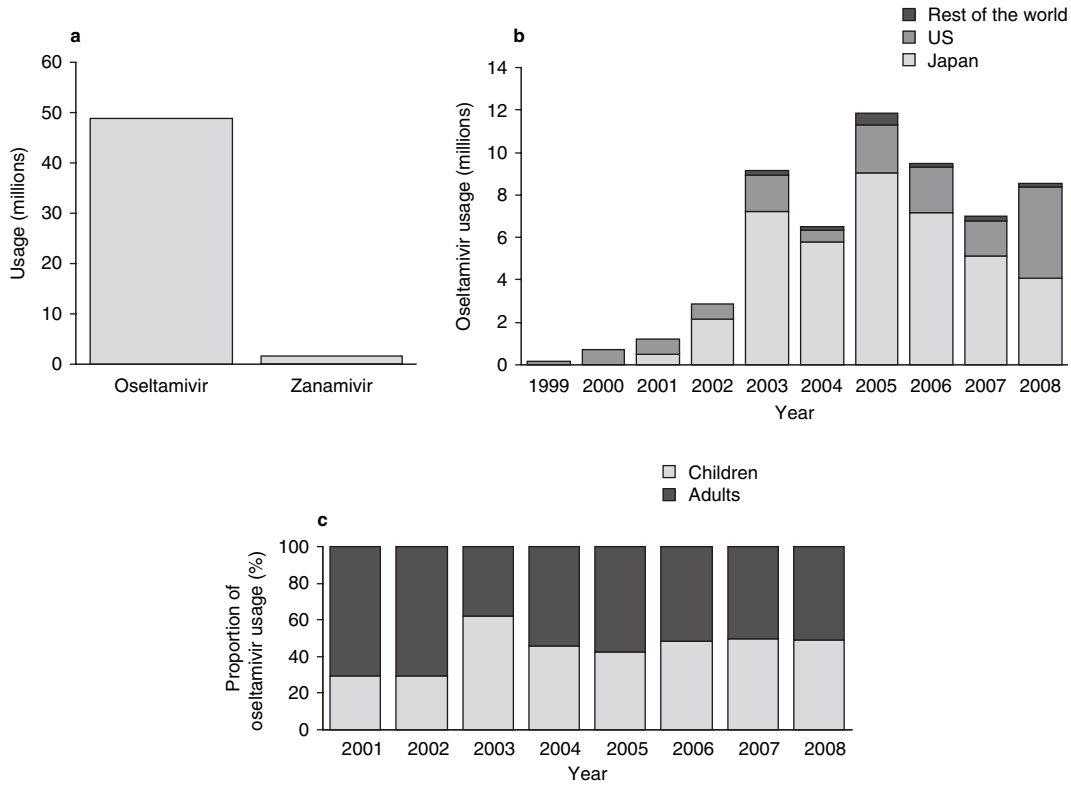

Fig. 2. Neuraminidase inhibitor usage. (**a**) The total use of oseltamivir compared with zanamivir, as measured by worldwide prescription data between 1999 and 2007. (**b**) Oseltamivir usage each year in Japan, the US and the rest of the world since its launch in 1999. (**c**) Proportion of children (aged 0–16 years) compared with adults using oseltamivir in Japan. All data derived from IMS Health,[2] kindly provided by F. Hoffmann-La Roche Ltd. IMS Health data represents prescription data and not necessarily consumption data.

appearance of A(H5N1) viruses in poultry, wild birds and occasionally in humans from 2003, many countries have acquired huge stockpiles of oseltamivir along with smaller amounts of zanamivir for use in potential pandemics.

1. Early Resistance Findings

Unlike the older class of anti-influenza drugs, the adamantanes, where viral resistance is rapidly generated in patients under treatment, resistance to the NAIs in patients under treatment has been found to be relatively low. While detection of zanamivir resistance has been very rare, with only one case observed in an immunocompromised patient, oseltamivir resistance has been detected in 1–2% of adults[8] and 5–6% of children[9] under treatment with oseltamivir, although more recent studies have suggested that resistance can be as high as 18% in treated children.[10]

In these studies, the most commonly observed resistance neuraminidase mutation in oseltamivir-resistant A(H3N2) viruses was in an Arg292Lys (R292K), whilst the predominant neuraminidase mutation in oseltamivir-resistant A(H1N1) viruses was a His274Tyr (H274Y) neuraminidase mutation (N2 neuraminidase amino acid numbering, equivalent to residue 275 based on N1 numbering). To assess the risk that these resistant viruses may spread in the community, the viral fitness of the mutants has been assessed. Viruses with an R292K neuraminidase mutation demonstrated compromised growth *in vitro*,[11] and in ferrets, were 2–3 logs less infectious and unable to

Table I. Pharmaceutical information for oseltamivir and zanamivir

Drug	Trade name	Form of drug	Delivery route	Licensed age (US)	Dosage[a] (adults)	Dosage[a] (children)
Oseltamivir	Tamiflu®	Prodrug	Oral	≥1 y	75 mg bid for 5 d	30–75 mg[b] bid for 5 d
Zanamivir	Relenza®	Active	Inhaled	>7 y	10 mg bid for 5 d	10 mg bid for 5 d

a Treatment dose: for prophylaxis one of these doses per day is recommended; children aged >5 years can be given zanamivir prophylactically.

b Oral suspension is available for children and adults who cannot swallow capsules and is usually given at a dose of 1.5–3 mg/kg until >40 kg when 75 mg doses are recommended.

bid = twice daily.

be transmitted.[12] In cell culture studies performed on a H274Y mutant A(H1N1) strain isolated from a patient under oseltamivir treatment, the virus had a compromised growth,[13] although a strain carrying the same mutation selected *in vitro* was found to replicate as well as the wild type.[14] These results suggested that resistant virus variants with the same neuraminidase mutation may differ in fitness depending on other viral components. In a ferret model, the infectivity and the transmissibility of a H274Y mutant were found to be restricted,[13] although in a different study, transmission of the mutant virus between ferrets was possible, but a greater viral dose of the mutant was required than for the wild type.[15]

Because of the compromised viral fitness of many NAI-resistant viruses, it was considered unlikely that such strains would spread throughout the community. Prior to 2007, surveillance studies that analysed community isolates (predominantly from untreated individuals) supported this hypothesis, as the frequency of resistant strains detected was <1%, even in countries such as Japan and the US, where the majority of oseltamivir was being used.

2. Recent Resistance Findings

Analysis of A(H1N1) viruses isolated during the northern hemisphere 2007–8 influenza season (October 2007–March 2008) in Europe and the US, revealed a striking increase in the frequency of circulating oseltamivir-resistant viruses with an H274Y mutation.[16,17] Of note was that these resistant strains were detected in patients who had not been treated with oseltamivir and occurred in countries with a relatively low usage of the drug. The H274Y mutant in Europe was first detected in week 46 of 2007, and subsequently increased to an average prevalence of 50% by week 16 of 2008.[17] The mutant strain continued to spread to the southern hemisphere, such that the oseltamivir-resistant strains were detected at a high frequency throughout South Africa, South East Asia and Oceania by mid 2008.[18] The prevalence of the H274Y mutant in South Africa, South East Asia and Oceania during the southern hemisphere influenza season demonstrates a near linear increase in the frequency of the mutant from <1% to >90% in less than 12 months (figure 3). Of importance is that the resistant strains appear to have a similar clinical impact and severity of illness to the oseltamivir-susceptible A(H1N1) strains.[16]

The rapid global spread of this strain clearly suggests that the virus has greater fitness than the previous sensitive A(H1N1) strain. The reasons why this strain has enhanced viral fitness, when previous studies demonstrated that the acquisition of an H274Y mutation led to a reduced viral fitness remain unclear, but is probably due to other compensatory mutations or reassortant events,[19] as was also observed following analysis of recent adamantane resistant A(H3N2) viruses.[20]

3. Structural Impact of the H274Y Mutation

The H274Y mutation causes approximately a 1500-fold reduction in susceptibility to oseltamivir compared with a wild-type virus. The mutation also confers a 500-fold reduction in susceptibly to peramivir, an NAI currently in

clinical trials, but has no effect on susceptibility to zanamivir.[18] The reasons for the differences in susceptibility are due to the structural differences between the inhibitors. It has been well documented that the H274Y mutation causes a large shift in the position of the side-chain of the neighbouring E276 relative to wild type.[21] This promotes significant movement of the oseltamivir diethoxy moiety, resulting in a weaker binding affinity and thus high resistance (figure 4a). This mutation has no effect on zanamivir as the alternate 3′ moiety of zanamivir (dihydroxyl) contains functionalities that prefer to hydrogen bond to the E276, and as such the movement of the side-chain of E276 is beneficial (figure 4b). Peramivir contains a similar 3′ diethyl moiety to oseltamivir, which would be similarly impacted by the E276 movement (figure 4c). However, the 5′ functionality of peramivir allows it to hydrogen bond to residues R156 and W180 as a result of the mutation, which may account for the partial retainment of activity (figure 4d).

The role of the H274Y mutation in conferring oseltamivir resistance appears to be subtype specific. The H274Y mutation has not been previously detected in N2 neuraminidases from community isolates, and when it was experimentally introduced into a N2 neuraminidase by site-directed mutagenesis and generated using reverse genetics, the mutation had no impact on oseltamivir sensitivity.[23] This is because residue 246 is a serine residue in N1 neuraminidase and an alanine residue in N2 neuraminidase. Therefore, although the mutation sterically clashes with one diethyl moiety of oseltamivir, the additional space and hydrophobic nature of the alanine residue in N2 neuraminidase allows favourable interactions to form with the other diethyl moiety of oseltamivir (figure 5).

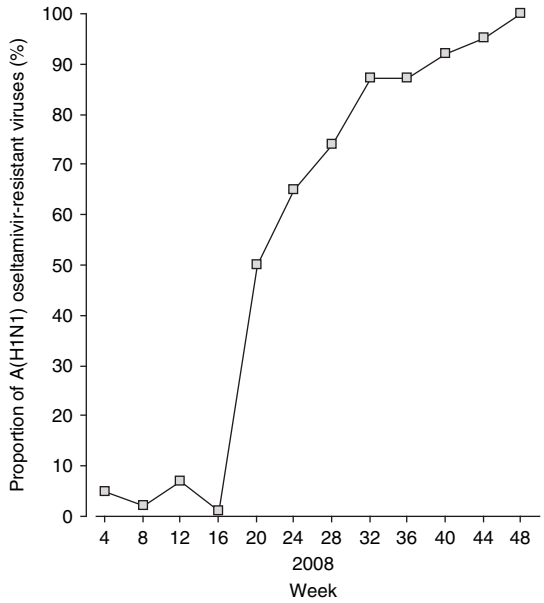

Fig. 3. The mean proportion of oseltamivir-resistant A(H1N1) viruses circulating in Oceania, South East Asia and South Africa in 2008.

4. Resistance in A(H5N1) and Pandemic (H1N1) 2009 Viruses

As there is a propensity for the H274Y mutation to occur under oseltamivir selective pressure in N1 neuraminidases, there has been considerable attention paid to monitoring for this resistance mutation in the potentially pandemic A(H5N1) viruses and the newly emerged pandemic (H1N1) 2009 viruses, given that both of these strains contain an N1 neuraminidase. Since 2003, strains of highly pathogenic A(H5N1) influenza have caused outbreaks in poultry and, on occasions, have infected humans across Asia, Africa, Europe and the Middle East.[24] Since 2003, 436 confirmed A(H5N1) human infections were reported, of which 262 have been fatal (case fatality rate of 60%).[25] Humans who acquire the infection develop a severe pneumonia that can progress to acute respiratory distress syndrome with high risk of mortality.

Oseltamivir has been shown in uncontrolled clinical trials to improve survival, although late initiation of treatment significantly reduced the effectiveness of the drug.[24] Because of the high viral loads and systemic nature of A(H5N1) infections, a double dose (150 mg twice daily) and an increased duration of therapy (10 days, compared with a normal course of 5 days) may be beneficial, as has been demonstrated in animal models.[26] However, oseltamivir-resistant A(H5N1) variants with an H274Y neuraminidase

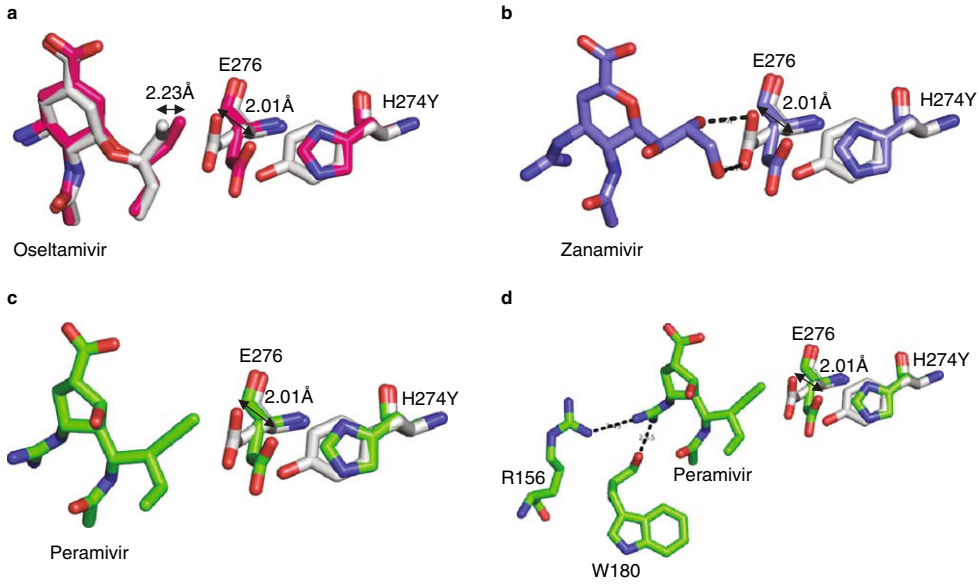

Fig. 4. Structural impact of H274Y mutation on the binding of oseltamivir, zanamivir and peramivir. The crystal structure of the H274Y N1 mutant (PDB code: 3CL0) is shown in grey sticks with the wild-type N1 structure depicted in pink (PDB code: 2HU4), purple (PDB code: 3B7E) and green (PDB code: 2HTU) for each inhibitor, respectively. All figures were created in Pymol.[22] (**a**) Crystallography has shown that the H274Y mutation causes a large shift in the side-chain of neighbouring E276. This in turn promotes movement of the oseltamivir diethoxy moiety, which is expected to be a contributing factor to the significant resistance of this mutation. (**b**) The H274Y mutation has no significant effect on zanamivir activity as the movement of E276 allows H-bonding to occur. (**c** and **d**) Peramivir shows partial resistance to H274Y mutation as the movement of E276 impinges on the diethoxy moiety of peramivir in a similar manner to oseltamivir. However, this effect may be partially offset due to the diaminomethylidene amino group, which can hydrogen bond to R156 and W180 at the opposite end of the molecule. **PDB** = Protein Data Bank.

mutation have been isolated from treated patients and may be associated with clinical deterioration and fatal outcomes.[27] In addition, an N294S neuraminidase mutation, which results in a minor (12- to 15-fold) reduction in oseltamivir susceptibility has also been detected in A(H5N1) viruses isolated from two patients in Egypt. The H274Y mutation has also been detected *in vitro* following serial cell culture passage of two antigenically different A(H5N1) viruses in increasing concentrations of oseltamivir. One of these strains, in addition to the H274Y mutation, also developed an I222M (N2 amino acid numbering) neuraminidase mutation, which, as a result of the synergistic dual mutation, increased oseltamivir resistance significantly (8000-fold compared with wild type) more than the already resistant single H274Y mutant (1000-fold compared with wild type).[28] Combination therapies using NAIs, adamantanes and ribavirin have been investigated in animal models, with results suggesting that such an approach may guard

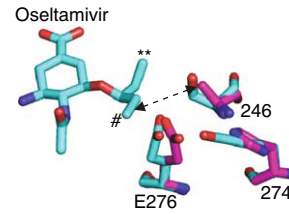

Fig. 5. Structural impact of the H274Y mutation in a N2 neuraminidase. Stick representation of oseltamivir in the H274Y N1 neuraminidase mutant (cyan, PDB code: 3CL0) and wild-type N2 neuraminidase (magenta, PDB code: 1INW). One of the differences between the two neuraminidases is residue 246, which is a serine residue in N1 and an alanine residue in N2. Thus, although the H274Y mutation would still be expected to clash with one arm of the diethyl moiety (marked **), the amino acid difference seen between the neuraminidases at residue 246 allows the alternate diethyl moiety (marked #) to create favourable hydrophobic interactions with A246 in N2 neuraminidase (highlighted by the dashed arrow), explaining the lack of oseltamivir resistance seen with a H274Y mutation in N2 neuraminidase. **PDB** = Protein Data Bank.

against the emergence of resistant strains,[29,30] although clinical trials are required to establish these findings.

While an A(H5N1) pandemic has not yet eventuated, the large stockpiles of oseltamivir, and to a lesser extent zanamivir, that were obtained by many countries in preparation for such an outbreak proved to be invaluable from March 2009 when a novel A(H1N1) virus of swine origin was unexpectedly detected in humans and subsequently spread throughout the world within a few months.[31]

Since the early detections of the A(H1N1) pandemic virus in the US in April 2009, in less than 3 months there were over 150 000 cases of infection with the virus around the world,[32] prompting WHO to declare the outbreak as a pandemic on 11 June 2009. With no specific vaccine available, NAIs, particularly oseltamivir, became the first line of defence to try to slow the spread of the virus. As a result, there have been unprecedented volumes of oseltamivir used, both for the treatment of infected patients and the prophylaxis of their contacts, although encouragingly, there have been less than thirty cases of resistant viruses reported to date worldwide (as of 14 October 2009), including strains detected in Japan, Hong Kong, Denmark, China, Singapore, Canada and Australia. All but one of the patients had received oseltamivir and all had the characteristic H274Y mutation. The majority of these cases were from patients receiving oseltamivir prophylaxis (75 mg once daily). This suggests that these patients are either already infected with the pandemic A(H1N1) strain prior to prophylaxis and are therefore receiving a suboptimal dose, or that the dose of 75 mg once daily is insufficient to prevent infection with the pandemic A(H1N1) strain. Although this dose has been considered sufficient for the prophylaxis of seasonal influenza, a higher dose may be necessary in the case of a novel virus, such as the pandemic A(H1N1) strain, where patients have little or no immune protection.

Encouragingly, there has not yet been any evidence of transmission of an oseltamivir-resistant pandemic A(H1N1) 2009 H274Y virus.

5. Conclusion

Oseltamivir remains the most widely used anti-influenza drug globally and while the use of NAIs had been relatively low against seasonal influenza (with the exception of Japan), very large stockpiles have been amassed in developed countries for use in pandemics. Whether the current pandemic warrants the widespread use of NAIs is debatable. Overuse of these agents has the potential to generate widespread resistance rendering antiviral stockpiles ineffective in combating future 'waves' of the pandemic strain that may have a greater virulence. In contrast, it would be an unpopular decision if Governments chose to not use their large antiviral stockpiles in a mild pandemic, such as the current A(H1N1) 2009 pandemic, particularly given that many countries' stockpiles are close to expiring.

History tells us that oseltamivir resistance in influenza viruses was rare before 2007, but within 12 months a resistant strain of seasonal H1N1 bearing the neuraminidase H274Y mutation had spread throughout the world and was the dominant strain in a number of countries. Oseltamivir resistant A(H5N1) viruses have also been found sporadically, as have resistant A(H3N2) viruses. Whether the pandemic (H1N1) 2009 viruses develops oseltamivir resistance in the future remains to be determined, although the need to develop and license new anti-influenza drugs such as favipiravir (T705) and laninamivir (CS-8958) will increase the potential treatment options should circulating strains become resistant to certain compounds. The finding that 100% of the pandemic (H1N1) viruses are resistant to adamantanes underlines the issue of drug resistance in viruses of significant public health concern. Close monitoring of the antiviral susceptibility of seasonal, highly pathogenic and pandemic (H1N1) influenza viruses both now and in the future remains a high priority.

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